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Transcutaneous immunization with phosphorylcholine induces antigen-specific mucosal and systemic immune responses in BALB/c mice

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ABSTRACT

Objective: Transcutaneous immunization (TCI) is a novel route of vaccination through application of a topical vaccine antigen on the skin. Phosphorylcholine (PC) is a structural component of a variety of pathogens and anti-PC immune responses protect mice against invasive bacterial diseases. The purpose of the study was to examine the effect of TCI using PC in BALB/c mice.

Methods: TCI was performed in BALB/c mice using PC–keyhole limpet hemocyanin (KLH) plus cholera toxin (CT). Immunogenicity was evaluated by measuring PC-specific IgG and specific IgG1, IgG2a, IgM, IgA, and secretory IgA antibodies by ELISA. The concentrations of IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ were also measured using ELISA for mouse.

Results: Six months after immunization, IgG after TCI using PC plus CT was significantly higher than in controls, but this was not found for IgA. In saliva, secretory IgA antibodies decreased with a peak level at 2–3 months. IgG1 was significantly higher than IgG2 after TCI. Production of IL-4 from $CD4^+$ cells was significantly higher after TCI than in controls, whereas production of IFN-γ, IL-5, IL-12 and IL-13 was not detected in either group.

Conclusion: These results suggest that TCI using PC plus CT with BALB/c mice is a simple approach for induction of systemic and mucosal immune responses that are shifted in the Th-2 direction.

1. Introduction

The pervasiveness of drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Pseudomonas aeruginosa* and the widespread and highly pathogenic avian influenza virus emphasize the urgency of development of vaccines for prevention and to reduce medical costs. Systemic administration of vaccines can be used, including subcutaneous injection and transmucosal administration of transnasal and sublingual vaccines, but facial nerve paralysis may occur as an adverse event after transmucosal delivery of a transnasal vaccine [1].

Transcutaneous immunization (TCI) is a new administration route that may have fewer associated adverse events [2,3]. This approach causes no pain upon inoculation, in contrast to injection of vaccines, and has no side effects such as fever and anaphylaxis because administration of the antigen is limited to the skin surface, where blood vessels are not distributed. We have described a mucosal immune response in TCI using cholera toxin (CT) as an antigen [4].

Phosphorylcholine (PC) is expressed on the cytomembrane surface of various bacteria. Thus, it is likely that bacterial infection could be controlled over a wide area of the membrane surface by an antibody against PC, and we have examined the mucosal immune response after nasal administration of PC [5,6]. For clinical use, there is a need to know how long the antibody titer can be maintained after administration of PC as a vaccine. Here, we describe the effects of TCI with PC on changes over time in blood serum and the mucosal immune response at the membrane surface.

2. Materials and methods

2.1. Mice

Six-week-old female BALB/c mice were obtained from CLEA Japan Inc. (Shizuoka, Japan) and maintained in the animal facility

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of Kagoshima University under specific pathogen-free conditions. All mice used in the study were 6 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 two groups (five mice per group). The TCI group received transcutaneous immunization with PC–keyhole limpet hemocyanin (KLH) (Biosearch, San Rafael, CA) (200 μg/mouse) and cholera toxin (CT) (2 μg/mouse) as a mucosal adjuvant. The antigens in 10 μl of phosphate-buffered saline (PBS) were dropped onto depilated back skin using a pipette. The control group received 10 μl of PBS with CT (2 μg/mouse) dropped onto depilated back skin. Inoculations were given once each week for six consecutive weeks. Saliva and serum samples were collected once each month after the last immunization. Saliva samples were obtained after inducing salivary gland secretion by intraperitoneal injection of 100 μl of 1 mg/ml pilocarpine (Sigma, St. Louis, MO) in sterile PBS.

2.3. Detection of PC-specific antibody production by ELISA

PC-specific antibody titers in serum and saliva were determined by an enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml of PC–bovine serum albumin (BSA) (Biosearch, San Rafael, CA) dissolved in PBS and the wells were blocked with 1% BSA dissolved in PBS (BSA-PBS). Each sample was then serially diluted in 1% BSA-PBS and transferred to an individual well. After incubation for 2 h, the plates were washed and reacted with 1:3000 diluted horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, and IgA (Southern Biotechnology Associates, Birmingham, AL). The reaction was developed with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, CA) for 5 min at room temperature, and then terminated by addition of 0.5 N HCl (50 μl/well). Optical density (OD) was recorded using a plate reader at 450 nm. OD > 0.3 was considered to be positive.

2.4. Detection of cytokines production by ELISA

A subset of $CD4^+$ T cells was obtained from single spleen cell suspensions by positive sorting with a magnetic bead separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Splenic mononuclear cells treated with mitomycin C (50 μg/ml, 37 °C, 45 min) were used as feeder cells. Purified $CD4^+$ T cells were incubated in culture medium with feeder cells and PC–BSA (10 μg/ml) for 72 h. Supernatants were collected to detect cytokine production. The concentrations of IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ were measured using ELISA kits for mouse (BioSource International Inc., Camarillo, CA).

2.5. Statistical analysis

Data were compared using one-factor ANOVA with $p < 0.05$ considered significant.

3. Results

3.1. PC-specific IgM, IgG and IgA in serum (reciprocal log_2 titer)

There was no significant difference in PC-specific IgM levels between the TCI (10.3 ± 0.33) and control groups (10.0 ± 0.0) before immunization. PC-specific IgM was significantly higher ($p = 1.3 \times 10^{-2}$) in the TCI group (13.0 ± 0.40) for 6 months after immunization, compared to the control group (11 ± 0.40) (Fig. 1).

There was no significant difference in PC-specific IgG levels between the TCI (6.6 ± 0.33) and control groups (7.0 ± 0.0) before immunization. The PC-specific IgG level was also significantly higher ($p = 8.8 \times 10^{-8}$) in the TCI group (15.0 ± 0.40) for 6 months after immunization, compared to the control group (7.0 ± 0.40) (Fig. 2).

There was no significant difference in PC-specific IgA levels between the TCI (7.0 ± 0.0) and control groups (7.5 ± 0.29) before immunization ($p = 0.13$). The PC-specific IgA level in serum was significantly higher ($p = 5.6 \times 10^{-3}$) in the TCI group (8.0 ± 0.32) for 5 months after immunization compared to the control group

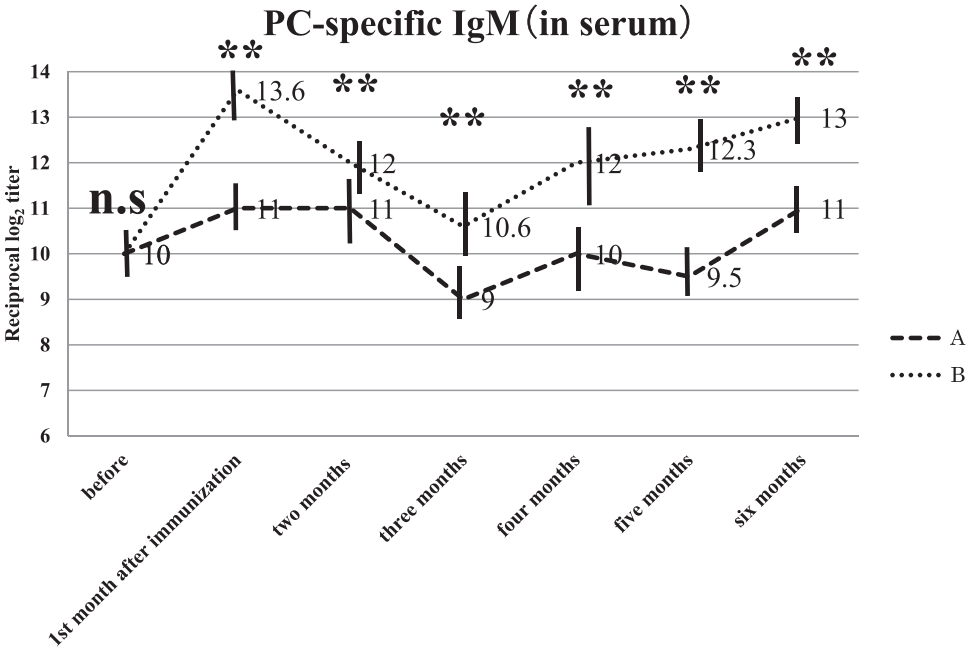

Fig. 1. PC-specific IgM immune responses in serum. There was no significant difference in PC-specific IgM levels between the TCI (10.3 ± 0.33) and control groups (10.0 ± 0.0) before immunization. PC-specific IgM was significantly higher ($p = 1.3 \times 10^{-2}$) in the TCI group (13.0 ± 0.40) for 6 months after immunization, compared to the control group (11.0 ± 0.40). The results were obtained from five mice per group and are expressed as the mean ± the standard error (S.E.). A: control group. B: TCI group. $^*p < 0.05$, $^{**}p < 0.01$.

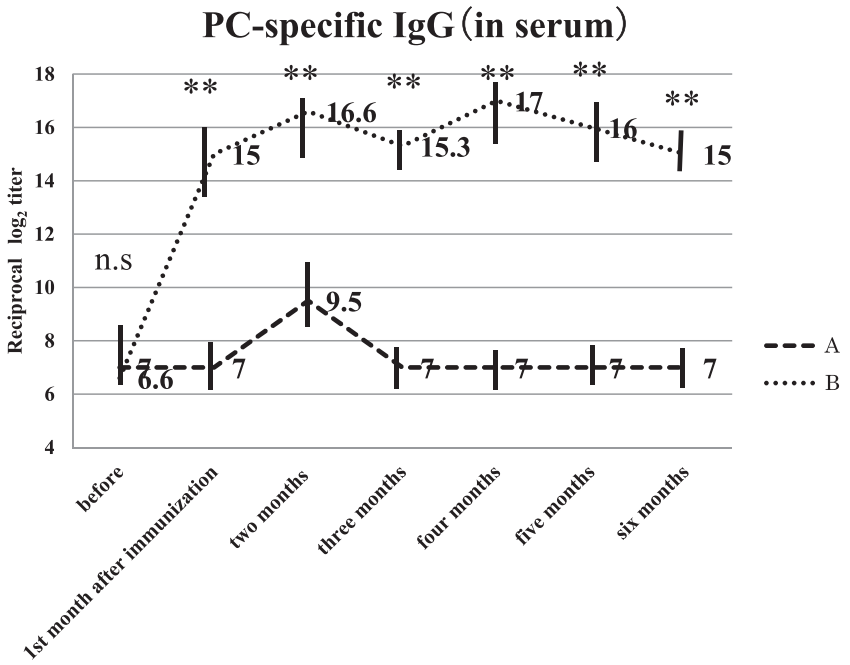

Fig. 2. PC-specific IgG immune responses in serum. There was no significant difference in PC-specific IgG levels between the TCI (6.6 ± 0.33) and control groups (7.0 ± 0.0) before immunization. The PC-specific IgG level was also significantly higher ($p = 8.8 \times 10^{-8}$) in the TCI group (15.0 ± 0.40) for 6 months after immunization, compared to the control group (7.0 ± 0.40). The results were obtained from five mice per group and are expressed as the mean ± S.E. A: control group. B: TCI group. $^{*}p < 0.05$, $^{**}p < 0.01$.

(6.0 ± 0.40), but the difference was not significant in the 6 months after immunization ($p = 0.09$) (Fig. 3).

3.2. PC-specific IgA in saliva (reciprocal $\log_2$ titer)

Before immunization, no PC-specific IgA was detected in the TCI (0.0 ± 0.0) and control groups (0.0 ± 0.0). The PC-specific IgA level in saliva was significantly higher ($p = 3.1 \times 10^{-3}$) in the TCI group (2.0 ± 0.70) in the 6 months after stimulation, compared to the control group (0.0 ± 0.0) (Fig. 4). However, PC-specific IgA in saliva in the TCI group decreased after reaching a peak in the second month.

3.3. IgG subclass (reciprocal $\log_2$ titer)

Regarding the IgG subclass in serum after 6 months, IgG1 levels (17.0 ± 0.82) were significantly higher ($p = 1.9 \times 10^{-5}$) than IgG2a levels (10.0 ± 0.82) in the TCI. IgG1 levels (7.0 ± 0.82) were significantly higher ($p = 1.3 \times 10^{-4}$) than IgG2a levels (2.0 ± 0.82) in the control groups (Fig. 5).

IgG1 levels in the TCI group (17.0 ± 0.82) were significantly higher ($p = 2.8 \times 10^{-7}$) than those in the control group (7.0 ± 0.82). IgG2a levels in the TCI group (10.0 ± 0.82) were significantly higher ($p = 8.8 \times 10^{-6}$) than those in the control group (2.0 ± 0.82) (Fig. 5).

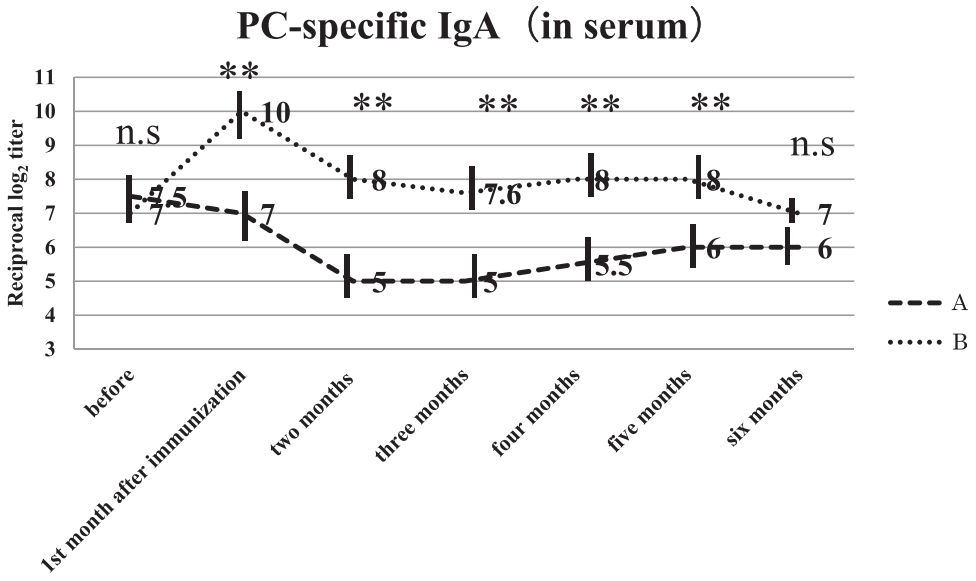

Fig. 3. PC-specific IgA immune responses in serum. There was no significant difference in PC-specific IgA levels between the TCI (7.0 ± 0.0) and control groups (7.5 ± 0.29) before immunization ($p = 0.13$). The PC-specific IgA level in serum was significantly higher ($p = 5.6 \times 10^{-3}$) in the TCI group (8.0 ± 0.32) for 5 months after immunization compared to the control group (6.0 ± 0.40), but the difference was not significant in the 6 months after immunization ($p = 0.09$). The results were obtained from five mice per group and are expressed as the mean ± S.E. A: control group. B: TCI group. $^{*}p < 0.05$, $^{**}p < 0.01$.

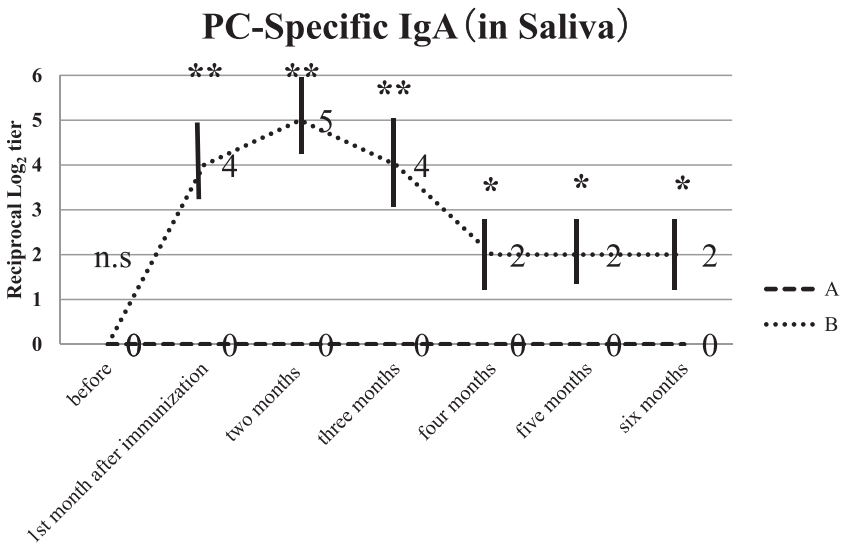

Fig. 4. PC-specific IgA immune responses in saliva. Before immunization, no PC-specific IgA was detected in the TCI (0.0 ± 0.0) and control groups (0.0 ± 0.0). The PC-specific IgA level in saliva was significantly higher ($p = 3.1 \times 10^{-3}$) in the TCI group (2.0 ± 0.70) in the 6 months after stimulation, compared to the control group (0.0 ± 0.0). However, PC-specific IgA in saliva in the TCI group decreased after reaching a peak in the second month. The results were obtained from five mice per group and are expressed as the mean ± S.E. A: control group. B: TCI group. $^{*}p < 0.05$, $^{**}p < 0.01$.

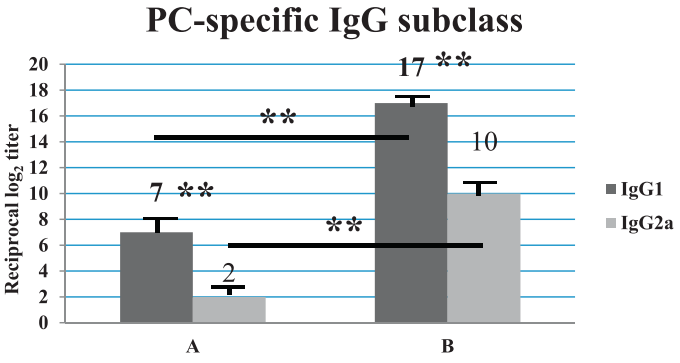

Fig. 5. PC-specific IgG1 and IgG2a immune responses. Regarding the IgG subclass in serum after 6 months, IgG1 levels (17.0 ± 0.82) were significantly higher ($p = 1.9 \times 10^{-5}$) than IgG2a levels (10.0 ± 0.82) in the TCI. IgG1 levels (7.0 ± 0.82) were significantly higher ($p = 1.3 \times 10^{-4}$) than IgG2a levels (2.0 ± 0.82) in the control groups. IgG1 levels in the TCI group (17.0 ± 0.82) were significantly higher ($p = 2.8 \times 10^{-7}$) than those in the control group (7.0 ± 0.82). IgG2a levels in the TCI group (10.0 ± 0.82) were significantly higher ($p = 8.8 \times 10^{-6}$) than those in the control group (2.0 ± 0.82). The results were obtained from five mice per group and are expressed as the mean ± S.E. A: control group. B: TCI group. $^{*}p < 0.05$, $^{**}p < 0.01$.

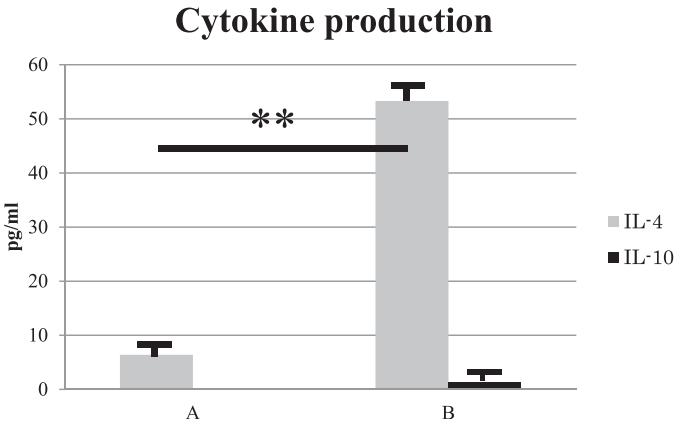

Fig. 6. Cytokine production from splenic $CD4^{+}$ T cells. The IL-4 level in the TCI group (53.3 ± 3.11 pg/ml) was significantly higher than that in the control group (6.4 ± 0.58 pg/ml) ($p = 1.81 \times 10^{-5}$). The IL-10 level in the TCI was 1.32 ± 1.0 pg/ml, not detected in the control group ($p = 0.26$). IFN-γ, IL-5, IL-12 and IL-13 were not detected in either group. The results were obtained from five mice per group and are expressed as the mean ± S.E. A: control group. B: TCI group. $^{*}p < 0.05$, $^{**}p < 0.01$.

3.4. Cytokine production

The IL-4 level in the TCI group (53.3 ± 3.11 pg/ml) was significantly higher than that in the control group (6.4 ± 0.58 pg/ml) ($p = 1.81 \times 10^{-5}$). The IL-10 level in the TCI was 1.32 ± 1.0 pg/ml, not detected in the control group ($p = 0.26$) (Fig. 6).

IL-5, IL-12, IL-13 and IFN-γ were lower than the detection limit in both groups.

4. Discussion

The administration routes for vaccines can largely be divided into systemic administration, such as hypodermic injection, and transmucosal administration, as used for transnasal and sublingual vaccines. The transmucosal route includes nasal, sublingual, oral, and transrectal administration. Transmucosal vaccines can evoke antigen-specific immune responses, even at a membrane surface far from the immunization site, showing anatomically clear zoning. Antigens can be induced in the small and large intestines by oral administration, and in the upper and lower airways and genitals by sublingual administration. However, adverse events of facial nerve palsy have been described after transmucosal administration of a transnasal inactivated influenza vaccine, and thus clinical use of the vaccine was abandoned [1]. Therefore, an administration route with fewer adverse events has been sought and this has led to interest in TCI.

Glenn et al. [2,3] found that TCI could be induced by systemic (serum IgG antibody) and mucosal (SIgA antibody) responses when an adjuvant and vaccine antigen were applied simultaneously. This approach causes no pain upon inoculation, in contrast to injection of vaccines, and has no side effects such as fever and anaphylaxis because administration of the antigen is limited to the skin surface, where blood vessels are not distributed. TCI also enables delivery of antigens to the upper and lower

Table 1
The advantages and disadvantages of each method of administration.

	Advantages	Disadvantages
Transcutaneous immunization (TCI)	No pain and no anaphylaxis. Diverse mucosal immune responses can be induced.	A high antigen dose is necessary. An adjuvant is required.
Subcutaneous immunization	Serum IgG is strongly induced.	Induction of antigen-specific IgA on the mucosa is not possible. The risk of anaphylaxis is high.
Transnasal immunization	The antigen dose is low. Diverse mucosal immune responses can be induced.	Transition to the brain via olfactory epithelium has been reported.
Sublingual immunization	Enterohepatic circulation can be avoided. Diverse mucosal immune responses can be induced.	Not frequently reported.
Oral immunization	Acts strongly on the intestine when induction of immunity is possible.	Influenced by enterohepatic circulation. A large amount of antigen is necessary. Ineffective for infants.

airways, and to the oral cavity and intestinal tract, as seen with sublingual administration [7].

A disadvantage of TCI is that it is necessary to increase the antigen dose for effective administration of the antigen to Langerhans cells and dendritic cells, compared to doses for other mucosal administrations (transnasal and subligual immunization) (Table 1).

The mechanism of TCI begins with incorporation of antigen and adjuvant by Langerhans and dendritic cells, which are antigen-presenting epidermal cells in the skin [8]. These antigen-presenting cells then migrate to regional lymph nodes (mainly cervical lymph nodes) to present antigen to T cells, inducing a systemic immune response. However, immune induction at the mucosal surface is unclear. We have described a mucosal immune response in TCI using cholera toxin (CT) as an antigen [4], but there have been no previous studies of TCI with PC and no follow-up studies over several months.

PC is an immunodominant determinant among cell wall carbohydrates of various bacteria, and a target antigen of a phylactic antibody [9]. PC–KLH, in which KLH is attached to the side chain, is widely used as an antigen in immune examinations. Production of an antibody for PC would allow development of a vaccine for various bacteria, and we have shown that a mucosal immune response by sublingual administration contributes to increased clearance of hemophilus influenza in the upper airway [5,6].

The results of the our study show that PC-specific IgM in serum reached a high level upon completion of immunization, which gradually decreased until the 3rd month, and then increased again in the 4th–6th months. Thus, IgM levels increased earlier than IgG and IgA after immunization, which gradually decreased, and then increased due to infection of intestinal bacteria. PC-specific IgG maintained a high antibody level from completion of immunization until the 6th month, suggesting that PC-specific IgG may be maintained in blood for a relatively long period. There was a significantly higher level of PC-specific IgA for 5 months in the TCI group compared to controls, but this difference disappeared in the 6th month. These findings show that the antibody titer can be maintained in serum for a certain period.

In a subclass analysis, there was a significant increase in IgG2a compared to IgG1 in the TCI group. In addition, IL-4 was significantly higher in TCI group, but IFN-γ was not detected. This suggests that TCI of PC with CT as adjuvant causes predominant induction of Th-2 type antibody-mediated immunity, rather than the Th-1 type. This is consistent with our findings using TCI with CT as the antigen [4]. There is a report on TCI using C57BL/6, but no report has discussed Th-1/Th-2. Thus, it cannot be concluded that Th-2 was induced because of TCI. We are unable to say anything except that Th-2 was induced in BALB/c mice when cholera toxin was used as an adjuvant in this study.

Regarding PC-specific IgA in saliva, a comparatively high antibody titer was maintained for 3 months. The titer then decreased, but a significantly higher antibody titer than that in controls persisted for 6 months.

These results suggest that TCI using PC plus CT with BALB/c mice is a simple approach for induction of systemic and mucosal immune responses for a long period that are shifted in the Th-2 direction.

Conflict of interest

None.

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