Transcutaneous Immunization with *P. gingivalis* Surface Protein Antigen Induces T Helper 2 Responses in Systemic Tissues

Satomi Maeba,¹ Shigeo Otake,^{1,2,5} Jun Namikoshi,¹ Mitsuo Hayakawa,^{3,5}
Yoshimitsu Abiko,^{4,5} and Masafumi Yamamoto^{1,2,5}

¹Clinical Pathology, Nihon University Graduate School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan
Departments of ²Oral Medicine, ³Chemistry, ⁴Biochemistry and ⁵Research Institute of Oral Science, Nihon University
School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to :

Satomi Maeba

E-mail : satomi@mascad.nihon-u.ac.

jp

Keywords :

anti-periodontitis vaccine, 40-kDa
outer membrane protein, cholera
toxin, transcutaneous immunization,
cytokine

Abstract

Porphyromonas gingivalis is a major pathogen of chronic periodontitis. An outer membrane protein with a molecular mass of 40-kDa (40k-OMP) is a highly immunogenic surface protein produced by *P. gingivalis*. In this study, to develop an effective vaccine against *P. gingivalis* infection, we assessed T helper (Th) cell responses in systemic and mucosal compartments after 40k-OMP was administered transcutaneously. When CD4⁺ T cells isolated from the spleens of mice immunized with 40k-OMP alone or 40k-OMP plus cholera toxin were restimulated with 40k-OMP *in vitro*, significant levels of proliferative responses were induced. In contrast, only low levels of CD4⁺ T cell proliferation were induced in cervical lymph nodes. Analysis of Th1 [interferon (IFN)- γ] and Th2 [interleukin (IL)-4, IL-5, and IL-6] cytokine responses showed that 40k-OMP-specific Th cells from the spleen produced significant levels of IL-4, IL-5, and IL-6 but did not trigger changes in IFN- γ production. These results suggest that transcutaneous administration of 40k-OMP can elicit 40k-OMP-specific Th2-type cytokine responses in systemic, but not mucosal, lymphoid tissues.

Introduction

Porphyromonas gingivalis has been shown to be one of the major pathogens of chronic periodontitis, which causes destruction of alveolar bone with subsequent tooth loss (1). *P. gingivalis* has several virulence factors that enable this organism to cause periodontal disease (1). In particular, the coaggregation of *P. gingivalis* with other gram-positive or gram-negative bacteria contributes in the formation and maturation of biofilm that in turn lead to the development of periodontal diseases (2-6).

An outer membrane protein (OMP) in the whole cell-associated outer membrane or in outer membrane vesicles mediates the coaggregation of *P. gingivalis* with other bacteria (7). It has been shown that the OMP with a molecular mass of 40-kDa (40k-OMP) produced by *P. gingivalis* is a key virulence

factor for coaggregation (8-10). This outer membrane protein has been found on many strains of *P. gingivalis* (8-10). In this regard, previous studies have shown that monoclonal antibodies to recombinant 40k-OMP inhibit the coaggregation of several strains of *P. gingivalis* with *A. viscosus* (8, 10, 11). Furthermore, these monoclonal antibodies possess complement-mediated bactericidal activity to *P. gingivalis* (12, 13). It has also been demonstrated that anti-40k-OMP antibody opsonizes *P. gingivalis* as a target for phagocytosis by a human neutrophil cell line (14). Taken together, these studies indicate that 40k-OMP appears to be an attractive candidate as an anti-periodontitis vaccine. In this study, to assess the potential for the application of 40k-OMP as an anti-periodontitis vaccine, we have analyzed 40k-OMP-specific T helper (Th) cell responses in both

mucosal and systemic tissues after transcutaneous administration of 40k-OMP.

Materials and Methods

Mice

We purchased BALB/c mice from Sankyo Lab Service (Tokyo, Japan) and maintained them under conventional conditions in the Nihon University School of Dentistry at Matsudo. All mice were provided sterile food and water *ad libitum* and used in this study at 8 to 12 weeks of age.

Antigens and adjuvants

The 40k-OMP was purified to homogeneity from a cell suspension prepared by sonication of *Escherichia coli* K-12 harboring the recombinant plasmid pMD125 as described previously (15). The purity of the 40k-OMP was determined by SDS-PAGE and no contaminating protein bands were noted (data not shown). Furthermore, possible residual endotoxin was assessed in the preparation with an LAL pyrochrome kit (Associates of Cape Cod Inc., Woods Hole, MA). The 40k-OMP contained only 0.4 pg of endotoxin. Cholera toxin (CT) was obtained from List Biologic Laboratories (Campbell, CA).

Immunization and sample collection

The 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. The skin was swabbed with 70% ethanol and allowed to dry before the application of an adhesive plaster containing a 150- μ l aliquot of phosphate-buffered saline (PBS) containing 25 μ g of 40k-OMP alone or combined with 10 μ g of CT on days 0, 7, and 14. Single cell suspensions were obtained from cervical lymph nodes (CLN) and spleen by gently teasing the tissue through a sterile stainless steel screen as described previously (16).

Stimulation of 40k-OMP-specific CD4⁺ T cells

The CD4⁺ T cells from CLN and spleens were purified by the use of a magnet-activated cell sorter

system (Miltenyi Biotec, Auburn, CA), as described elsewhere (16). Purified CD4⁺ T cells (2.5×10^6 cells/ml) were cultured with 2 μ g/ml of 40k-OMP in the presence of T cell-depleted, mitomycin-treated splenic feeder cells (2.5×10^6 cells) in RPMI 1640 medium (Gibco BRL, Rockville, MD) containing 10% fetal bovine serum, 50 μ M 2-mercaptoethanol, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 U/ml of recombinant interleukin (IL)-2 (Genzyme, Cambridge, MA). The cultures were incubated for 4 days at 37°C in 5% CO₂ in air. To measure 40k-OMP-specific cell proliferation, 1.0 μ Ci of [³H] thymidine was added to the culture 18 hour before harvesting and the incorporated radioactivity was measured by scintillation counting.

Analysis of cytokine profiles

Cytokine levels in culture supernatants were determined by a cytokine-specific enzyme-linked immunosorbent assay (ELISA) as described previously (17). Ninety-six well plates (NUNC, Inc., Naperville, IL) were coated with monoclonal anti-IFN- γ (BD PharMingen, San Diego, CA), anti-IL-4 (BD PharMingen), anti-IL-5 (BD PharMingen), or anti-IL-6 antibodies (BD PharMingen). After blocking with PBS containing 1% bovine serum albumin, samples and serial twofold dilutions of standards were added to duplicate wells and incubated overnight at 4°C. The wells were washed and incubated with the biotinylated monoclonal anti-IFN- γ (BD PharMingen), anti-IL-4 (BD PharMingen), anti-IL-5 (BD PharMingen), or anti-IL-6 antibodies (BD PharMingen), which recognize different antigen molecules from the coating antibodies. After incubation, horseradish peroxidase-labeled anti-biotin antibody (Vector Laboratories, Burlingame, CA) was added and developed with 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) containing H₂O₂ (Moss, Inc., Pasadena, MD). Standard curves were generated using mouse recombinant IFN- γ (Genzyme), recombinant IL-5 (Genzyme), recombinant IL-6 (Genzyme), and recombinant IL-4 (Endogen, Boston, MA).

Statistical Analysis

The data are expressed as the mean $\pm$ standard error, and statistical significance ($p < 0.05$) was determined by Student's *t* test.

Results

Proliferative responses of 40k-OMP-specific CD4⁺ T cells

To determine whether CD4⁺ T cells in mucosal as well as systemic compartments become 40k-OMP-specific T cells following transcutaneous 40k-OMP vaccination, CD4⁺ T cells from the CLN and spleen of immunized mice were cultured with 40k-OMP in the presence of mitomycin-treated splenic feeder cells. When CD4⁺ T cells isolated from the spleen of mice given 40k-OMP alone were restimulated with 40k-OMP, significant levels of proliferative responses were induced (Fig. 1). In contrast, essentially no increased proliferative responses were found in the CLN, which drain the lymph nodes of the

Figure 1. The 40k-OMP-specific cell proliferative responses in mice transcutaneously immunized with 40k-OMP. Groups of BALB/c mice were transcutaneously immunized with 25 μ g of 40k-OMP plus 10 μ g of CT as an adjuvant (closed bars), 25 μ g of 40k-OMP alone (dotted bars), or PBS (open bars) on days 0, 7, and 14. The CD4⁺ T cells were isolated from the spleen 7 days after the last immunization and were cultured with 2 μ g of 40k-OMP in the presence of splenic feeder cells for 4 days. To measure 40k-OMP-specific cell proliferation, 1.0 μ Ci of [³H]thymidine was added into the culture 18 hours before harvesting and the incorporated radioactivity was measured by scintillation counting. The results are expressed as the mean $\pm$ standard error values from four mice per group and were taken from a total of three separate experiments. * $p < 0.05$ when compared with mice given PBS. ** $p < 0.05$ when compared with mice given 40k-OMP alone.

maxillofacial mucosal compartment, taken from mice immunized transcutaneously with 40k-OMP alone (Fig. 1).

It has been shown that mucosal (e.g., nasal or oral) administration of CT with soluble protein antigen is an effective regimen for the induction of antigen-specific immune responses in both mucosal and systemic compartments (16, 18-25). We next examined the effect of the transcutaneous administration of CT as an adjuvant on 40k-OMP-specific T cell proliferative responses. The CD4⁺ T cells isolated from spleen of mice transcutaneously immunized with 40k-OMP plus CT as adjuvant showed significantly higher proliferative responses than did those immunized with 40k-OMP alone (Fig. 1). However, marginal levels of CD4⁺ T cell proliferation were induced in the CLN of mice given 40k-OMP plus CT. These results indicated that transcutaneous administration of 40k-OMP was capable of inducing 40k-OMP-specific CD4⁺ T cell responses in the systemic, but not mucosal, lymphoid tissues. Furthermore, the responses could be induced without adjuvants such as CT.

40k-OMP-specific Th cytokine response

Our results indicated that CD4⁺ T cells in systemic

Figure 2. The production of IFN- γ by 40k-OMP-specific cells from mice transcutaneously immunized with 40k-OMP. The CD4⁺ T cells were isolated from spleen of mice immunized with 40k-OMP plus CT (closed bars), 40k-OMP alone (dotted bars), or PBS (open bars) and were cultured with 40k-OMP as described in the legend of Figure 1. After *in vitro* restimulation with 40k-OMP, culture supernatants were subjected to an IFN- γ -specific ELISA. The results are expressed as the mean $\pm$ standard error values from four mice per group and were taken from a total of three separate experiments.

lymphoid tissues were primed after transcutaneous immunization with the 40k-OMP vaccine. In the next study, we determined the cytokine profile of those 40k-OMP-specific Th cells. The CD4⁺ T cells were isolated from the spleens of mice transcutaneously immunized with 40k-OMP alone or 40k-OMP plus CT and were restimulated with 40k-OMP *in vitro* in the presence of splenic feeder cells. To characterize 40k-OMP-specific cytokine responses, culture supernatants were harvested and examined for Th1-type and Th2-type cytokine production by a cytokine-specific ELISA. Analysis of Th1-type cytokine responses revealed that a slight but not statistically

significant increase in IFN- γ production was detected in the supernatant harvested from wells containing CD4⁺ T cells from the spleens of mice immunized with 40k-OMP alone or 40k-OMP plus CT compared with nonimmunized CD4⁺ T cells (Fig. 2).

On the other hand, splenic CD4⁺ T cells from mice given 40k-OMP alone produced significant levels of Th2-type cytokines (e.g., IL-4, IL-5, and IL-6) when stimulated with 40k-OMP *in vitro*. The amounts of those Th2-type cytokines were greater in culture supernatants obtained from 40k-OMP-activated splenic CD4⁺ T cells of mice given 40k-OMP plus CT (Fig. 3). As expected, CD4⁺ T cells from CLN of mice

Figure 3. The production of IL-4, IL-5, and IL-6 by 40k-OMP-specific cells from mice transcutaneously immunized with 40k-OMP. The CD4⁺ T cells were isolated from the spleens of mice immunized with 40k-OMP plus CT (closed bars), 40k-OMP alone (dotted bars), or PBS (open bars) and were cultured with 40k-OMP as described in the legend of Figure 1. After *in vitro* restimulation with 40k-OMP, culture supernatants were subjected to IL-4, IL-5, or IL-6-specific ELISA. The results are expressed as the mean $\pm$ standard error values from four mice per group and were taken from a total of three separate experiments. * $p < 0.05$ when compared with mice given PBS. ** $p < 0.05$ when compared with mice given 40k-OMP alone.

given 40k-OMP alone or 40k-OMP plus CT produced only low levels of IL-4, IL-5, and IL-6 (data not shown). These results indicated that transcutaneous administration of 40k-OMP elicited predominantly CD4⁺ Th2-type cytokine responses in systemic compartments.

Discussion

Our previous studies have shown that nasal delivery of 40k-OMP with CT as an adjuvant induces 40k-OMP-specific Th2-type responses that in turn lead to the induction of serum IgG and IgA as well as salivary IgA antibody responses (18, 26). Furthermore, monoclonal antibodies to 40k-OMP inhibit coaggregation by *P. gingivalis*, as well as exhibit complement-mediated bactericidal and opsonic activity for the phagocytosis of *P. gingivalis* (12-14). These studies clearly indicate that 40k-OMP can be considered as a candidate antigen for the development of an anti-periodontal disease vaccine. Thus, in the present study, we assessed the potential of a transcutaneous 40k-OMP vaccine for the induction of T cell-dependent immune responses in mucosal and systemic compartments. Our results indicated that transcutaneous immunization with 40k-OMP induced CD4⁺ T cell proliferative responses in systemic, but not mucosal, lymphoid tissues. Furthermore, the 40k-OMP-specific CD4⁺ T cells were Th2-type cells producing IL-4, IL-5, and IL-6. Importantly, 40k-OMP-specific CD4⁺ T cell responses could be induced by transcutaneous administration of 40k-OMP alone. These results suggest that transcutaneous 40k-OMP vaccination induces Th2-type responses in the systemic compartment. Furthermore, transcutaneous 40k-OMP vaccination may not require adjuvants such as CT for the induction of a sufficient level of antigen-specific immunity.

Several studies have demonstrated that transcutaneous immunization is capable of inducing both mucosal and systemic immunity (27-29). However, our results indicate that antigen-specific CD4⁺ T cell responses were induced only in the spleen after transcutaneous immunization with 40k-OMP. For transcutaneous immunization, the site of application as

well as the method of administration needs to be carefully considered. In this regard, a variety of application methods for transcutaneous immunization has been demonstrated (27-35). For example, antigens have been directly applied to the lower back after swabbing with acetone (28). In another study, the skin was hydrated with saline before immunization (29). Thus, there may be more appropriate ways for transcutaneous application of 40k-OMP to induce immune responses in the mucosal areas. We are currently improving transcutaneous 40k-OMP vaccination methods for optimal induction of mucosal immune responses.

Our results showed that 40k-OMP-specific CD4⁺ T cells from the spleen produced significant levels of IL-4, IL-5, and IL-6, but not IFN- γ , indicating that transcutaneous immunization with 40k-OMP induced predominant Th2-type cytokine responses. In this regard, it is well established that Th2-type cells provide effective assistance to antibody responses, especially noncomplement-fixing IgG1 antibody synthesis. Indeed, in a separate study, we have shown that transcutaneous immunization with 40k-OMP induced dominantly IgG1 antibody responses (Maeba et al., unpublished observation). The induction of Th2-type cytokine responses may be explained by the route of immunization. Transcutaneous immunization may particularly induce Th2-type responses. However, a recent study has demonstrated that transcutaneous administration of an human immunodeficiency virus peptide vaccine induces a cytotoxic T lymphocyte response (29), indicating that cytotoxic T cell responses are also induced by transcutaneous immunization.

On the other hand, 40k-OMP may direct CD4⁺ T cells to Th2-type cells. However, our previous study has shown that the monoclonal antibodies against 40k-OMP possess complement-mediated bactericidal activity against *P. gingivalis* (12, 13). Taken together, these findings suggest that a combination of transcutaneous immunization and 40k-OMP seems to be a particularly effective vaccine regimen for induction of Th2-type cytokine responses.

It has been reported that protein antigen given via

the mucosal route without adjuvant is only a weak immunogen and thus requires a mucosal adjuvant such as CT to induce antigen-specific immune responses (16, 18-25). Our results also showed that transcutaneous immunization with 40k-OMP plus CT as an adjuvant induced significantly higher levels of 40k-OMP-specific immune responses than immunization with 40k-OMP alone. These studies, together with our present results, clearly indicate that CT is an effective adjuvant for transcutaneous immunization. Despite its efficacy, however, CT is unsuitable for use in humans because it causes severe diarrhea by the following mechanisms. CT consists of one A subunit (CT-A) and five B subunits (CT-B). The CT-B selectively binds GM1 cell surface receptors and promotes the entry of CT-A. Following entry into cells, CT-A catalyzes ADP-ribosylation of Gs α , resulting in elevated intracellular cyclic AMP levels. In epithelial cells, those levels trigger the secretion of water and chloride ions into the small intestine, producing the characteristic watery diarrhea (36). Furthermore, when CT is administered nasally, it accumulates in the olfactory nerves and epithelium regions via the GM1 cell surface receptor (37). These findings imply a potential severe side effect of CT when used as adjuvant. However, our results showed that even without CT, the transcutaneous administration of 40k-OMP induced significant 40k-OMP-specific CD4⁺ T cell proliferative responses. In support of this, in a separate study we have shown that transcutaneous immunization with 40k-OMP alone induced significant saliva IgG and serum IgG antibody responses (Maeba et al., unpublished observation). These findings suggest that transcutaneous immunization with 40k-OMP could be a safe and effective vaccine delivery system for use in humans.

In summary, our present study provides evidence that transcutaneous administration of a 40k-OMP vaccine elicits 40k-OMP-specific Th2-type cytokine responses in systemic lymphoid tissues. It is important to note that these Th2-type responses were induced without the use of CT as an adjuvant. These findings suggest that transcutaneous immunization

with 40k-OMP may act as an effective and safe vaccine for induction of *P. gingivalis*-specific immune responses.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (1437681) from JSPS and a Grant from The Ministry of Education, Culture, Sports, Science, and Technology to promote 2001-Multidisciplinary Research Projects. This work was also supported by the Suzuki Memorial Grant of the Nihon University School of Dentistry at Matsudo (02-0006, 02-1016, 03-1027) and a grant (A-04) from the Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo.

References

1. Cutler CW, Kalmar JR, Genco CA : Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. Trends Microbiol, 3 : 45-51, 1995.
2. Gibbons RJ, Nygaard M : Interbacterial aggregation of plaque bacteria. Arch Oral Biol, 15 : 1397-1400, 1970.
3. Van Houte J, Gibbons RJ, Banghart SB : Adherence as a determinant of the presence of *Streptococcus salivarius* and *Streptococcus sanguis* on the human tooth surface. Arch Oral Biol, 15 : 1025-1034, 1970.
4. Van Houte J, Gibbons RJ, Pulkkinen AJ : Adherence as an ecological determinant for streptococci in the human mouth. Arch Oral Biol, 16 : 1131-1141, 1971.
5. Kolenbrander PE, Andersen RN : Cell to cell interactions of Capnocytophaga and Bacteroides species with other oral bacteria and their potential role in development of plaque. J Periodontal Res, 19 : 564-569, 1984.
6. Theilade E, Theilade J, Mikkelsen L : Microbiological studies on early dento-gingival plaque on teeth and Mylar strips in humans. J Periodontal Res, 17 : 12-25, 1982.
7. Grenier D, Mayrand D : Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect Immun, 55 : 111-117, 1987.
8. Hiratsuka K, Abiko Y, Hayakawa M, Ito T, Sasahara H, Takiguchi H : Role of *Porphyromonas gingivalis* 40-kDa outer membrane protein in the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus*. Arch Oral Biol, 37 : 717-724, 1992.
9. Hiratsuka K, Yoshida W, Hayakawa M, Takiguchi H, Abiko Y : Polymerase chain reaction and an outer membrane protein gene probe for the detection of *Porphyromonas gingivalis*. FEMS Microbiol Lett, 138 :

- 167-172, 1996.
10. Saito S, Hiratsuka K, Hayakawa M, Takiguchi H, Abiko Y: Inhibition of a *Porphyromonas gingivalis* colonizing factor between *Actinomyces viscosus* ATCC 19246 by monoclonal antibodies against recombinant 40-kDa outer-membrane protein. *Gen Pharmacol*, 28 : 675-680, 1997.
11. Abiko Y, Ogura N, Matsuda U, Yanagi K, Takiguchi H: A human monoclonal antibody which inhibits the coaggregation activity of *Porphyromonas gingivalis*. *Infect Immun*, 65 : 3966-3969, 1997.
12. Katoh M, Saito S, Takiguchi H, Abiko Y: Bactericidal activity of a monoclonal antibody against a recombinant 40-kDa outer membrane protein of *Porphyromonas gingivalis*. *J Periodontol*, 71 : 368-375, 2000.
13. Saito S, Hayakawa M, Hiratsuka K, Takiguchi H, Abiko Y: Complement-mediated killing of *Porphyromonas gingivalis* 381 by the immunoglobulin G induced by recombinant 40-kDa outer membrane protein. *Biochem Mol Med*, 58 : 184-191, 1996.
14. Saito S, Hayakawa M, Takiguchi H, Abiko Y: Opsonophagocytic effect of antibody against recombinant conserved 40-kDa outer membrane protein of *Porphyromonas gingivalis*. *J Periodontol*, 70 : 610-617, 1999.
15. Kawamoto Y, Hayakawa M, Abiko Y: Purification and immunochemical characterization of a recombinant outer membrane protein from *Bacteroides gingivalis*. *Int J Biochem*, 23 : 1053-1061, 1991.
16. Yamamoto M, Briles DE, Yamamoto S, Ohmura M, Kiyono H, McGhee JR: A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *J Immunol*, 161 : 4115-4121, 1998.
17. VanCott JL, Staats HF, Pascual DW, Roberts M, Chatfield SN, Yamamoto M, Coste M, Carter PB, Kiyono H, McGhee JR: Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunization with live recombinant *Salmonella*. *J Immunol*, 156 : 1504-1514, 1996.
18. Namikoshi J, Otake S, Maeba S, Hayakawa M, Abiko Y, Yamamoto M: Specific antibodies induced by nasally administered 40-kDa outer membrane protein of *Porphyromonas gingivalis* inhibits coaggregation activity of *P. gingivalis*. *Vaccine*, 22 : 250-256, 2003.
19. Yamamoto M, McDaniel LS, Kawabata K, Briles DE, Jackson RJ, McGhee JR, Kiyono H: Oral immunization with PspA elicits protective humoral immunity against *Streptococcus pneumoniae* infection. *Infect Immun*, 65 : 640-644, 1997.
20. Marinaro M, Staats HF, Hiroi T, Jackson RJ, Coste M, Boyaka PN, Okahashi N, Yamamoto M, Kiyono H, Bluethmann H, et al.: Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J Immunol*, 155 : 4621-4629, 1995.
21. Saito M, Otake S, Ohmura M, Hirasawa M, Takada K, Mega J, Takahashi I, Kiyono H, McGhee JR, Takeda Y, Yamamoto M: Protective immunity to *Streptococcus mutans* induced by nasal vaccination with surface protein antigen and mutant cholera toxin adjuvant. *J Infect Dis*, 183 : 823-826, 2001.
22. Kurono Y, Yamamoto M, Fujihashi K, Kodama S, Suzuki M, Mogi G, McGhee JR, Kiyono H: Nasal immunization induces *Haemophilus influenzae*-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity. *J Infect Dis*, 180 : 122-132, 1999.
23. Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, Noda M, Takeda Y, McGhee JR: A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc Natl Acad Sci USA*, 94 : 5267-5272, 1997.
24. Kweon MN, Yamamoto M, Watanabe F, Tamura S, Van Ginkel FW, Miyauchi A, Takagi H, Takeda Y, Hamabata T, Fujihashi K, McGhee JR, Kiyono H: A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. *J Infect Dis*, 186 : 1261-1269, 2002.
25. Yamamoto M, Kiyono H, Kweon MN, Yamamoto S, Fujihashi K, Kurazono H, Imaoka K, Bluethmann H, Takahashi I, Takeda Y, Azuma M, McGhee JR: Enterotoxin adjuvants have direct effects on T cells and antigen-presenting cells that result in either interleukin-4-dependent or -independent immune responses. *J Infect Dis*, 182 : 180-190, 2000.
26. Namikoshi J, Maeba S, Yamamoto M, Hayakawa M, Abiko Y, Otake S: Nasal immunization with *P. gingivalis* surface protein antigen and cholera toxin adjuvant induces T helper 2 responses in both mucosal and systemic compartments. *Int. J. Oral-Med. Sci.*, 1 : 90-96, 2003.
27. Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, Alving CR: Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med*, 6 : 1403-1406, 2000.
28. Gockel CM, Bao S, Beagley KW: Transcutaneous immunization induces mucosal and systemic immunity: a potent method for targeting immunity to the female reproductive tract. *Mol Immunol*, 37 : 537-544, 2000.
29. Belyakov IM, Hammond SA, Ahlers JD, Glenn GM, Berzofsky JA: Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells. *J Clin Invest*, 113 : 998-

- 1007, 2004.
30. Anjuere F, George-Chandy A, Audant F, Rousseau D, Holmgren J, Czerkinsky C : Transcutaneous immunization with cholera toxin B subunit adjuvant suppresses IgE antibody responses via selective induction of Th1 immune responses. *J Immunol*, 170 : 1586-1592, 2003.
31. Berry LJ, Hickey DK, Skelding KA, Bao S, Rendina AM, Hansbro PM, Gockel CM, Beagley KW : Transcutaneous immunization with combined cholera toxin and CpG adjuvant protects against *Chlamydia muridarum* genital tract infection. *Infect Immun*, 72 : 1019-1028, 2004.
32. Glenn GM, Rao M, Matyas GR, Alving CR : Skin immunization made possible by cholera toxin. *Nature*, 391 : 851, 1998.
33. Glenn GM, Scharton-Kersten T, Vassell R, Matyas GR, Alving CR : Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. *Infect Immun*, 67 : 1100-1106, 1999.
34. Godefroy S, Goestch L, Plotnicky-Gilquin H, Nguyen TN, Schmitt D, Staquet MJ, Corvaia N : Immunization onto shaved skin with a bacterial enterotoxin adjuvant protects mice against respiratory syncytial virus (RSV). *Vaccine*, 21 : 1665-1671, 2003.
35. Hammond SA, Walwender D, Alving CR, Glenn GM : Transcutaneous immunization : T cell responses and boosting of existing immunity. *Vaccine*, 19 : 2701-2707, 2001.
36. Spangler BD : Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.*, 56 : 622-647, 1992.
37. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR : Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J Immunol*, 165 : 4778-4782, 2000.