Transcutaneous Immunization Induces Antigen–Specific Antibody Responses in the Oral Cavity

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Abstract
Transcutaneous immunization is a new method of vaccination that utilizes a topical application of antigen to intact skin for induction of immune responses. In this study, we have assessed the potential for the application of transcutaneous immunization for the development of a novel vaccine–delivery system. When BALB/c mice were immunized with ovalbumin (OVA) by direct application to shaved skin, OVA–specific serum IgG antibody responses were induced; however, none was induced in saliva. On the other hand, when OVA was given with cholera toxin (CT) as adjuvant, higher levels of OVA–specific serum IgG antibody responses were induced than that of OVA alone. Furthermore, OVA–specific IgG antibody responses were detected in the saliva of mice immunized with OVA plus CT. Antibody–forming cell (AFC) analysis confirmed the antibody titers by revealing significant numbers of OVA–specific IgG AFCs in the spleen and salivary gland. In addition, mononuclear cells from the spleen and cervical lymph nodes of mice immunized with OVA plus CT exhibited significant levels of proliferative responses when restimulated with OVA in vitro. These results indicate that transcutaneous immunization may be an effective vaccine delivery system for the induction of protein antigen–specific antibody responses in the oral cavity.

Keywords:
transcutaneous immunization, oral cavity, saliva, IgG antibody

Introduction
The oral cavity is the portal of entry for numerous pathogens that range from viruses to bacterium. The oral mucosa is protected by both nonspecific and specific defense mechanisms. The former include mucins, lysozyme, lactoferin, lactoperoxidase, and various antimicrobial peptides. In contrast, an important aspect of the specific defense mechanism of the oral cavity is the immune system (1). Although antibiotic therapy has been used to treat bacterial infections, the increasing number of resistant strains and the inefficiency of anti–viral drugs to resolve viral infections have accelerated the development of other strategies to prevent infections. Immunoprophylaxis achieved by the administration of vaccines represents a feasible tool since it can provide targeted immune protection and reduce health–care costs. Effective protection against oral pathogens requires both a mucosal immune response for induction of secretory IgA (S–IgA) antibodies in saliva and a systemic immune response for induction of IgG antibodies in crevicular fluid within the gingival and mucosal tissues (1).

A rational design of vaccines for the induction of effective immune responses in the oral cavity should carefully consider the types of immune responses and the antigen delivery system. Needle–free delivery has become a global priority because of the risk of needle–borne diseases associated with re–use and improper disposal of needles. Transcutaneous immunization (TCI), which introduces antigens by topical application to intact skin, offers several advantages over
other antigen delivery systems. First, TCI is capable of inducing both mucosal and systemic immune responses, which result in two layers of host protection against infectious diseases (2–4). Second, this needle–free vaccination decreases the risk of needle–borne diseases, reduces the complications that can accompany physical skin penetration, and improves access to vaccination because it eliminates the need for medical doctors and sterile equipment. These are extremely beneficial, especially in developing countries. Thus, the major aim of the present study was to assess the potential of a transcutaneous vaccine to induce an immune response in the oral cavity.

**Materials and Methods**

**Mice**

BALB/c mice were purchased from Japan SLC and were maintained in an experimental facility under pathogen–free conditions in the Nihon University School of Dentistry at Matsudo. All mice were provided with sterile food and water *ad libitum* and were used in this study at 8–12 weeks of age.

**Antigen and adjuvant**

Ovalbumin (OVA) was obtained from Sigma (St. Louis, MO). CT was obtained from List Biologic Laboratories (Campbell, CA).

![Mice](image1)

**Immunization and sample collection**

Mice were anaesthetized intraperitoneally with Ketamine (Sigma, St. Louis, MO). Fur was shaved from a section of the upper back with care taken not to break the skin (Fig. 1A). The skin was swabbed with 70% ethanol and allowed to dry. An adhesive plaster, into which a 150 µl aliquot of phosphate-buffered saline (PBS) containing 1 mg of OVA alone or combined with 10 µg of CT had been absorbed, was applied on day 0, 7, and 14 (Fig. 1B). Serum and saliva samples were collected as described elsewhere (5) to examine OVA–specific antibody responses.

**Detection of antigen–specific antibody responses**

The antibody titers in serum, saliva and nasal washes were determined by an ELISA (6, 7). Briefly, plates were coated with OVA (1 mg/ml) and blocked with 1% bovine serum albumin, and analyses were performed in duplicate. After blocking, serial dilutions of serum or saliva samples were added in duplicate. Starting dilutions of serum and saliva were 1: 2º and 1: 2º, respectively. Following incubation, the plates were washed, and peroxidase–labeled goat anti–mouse γ or α heavy chain–specific antibodies (Southern Biotechnology Associates (SBA), Birmingham, AL) were added to appropriate wells. Finally, 2,2’–azino–bis (3–ethylbenz–thiazoline–6–sulfonic acid) (ABTS) with H₂O₂ (Moss, Ins., Pasadena, MA) was added for color development. Endpoint titers were expressed as the reciprocal log₂ of the last dilution, which gave an optical density at 414 nm of 0.1 greater than background after 15 min of incubation.

**ELISPOT employed for assessment of antibody–forming cells (AFCs)**

The salivary glands and nasal passages were carefully excised and teased apart, and dissociated using 0.3 mg/ml collagenase type IV (Sigma) in RPMI 1640. The mononuclear cells were obtained at the interface of the 50% and 75% layers of a discontinuous percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ) (8, 9). To assess numbers of antigen–specific AFCs, an ELISPOT assay was performed as
previously described (10, 11). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, MA) were coated with OVA (1 mg/ml), incubated for 20h at 4°C, and the plates were then washed extensively and blocked with 10% goat serum. The blocking solution was discarded, and lymphoid-cell suspensions at various dilutions were added to wells and were incubated for 4h at 37°C in 5% CO₂ in moist air. The detection antibodies consisted of goat horseradish peroxidase-conjugated anti-mouse α or γ heavy chain-specific antibodies (SBA). Following overnight incubation, plates were washed with PBS and developed by addition of 3- amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H₂O₂ (Moss) to each well. Plates were incubated at room temperature for 15–20 min and washed with water, and AFCs were counted with the aid of a stereomicroscope.

**Measurement of OVA-specific cell proliferation**

Single-cell suspensions were obtained from cervical lymph nodes (CLN) and spleens by gently teasing the tissue through a sterile stainless steel screen. Purified cells (5 × 10⁶ cells/ml) were cultured with 1 mg/ml of OVA in RPMI 1640 (Gibco BRL) containing 10% fetal bovine serum (FBS), 50 µM 2 mercaptoethanol, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were incubated for 4 days at 37°C in 5% CO₂ in air. To measure OVA-specific cell proliferation, 1.0 µCi of (6)-thymidine was added into the culture 18 hours before harvesting, and incorporated radioactivity was measured by scintillation counting.

**Statistics**

The data are expressed as the mean±standard error, and statistical significance (p<0.05) was determined by Student’s t test.

**Results**

**Analysis of OVA-specific serum antibody responses by TCI with OVA**

To evaluate the ability of TCI with OVA to induce serum-antibody responses, a group of mice was tran-cutaneously immunized with OVA alone. When the OVA-specific antibody responses were analyzed by ELISA, IgG antibodies were detected after secondary immunization and increased by a third immunization; however, the responses were low (Fig. 2A). OVA-specific IgA responses were not detected during the analysis period (data not shown). Since nasal administration of CT with soluble protein proved to be an effective regimen for the generation of antigen-specific antibody responses in both systemic and mucosal compartments (5, 11–15), we sought to determine whether the transcutaneous administration of OVA plus CT as adjuvant could induce OVA-specific antibody responses. Mice transcutaneously immunized with OVA plus CT showed OVA-specific serum IgG responses that are significantly higher than those induced by OVA alone (Fig. 2A). After the third immunization, these OVA-specific IgG antibody responses were found to be further elevated to the maximum level (Fig. 2A). However, no IgA antibodies were detected (data not shown). The results of these serum IgG titers were confirmed by AFC responses, which indicated significant numbers of OVA-specific IgG-forming cells in cells isolated from the spleens of mice given OVA plus CT as

![Fig. 2. OVA-specific serum IgG antibody responses and a number of IgG AFC in the spleens of mice transcutaneously immunized with OVA. Groups of BALB/c mice were transcutaneously immunized with 1 mg of OVA alone (open square) or 1 mg of OVA plus 10 µg of CT (closed square) on days 0, 7, and 14. Serum samples were collected at weekly intervals and were assessed for OVA-specific IgG antibody responses (A). Mononuclear cells were isolated from the spleens of mice on day 21 and examined for OVA-specific IgG AFCs (B). The results are expressed as the mean±standard error obtained for six mice per group. ND: not detectable. *p<0.05, compared with that of mice immunized with OVA alone.](image_url)
adjuvant. In contrast, only low numbers of IgG were detected in the spleens of mice given OVA alone (Fig. 2B).

**OVA-specific IgG antibody responses were detected by TCI with OVA**

The transcutaneous administration of OVA plus CT induced high levels of OVA-specific IgG antibody responses in saliva obtained a week after the third immunization while no such responses ($< \log_2 2$) were detected in mice given OVA alone (Fig. 3A). To determine whether IgG antibody responses in external secretions were mucosa-associated or, alternatively, were exudates from serum, the numbers of OVA-specific IgG AFCs were counted. As shown in Fig. 3B, high numbers of IgG AFCs were found in the salivary glands of mice given OVA plus CT, whereas only a few AFCs were detected in the salivary glands and nasal passages of mice given OVA alone. Further, it is important to note that although no IgA anti-OVA antibody titer was detected in the saliva, IgA AFCs were found in the salivary glands of mice transcutaneously immunized with OVA plus CT (Fig. 3B). This result indicated that TCI with OVA plus CT was capable of inducing OVA-specific mucosal IgA antibody responses in the saliva. This issue is currently under investigation.

**OVA-specific cell proliferative responses**

In order to determine whether OVA-specific IgG responses induced by TCI with OVA were T-cell-dependent responses, we next examined OVA-specific cell-proliferative responses. As Fig. 4 shows, mononuclear cells isolated from the spleens and CLN of mice transcutaneously immunized with OVA plus CT as adjuvant showed significant levels of proliferation when re-stimulated with OVA in vitro. Further, the proliferative response of lymphocytes isolated from CLN, which is draining lymph nodes of maxillofacial mucosal compartment is higher, but not statistically significant, than that seen in splenocytes. In contrast, marginal levels of cell-proliferative responses were detected in the spleens and CLN taken from mice immunized transcutaneously with OVA alone (Fig. 4). These results indicated that transcutaneously administered OVA plus CT could elicit T cell-dependent immune responses in both mucosal and systemic compartments.

**Discussion**

In the present study, we have assessed the potential

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![Fig. 3. Detection of OVA-specific antibody responses in saliva and numbers of AFCs in salivary glands. Groups of BALB/c mice were transcutaneously immunized with OVA alone (open square) or OVA plus CT (closed square) as described in the legend of Fig. 1. Saliva samples were collected on day 21 and were assessed for OVA-specific IgG and IgA antibody titers (A). Mononuclear cells were isolated from salivary glands of mice on day 21 and examined for OVA-specific IgG AFCs (B). The results are expressed as the mean±standard error values obtained for six mice per group. *p<0.05 compared with that of mice immunized with OVA alone.](image)

![Fig. 4. OVA-specific cell-proliferative responses in mice immunized nasally with OVA. Groups of BALB/c mice were transcutaneously immunized with OVA alone (open square) or OVA plus CT (closed square) as described in the legend of Fig. 1. Mononuclear cells were isolated from CLN and the spleen 7 days after the last immunization and were cultured with 1 mg/ml of OVA in the presence for 4 days. To measure OVA-specific cell proliferation, 1.0 μCi of (8) thymidine was added into the culture 18 hours before harvesting, and incorporated radioactivity was measured by scintillation counting. The results are expressed as the mean±standard error values from four mice per group and were taken from a total of three separate experiments.](image)
of a transcutaneous vaccine to induce an immune response in the oral cavity. For TCI, we used OVA as an antigen to study the nature and kinetics of antigen–specific antibody responses since OVA has been widely used as a model antigen to assess antigen–specific immune responses (6, 16–18). Our results showed that TCI with OVA could induce significant OVA–specific IgG responses in serum; however, the responses were low. Further, no OVA–specific antibodies were detected in the saliva. Indeed, previous studies have also reported that OVA without adjuvant is a weak immunogen when it is given orally or nasally (6, 16–18). These earlier studies together with our findings suggest that an adjuvant is required for effective induction of OVA–specific antibody responses effectively when OVA is given transcutaneously.

CT has been widely used as adjuvant for mucosal immunization such as oral or nasal immunization (6, 10, 16–20). Thus, we have used CT as an adjuvant for TCI. Our results indicated that transcutaneous administration of CT possessed adjuvant effect and induced significantly higher levels of OVA–specific IgG antibody responses in serum when compared with mice given OVA alone. Further, OVA–specific IgG antibodies were detected in the saliva. These results clearly indicate that CT is an effective adjuvant for TCI. However, despite these beneficial attributes, CT is unsuitable for humans since it causes severe diarrhea (21). CT consists of one A subunit (CT–A) and five B subunits (CT–B). CT–B selectively binds GM1 cell surface receptor and promotes the entry of CT–A. Following entry into cells, CT–A catalyzes ADP–riboseylation of Gsα, resulting in elevated intracellular cyclic AMP levels, which in epithelial cells cause secretion of water and chloride ions into the small intestine with a characteristic watery diarrhea (21). Further, CT accumulates in the olfactory nerves/epithelium (ON/E) regions via GM1 cell surface receptors when given nasally (22). Moreover, CT as mucosal adjuvant redirects co–administered protein antigen into these neuronal tissues (22). The finding raised some concerns about a potential role for GM1 binding molecules that target neural tissues including the central nerve system (CNS) in nasal immunization. However, a recent study has demonstrated that TCI of human volunteers with heat–labile enterotoxin (LT) of enterotoxigenic Escherichia coli elicits robust LT–specific antibody responses without any significant side effect (2). Further, application of CT to the skin induces potent immune responses without evidence of the systemic toxicities that accompany its use via oral, nasal or parenteral routes (23). These studies together with our results suggest that CT can be used as an adjuvant for TCI of humans.

One of the unique features of immune responses at mucosal surfaces is the production of secretory IgA antibodies and their transport across the epithelium. This IgA response presents the first line of defense against the invasion by viral and bacterial pathogens through oral as well as other mucosal tissues. The mucosal immune system is an integrated network of tissues, cells and effector molecules, which function to protect the host from those pathogens. Further, mucosal lymphocytes exhibit unique homing receptors, the integrins, which recognize ligands expressed on mucosal endothelial cells, allowing their retention in mucosal tissues for the delivery of cellular and humoral immune responses (24). Thus, the mucosal immune system is separate from the peripheral lymphoid tissues, which provide immune protection for internal organs and tissues. Not surprisingly, the induction of peripheral immune responses by parenteral immunization does not result in significant mucosal immunity. However, mucosal immunization, such as oral or nasal, does result in effective immunity in external secretions and in the peripheral compartment as well (5, 10, 11, 15, 20). In this regard, previous studies have demonstrated that TCI is capable of inducing mucosal IgA antibody responses as well as serum IgG antibodies (2, 3). However, our results indicated that OVA–specific IgG, but not IgA, antibody responses were induced in saliva after TCI with OVA plus CT. Thus, it is possible that these salivary IgG antibodies may be exudates from serum. However, AFC analysis indicated that a high number of OVA–specific IgG AFCs were found in the sali-
vary gland. Further, significant levels of OVA-specific proliferative responses were seen in the CLN in addition to the spleen after TCI with OVA plus CT. In this regard, it has been shown that local production of IgG occurs in the female reproductive tract in rabbit (25). Local production of IgG in saliva and vaginal secretions has also been proposed during acquired immunodeficiency syndrome (AIDS) (26). Further, recent studies examining patterns of antibody reactivity to streptococcal antigens demonstrated that IgG purified from secretions exhibit reactivity patterns that are different from that of autologous serum IgG (27). These studies suggest that OVA-specific salivary IgG antibodies induced by TCI with OVA plus CT are due to local synthesis, rather than transudation from serum.

The oral cavity is an important and characteristic component of the mucosal immune system. However, it differs from other mucosal components because its local immune responses are both mucosal and systemic. Salivary antibodies are produced from the salivary glands, which are part of the mucosal immune system. On the other hand, antibodies in crevicular fluid are derived from tissue fluids in blood capillaries which are part of the systemic immune system (1). Thus, although the main immune system in the oral cavity is mucosal, it is clear that systemic-derived antibodies in crevicular fluid are also biologically active within the oral cavity. Thus, effective protection against oral infectious diseases requires both mucosal and systemic antibody responses. Our results showed that TCI with OVA plus CT induced OVA-specific IgG in both saliva and serum. Further, AFC analysis indicated that salivary IgG responses were mucosa-associated. These findings suggest that the TCI can be a practical and effective route of immunization for the induction of specific immunity against oral infectious diseases.

In summary, our present study provides evidence that transcutaneous administration of a vaccine containing OVA plus CT elicits OVA-specific IgG antibody responses in both serum and saliva. These findings indicate that TCI could be an effective vaccine delivery system for the prevention of oral infectious diseases.

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