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Transcutaneous immunization in auricle skin 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 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 back skin or auricle skin in BALB/c mice.

Methods: TCI was performed in BALB/c mice in back skin or auricle skin using PC-keyhole limpet hemocyanin (KLH) plus cholera toxin (CT). Inoculations were given once each week for six consecutive weeks. Immunogenicity was evaluated by measuring PC-specific IgG and specific IgG1, IgG2a, IgM, IgA, and secretory IgA antibodies by ELISA. IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-? levels were also measured by ELISA.

Results: Serum IgG after TCI in auricle skin was significantly higher than after TCI in back skin and in controls. Secretory IgA antibodies after TCI in auricle skin were also significantly higher than after TCI in back skin and in controls in nasal, BALF, vaginal and fecal samples. PC-specific IgG1 and IgG2a were significantly higher after TCI in auricle skin compared to controls and compared to TCI in back skin. IgG1 was significantly higher than IgG2a after TCI in auricle skin. Production of IFN-?, IL-4 and IL-10 from $CD4^+$ cells was significantly higher after TCI in auricle skin than after TCI in back skin and in controls, whereas IL-5, IL-12 and IL-13 were not detected in any mice.

Conclusion: These results suggest that TCI in auricle skin using PC plus CT in BALB/c mice is a simple approach for induction of systemic and mucosal immune responses that are shifted in the Th2 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 urgent need for development of vaccines for prevention of infection and reduction of medical costs.

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

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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 [1]. However, adverse events of facial nerve palsy can occur after transmucosal administration of a transnasal inactivated influenza vaccine, and thus clinical use of this vaccine was abandoned [2].

Transcutaneous immunization (TCI) is a new administration route that may have fewer associated adverse events [3,4]. This approach causes no pain upon inoculation, in contrast to injection of vaccines, and does not have 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 in back skin using cholera toxin (CT) as an antigen [5].

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 [6,7] and TCI in back skin [1]. It is important to understand which site is best for application of TCI in clinical use. Therefore, in this study, we compared transcutaneous immunization in back and auricle skin. Here, we describe the effects of TCI with PC on changes in 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 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 three groups (five mice per group). TCI in back skin or auricle skin was performed by transcutaneous immunization with PC-keyhole limpet hemocyanin (KLH) (Biosearch, San Rafael, CA) (200 ?g/mouse) and cholera toxin (CT) (2 ?g/mouse) as a mucosal adjuvant. The antigens in 10 ?l of phosphate-buffered saline (PBS) were dropped onto depilated back skin or auricle skin using a pipette. The control group received 10 ?l of PBS with CT (2 ?g/mouse) dropped onto depilated auricle skin. Inoculations were given once each week for six consecutive weeks. Nasal wash, saliva, lung wash, vaginal wash, and fecal and serum samples were collected one week after the final immunization. After bloodletting and removal of the mandible, the nasal cavity was gently flushed with 200 ?l of PBS from the posterior opening of the nose. The nasal washes were collected from the anterior openings of the nose [8]. Saliva samples were obtained after inducing salivary gland secretion by intraperitoneal injection of 100 ?l of 1 mg/ml pilocarpine (Sigma, St. Louis, MO) in sterile PBS. Bronchoalveolar lavage fluid (BALF) was obtained by irrigation with 200 ?l of PBS, using a blunted needle inserted into the trachea after tracheotomy. Vaginal washes were collected with 200 ?l of PBS. Fecal extract samples were obtained by adding weighed pellets to PBS containing 0.01% sodium azide (1 ml/100 mg fecal sample) [9].

2.3. Detection of PC-specific antibody production by ELISA

PC-specific antibody titers in serum, nasal, saliva, BALF, vaginal, and fecal samples were measured by enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 ?g/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 ?l/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 ?l/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 cytokine production by ELISA

A subset of $CD4^+$ T cells was obtained from spleen, neck lymphoid, and abdominal lymphoid cell suspensions by positive sorting with a magnetic bead separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Splenic mononuclear cells treated with mitomycin C (50 ?g/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 ?g/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-? 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 before TCI in back skin (10.0 ± 0.40), TCI in auricle skin (10.0 ± 0.40), and in controls (11.0 ± 0.40). Compared to controls (9.75 ± 0.50), PC-specific IgM was significantly higher after TCI in back skin (11.8 ± 0.83) and auricle skin (11.0 ± 0.0). There was

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