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Transcutaneous immunization with an outer membrane protein of *Porphyromonas gingivalis* without adjuvant elicits marked antibody responses

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Background/aims: We have previously reported that specific immunoglobulin G (IgG) antibodies induced by transcutaneous immunization (TCI) with a 40-kDa outer membrane protein (40k-OMP) of *Porphyromonas gingivalis*, with cholera toxin (CT) as adjuvant, inhibited coaggregation by *P. gingivalis*. In this study, we further pursue the potential of the 40k-OMP as a transcutaneous vaccine.
Methods/results: TCI of rats administered 40k-OMP elicited significant 40k-OMP-specific serum IgG and IgA, as well as salivary IgG antibody titers. Importantly, these antibody responses were induced without adjuvant. Thus, both serum and saliva antibody titers induced by TCI with the 40k-OMP alone were identical to those of 40k-OMP plus cholera toxin as adjuvant. The serum antibody responses induced by 40k-OMP persisted for more than 140 days. On the other hand, salivary IgG anti-40k-OMP antibodies were gradually decreased. Analysis of antibody-forming cells (AFCs) confirmed the antibody titers by detecting high numbers of 40k-OMP-specific IgG AFCs in spleen and cervical lymph node.
Conclusion: Since 40k-OMP-specific IgG inhibited the coaggregation of *P. gingivalis* with *Streptococcus gordonii*, and the hemagglutinin activity of *P. gingivalis*, TCI with the 40k-OMP may be important as an adjuvant-free immunogen for the prevention of chronic periodontitis.

Key words: outer membrane protein; *Porphyromonas gingivalis*; transcutaneous immunization

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Porphyromonas gingivalis, a gram-negative anaerobic bacterium, has been shown to be one of the major pathogens of chronic periodontitis, a disease that can progress to alveolar bone resorption and eventual tooth loss (8). Since the process of destruction is irreversible, the damage, once done, is permanent (8). Moreover, recent studies have suggested that chronic periodontitis is linked to a number of systemic diseases including cardiovascular diseases, diabetes and osteoporosis (24, 32). Thus, the prevention of periodontitis might be relevant not only for oral but for systemic health as well.

The coaggregation of *P. gingivalis* with gram-positive bacteria as well as its hemagglutinin adhesins are thought to be involved in the colonization of the oral cavity by *P. gingivalis* (9, 16, 25, 39). The coaggregation of *P. gingivalis* with gram-positive and other gram-negative bacteria is mediated by a 40-kDa outer membrane protein (40k-OMP) produced by *P. gingivalis* (18, 19, 34). The 40k-OMP, found in many strains of *P. gingivalis*, resides both on the cell surface and in extracellular vesicles (18, 19, 32). Furthermore, because antibody against recombinant (r) 40k-OMP inhibited the hemagglutinating

activity, and the polymeric form of r40k-OMP itself expressed hemagglutinating activity, the 40k-OMP is thought to be one of the *P. gingivalis* hemagglutinins (5). Moreover, monoclonal antibodies (mAbs) to r40k-OMP possess a complement-mediated bactericidal activity against *P. gingivalis* (21, 33). Our previous studies have shown that specific immunoglobulin G (IgG) antibodies induced by nasal or transcutaneous administration of 40k-OMP with cholera toxin (CT) as adjuvant inhibited coaggregation by *P. gingivalis* (27, 31). These studies indicate that the 40k-OMP could be an effective vaccine antigen for the prevention of *P. gingivalis* infection.

If an effective vaccine for oral cavity is to be designed, careful consideration must be given to the various immune responses and antigen delivery systems. Because of the risk of needle-borne diseases associated with reuse and improper disposal of needles, needle-free delivery has become a global priority. Transcutaneous immunization (TCI), which introduces antigens by topical application to intact skin (11), offers several advantages over other antigen delivery systems. This needle-free vaccine administration has the potential to lead to the following significant advances in immunization delivery: improved safety for the vaccinator, vaccinee, and community; better compliance with immunization schedules; decreased or eliminated injection site pain; easier and speedier vaccine delivery; and reduced costs. For these reasons, needle-free vaccine delivery is supported by many prominent public health organizations involved in the delivery of vaccines, including the World Health Organization, the Global Alliance for Vaccines and Immunization, and the Centers for Disease Control and Prevention.

The major aim of the present study was to assess the potential of a transcutaneous vaccine containing 40k-OMP for inducing an immune response that would inhibit coaggregation and hemagglutination by *P. gingivalis*.

Materials and methods

Rats

Sprague–Dawley (SD) rats were purchased from Sankyo Lab Service Inc. (Tokyo Japan) and were maintained in an experimental facility under pathogen-free conditions at the Nihon University School of Dentistry at Matsudo. All rats were provided with sterile food and water *ad libitum* and were used in this study at 5–8 weeks of age.

Antigen and adjuvant

A recombinant plasmid containing the *40k-OMP* gene (pMD125) was kindly provided by Dr Abiko (Nihon University). The 40k-OMP was purified to homogeneity from a cell suspension prepared by sonication of *Escherichia coli* K-12 harboring the pMD125, as described previously (22). The purity of the 40k-OMP was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and no contaminating protein bands were noted (data not shown). Further, a LAL pyrochrome kit (Associates of Cape Cod, Inc., Woods Hole, MA) was used to determine the levels of any residual endotoxins. The preparation (1 μg) with the 40k-OMP contained less than 0.4 pg endotoxin. CT was obtained from List Biologic Laboratories (Campbell, CA).

Immunization and sample collection

Rats were anesthetized 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. An adhesive plaster, into which 300-μl phosphate-buffered saline (PBS) containing 200 μg 40k-OMP alone or combined with 50 μg CT had been absorbed, was applied on days 0, 7, and 14. Serum and saliva samples were collected, as described elsewhere (45), to examine 40k-OMP-specific antibody responses.

Detection of antigen-specific antibody responses

Antibody titers in serum and saliva samples were determined by an enzyme-linked immunosorbent assay (28, 41). Briefly, plates were coated with 40k-OMP (5 μg/ml) and blocked with PBS containing 2% bovine serum albumin. After blocking, serial dilutions of serum or saliva samples were added in duplicate. Starting dilutions of serum and saliva samples were $1 : 2^5$ and $1 : 2^2$, respectively. Following incubation, the plates were washed and peroxidase-labeled goat anti-rat γ or α heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. Finally, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) with H_2O_2 (Moss, Ins, Pasadena, MA) was added for color development. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density at 414 nm of 0.1 greater than background after 15 min of incubation.

Antibody-forming cell (AFC) analysis

The spleens and cervical lymph nodes (CLN) were carefully excised, teased apart, and dissociated using 0.3 mg/ml collagenase type IV (Sigma) in RPMI-1640 (Invitrogen, Carlsbad, CA). The mononuclear cells were obtained at the interface of the 50 and 75% layers of a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ). To assess the numbers of antigen-specific AFCs, an ELISPOT assay was performed as previously described (47). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore Cop., Bedford, MA) were coated with 40k-OMP (5 μg/ml), incubated for 20 h at 4°C, washed extensively and blocked with RPMI-1640 containing 10% fetal calf serum. The blocking solution was discarded, lymphoid cell suspensions at various dilutions were added to the wells and they were incubated for 4 h at 37°C in 5% CO_2 containing moist air. The detection antibodies consisted of goat horseradish peroxidase-conjugated anti-rat γ or α heavy chain-specific antibodies (Southern Biotechnology Associates). 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 H_2O_2 (Moss) to each well. Plates were incubated at room temperature for 15–20 min, washed with water, and AFCs were counted with the aid of a stereomicroscope.

Coaggregation assay

Serum IgG antibodies from immunized rats were purified using a Hi TrapTM protein G HP column (Amersham Biosciences). *P. gingivalis* 381 and *Streptococcus gordonii* Challis were grown in brain–heart infusion (BBL Microbiology Systems, Cockeysville, MD) containing hemin (10 μg/ml) and vitamin K (1 μg/ml). The bacterial cells were incubated at 37°C in an anaerobic chamber containing N_2 (80%), H_2 (10%), and CO_2 (10%). Coaggregation was determined by the visual assay method as described previously (9, 31). Briefly *P. gingivalis* cells were preincubated with purified IgG antibodies at 37°C for 30 min. The cell suspension (100 μl) was then mixed with equal volumes of *S. gordonii* suspension (approximately 10^{10} cells/ml) on a floccu-

lation slide. The mixture was incubated at 37°C for 10 min with rotation. A score of zero to three was assigned to denote the degree of coaggregation as described previously (7, 31). No visible coaggregation was scored as zero and small uniform coaggregates were scored as one. Scores of two or more were determined largely by the settling rate of coaggregates. Coaggregates which were easily seen but which did not settle immediately, leaving the suspension turbid, were scored as two. Large coaggregates which settled rapidly leaving the supernatant fluid clear, were scored as three.

Hemagglutinin assay

Vesicles were isolated from *P. gingivalis* as described previously (16) with minor modifications (18). Briefly, *P. gingivalis* 381 cells from a 1-l diffusate culture were removed from the growth medium by centrifugation. The vesicle-containing supernatant was concentrated to 250 µl by passage through an ultrafiltration system. (Millipore Co.). The sample was then dialyzed against 50 mM Tris–HCl, pH7.2, containing 0.5 mM dithiothreitol at 4°C overnight to solubilize the pilli. The vesicles were collected by centrifugation (27,000 *g* for 60 min) and suspended in PBS. The hemagglutinating activity of *P. gingivalis* was assayed using human erythrocytes in round-bottomed microtiter plates (33). Fifty microliters of vesicle suspension (0.625 µg) in PBS was transferred into microtiter wells and then 100 µl 2% human erythrocytes was added. After incubation for 1 h at 37°C at high humidity, the hemagglutinating activity of the vesicles was observed. The inhibition of hemagglutinating activity induced by adding purified IgG antibodies was carried out under the same conditions as mentioned above, except for the preincubation of *P. gingivalis* vesicles with purified IgG antibodies for 1 h at 37°C.

Rat abscess experiments

To examine the protective effect of TCI with 40k-OMP against *P. gingivalis* 381 challenge, a rat lesion model like the one described by Tzianabos et al. (40) was utilized. Overnight cultures of *P. gingivalis* 381 were harvested, washed three times with PBS and resuspended in PBS. After the 21 days of the first immunization, eight rats immunized with 40k-OMP were challenged with 2×10^{10} cells of *P. gingivalis* 381 subcutaneously in the abdomen and the lesion sizes were measured after 3 days. Non-immunized rats were also challenged, as a control.

Statistical analysis

Differences in antibody activity and abscess-inducing potential between the experimental and control groups were assessed by the paired Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Results

Induction of 40k-OMP-specific antibody responses by TCI with 40k-OMP

To evaluate the efficacy of TCI with 40k-OMP to induce serum antibody responses, a group of rats were transcutaneously immunized with the 40k-OMP alone. The 40k-OMP-specific IgG and IgA responses were detected 7 days after primary immunization and were enhanced by a second immunization. After the third immunization, these 40k-OMP-specific IgG and IgA responses were further elevated on day 21 and persisted through to day 140 (Fig. 1A). The values in immunized groups were significantly higher than those in the non-immunized group ($P < 0.01$). To determine whether TCI with 40k-OMP also induces antibody responses in saliva, we examined 40k-OMP-specific salivary IgG and IgA responses. The 40k-OMP-specific salivary IgG titers were increased within 21 days after the third immunization with 40k-OMP alone, and gradually decreased (Fig. 1B). In contrast, essentially no IgA responses were detected in the saliva of rats transcutaneously immunized with the 40k-OMP alone (Fig. 1B).

Since mucosal (e.g. nasal or oral) administration of CT with soluble protein has proven to be an effective regimen for the generation of antigen-specific antibody responses (20, 28, 30, 45, 47), we next examined the effect of the transcutaneous administration of CT as adjuvant on 40 K-OMP-specific antibody responses. Rats transcutaneously immunized with 40k-OMP plus CT showed almost the same 40k-OMP-specific serum IgG and IgA responses as those immunized with 40k-OMP alone (Fig. 2A). There were no significant differences in the levels of these responses between immunized groups. TCI with 40k-OMP plus CT also did not change salivary IgG anti-40k-OMP antibody titers after the primary immunization with 40k-OMP alone. Furthermore, even when CT was used as adjuvant with

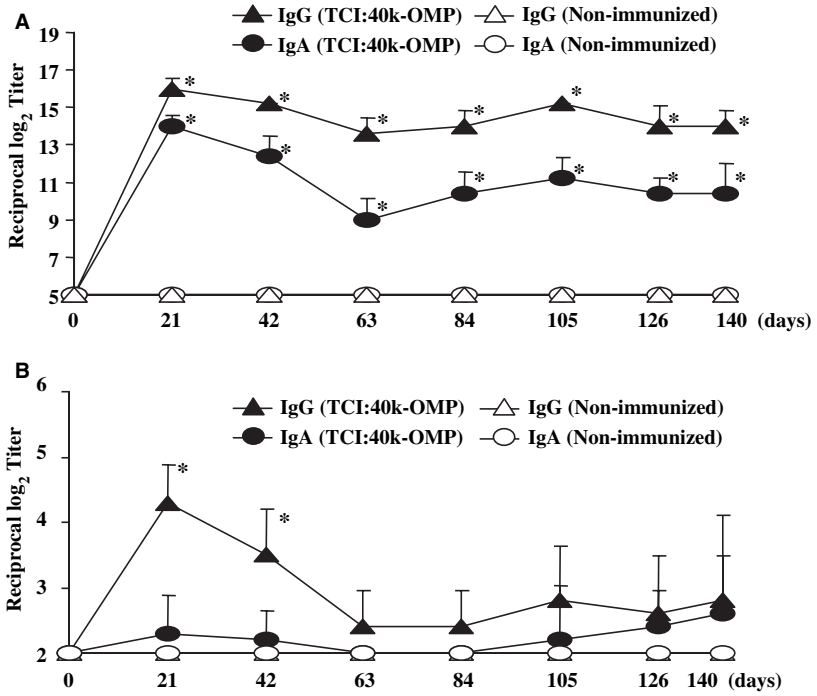

Fig. 1. The 40k-OMP-specific antibody responses in serum and saliva. (A,B) Groups of SD rats were transcutaneously immunized with 200 µg 40k-OMP alone on days 0, 7 and 14. Serum (A) and saliva (B) samples were collected at weekly intervals and assessed for 40k-OMP-specific IgG and IgA antibodies. The results are expressed as the mean ± SE, obtained from six rats per group. *$P < 0.01$; significant differences from non-immunized group.

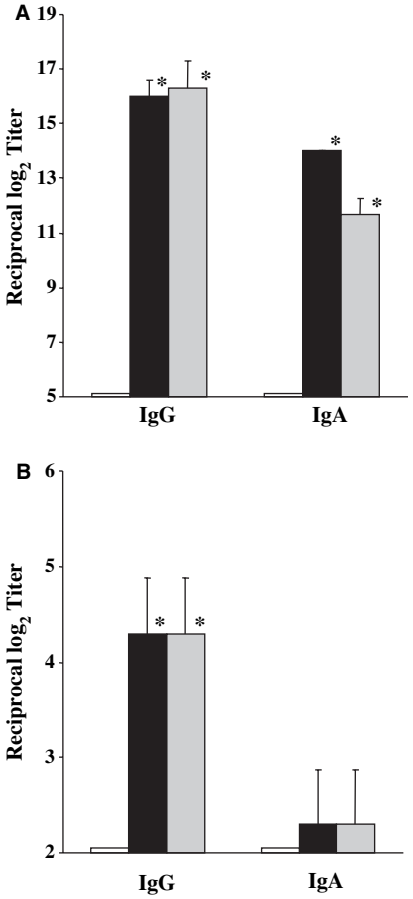

Fig. 2. 40k-OMP-specific antibody responses following transcutaneous vaccination with 40k-OMP alone or 40k-OMP with CT as adjuvant. (A,B) Groups of D rats were transcutaneously immunized with 200 μg 40k-OMP alone (black bars) or 200 μg 40k-OMP plus 50 μg CT (grey bars) on days 0, 7 and 14, or not immunized (open bars). Serum (A) and saliva (B) samples were collected on day 21 and assessed for 40k-OMP-specific IgG and IgA antibodies. The results are expressed as the mean ± SE obtained from six rats per group. $*P < 0.01$; significant differences from non-immunized group.

TCI, no 40k-OMP-specific salivary IgA antibodies were detected.

When the serum antibody titers were confirmed by AFC responses, significant numbers of 40k-OMP-specific IgG-forming and IgA-forming cells were seen in the spleens and the CLN of rats given 40k-OMP alone (Fig. 3A,B). The values in immunized groups were significantly higher than those in the non-immunized group ($P < 0.01$). AFC responses also showed similar numbers of IgG-forming and IgA-forming cells in the spleens and the CLN of rats immunized with 40k-OMP plus CT as in those of rats given 40k-OMP alone (Fig. 3A,B). There were no significant differences in the levels of these responses between immunized groups.

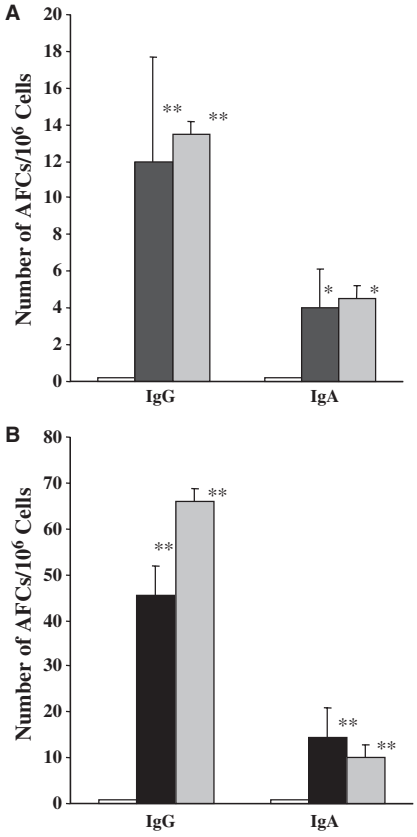

Fig. 3. 40k-OMP-specific B-cell responses in spleen and cervical lymph nodes. Groups of SD rats were transcutaneously immunized with 200 μg 40k-OMP alone (black bars) or 200 μg 40k-OMP plus 50 μg CT (grey bars) on days 0, 7 and 14, or not immunized (open bars). Mononuclear cells were isolated from spleen (A) and cervical lymph nodes (B) of rats on day 21 and examined for 40k-OMP-specific IgG and IgA AFCs. $**P < 0.01$; $*P < 0.05$; significant differences from non-immunized group.

Transcutaneously induced 40k-OMP-specific IgG suppresses coaggregation of *P. gingivalis* with *S. gordonii*

We next examined whether antibodies induced by transcutaneously administered 40k-OMP were capable of suppressing coaggregation by *P. gingivalis*. The *P. gingivalis* cells were pretreated with serum IgG from rats exposed to 40k-OMP alone, 40k-OMP plus CT or PBS as control and then incubated with *S. gordonii*. The flocculation slide assay showed that serum IgG antibodies from rats administered 40k-OMP alone, inhibited coaggregation of *P. gingivalis* with *S. gordonii* cells (Fig. 4). The serum IgG antibody from rats transcutaneously immunized with 40k-OMP plus CT also similarly inhibited coaggregation. As expected, serum IgG from rats given PBS failed to suppress the coaggregation of *P. gingivalis* with *S. gordonii* (Fig. 4).

Transcutaneously induced 40k-OMP-specific IgG inhibits hemagglutinin activity of *P. gingivalis* vesicles

We also examined whether antibodies induced by transcutaneously administered 40k-OMP were capable of suppressing hemagglutination by *P. gingivalis* vesicles. As shown in Fig. 5, the serum IgG from rats given 40k-OMP alone or 40k-OMP plus CT significantly inhibited the hemagglutinating activity of *P. gingivalis* vesicles in a dose-dependent manner. However, serum IgG from rats administered PBS failed to suppress the hemagglutinating activity of vesicles from *P. gingivalis* (data not shown).

Protection against *P. gingivalis*-induced abscess

To evaluate the protective effect of TCI with 40k-OMP, we compared the lesion sizes produced by *P. gingivalis* 381 infection in immunized and non-immunized rats. The rats were challenged subcutaneously with 2×10^{10} viable cells of *P. gingivalis* 381. The rats (eight per group) were monitored 3 days after challenge for the development of lesions (Fig. 6). Although all the rats developed lesions when challenged with *P. gingivalis* 381, the rats that had received a 40k-OMP by TCI were found to have significantly smaller lesions than those in the non-immunized control groups ($P < 0.01$).

Discussion

Previous studies have shown that mAbs to 40k-OMP inhibited coaggregation by *P. gingivalis*, as well as exhibiting complement-mediated bactericidal and opsonic activities for the phagocytosis of *P. gingivalis* (1, 18, 34). The development of a transcutaneous 40k-OMP vaccine for human use may be a significant milestone in the quest for an effective vaccine against *P. gingivalis* infection. In this study, we found that the 40k-OMP alone induced significant 40k-OMP-specific IgG responses in both serum and saliva samples, and 40k-OMP-specific IgG responses were maintained up to day 140. Furthermore, the 40k-OMP-specific IgG responses induced by the transcutaneous vaccine significantly diminished coaggregation and hemagglutination by *P. gingivalis*. In addition, the rats immunized with the transcutaneous vaccine were resistant to

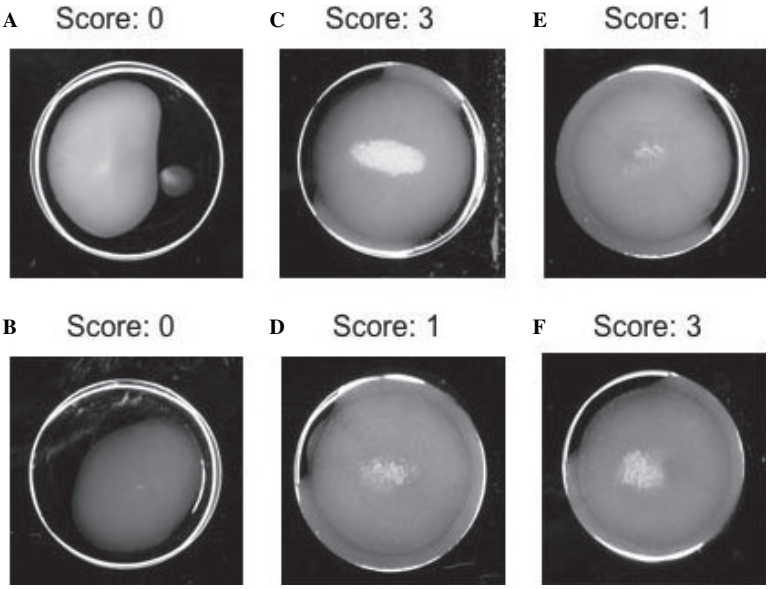

Fig. 4. Inhibition of coaggregation of *Porphyromonas gingivalis* with *Streptococcus gordonii* by 40k-OMP-specific IgG antibodies. *P. gingivalis* cells were preincubated with 40k-OMP-specific IgG and then mixed with *S. gordonii* on the flocculation slide. The results of the flocculation slide assay with (A) *S. gordonii* cells alone, (B) *P. gingivalis* cells alone, (C) a mixture of *S. gordonii* cells and *P. gingivalis* cells, (D) a mixture of *S. gordonii* cells and *P. gingivalis* cells with 200 μg/ml IgG derived from the serum of rats administered 40k-OMP alone, (E) a mixture of *S. gordonii* and *P. gingivalis* cells with 200 μg/ml IgG derived from the serum of rats administered 40k-OMP plus CT, (F) a mixture of *S. gordonii* cells and *P. gingivalis* cells with 200 μg/ml of IgG derived from the serum of mice administered PBS. IgG antibodies used in this experiment were purified from pooled serum of three rats per group.

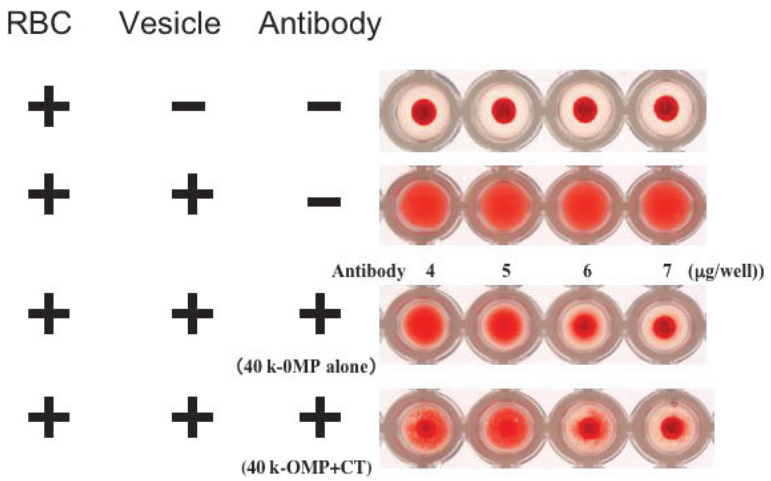

Fig. 5. Inhibition of hemagglutination of *Porphyromonas gingivalis* vesicles by 40k-OMP-specific IgG antibodies. The hemagglutinating activity of *P. gingivalis* was assayed with washed erythrocytes in round-bottomed microtiter plates. Fifty microliters of vesicle suspension (0.625 μg) was preincubated with several concentrations of 40k-OMP-specific IgG and then incubated with 100 μl 2% human erythrocytes.

P. gingivalis 381 challenge. These results suggest that TCI with 40k-OMP may be an effective antigen delivery system for the induction of protective immune responses against *P. gingivalis* infection.

It is important to note that TCI with 40k-OMP alone elicited significant serum responses as well as salivary IgG responses. Normally, protein antigen given via the mucosal route without adjuvant has been generally reported to be only a weak immunogen (28, 45–47) and therefore, has been presumed to require a mucosal adjuvant such as CT to induce antigen-specific antibody responses (28, 31, 45, 47). Our previous findings also indicated that nasal

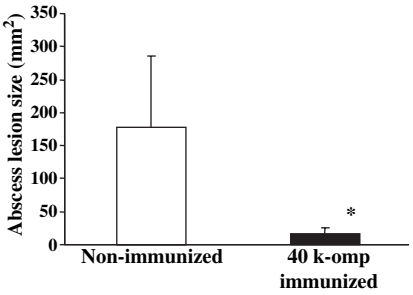

Fig. 6. Protection of *Porphyromonas gingivalis*-induced abscess by TCI with 40k-OMP. Lesion sizes are expressed as mean ± SE in square millimeters obtained from eight rats per group. $*P < 0.01$; significant differences from non-immunized group.

delivery of 40k-OMP with CT as an adjuvant induced 40k-OMP-specific Th2-type responses that in turn led to the induction of serum IgG and IgA as well as salivary IgA antibodies (30, 31). Despite its efficacy, however, CT is unsuitable for use in humans because it causes severe diarrhea (37). Furthermore, when given nasally, CT accumulates in the olfactory nerves/epithelium regions via GM1 cell surface receptors (42). When used as a mucosal adjuvant, it can even redirect co-administered protein antigens into these neuronal tissues (42). These findings raise some concerns about nasal administration and the potential threat posed by GM1-binding molecules targeting neural tissues including the central nervous system. Since TCI represents a novel and completely independent antigen delivery system, it may significantly reduce the negative effects produced by nasal or oral administration of CT.

Though some studies suggest that CT could be used as an adjuvant for transcutaneous vaccines, it would be more prudent, given its toxicity, not to use it in humans. Even without CT, TCI with the 40k-OMP induced significant 40k-OMP-specific serum and salivary IgG responses. Furthermore, serum IgG antibodies induced by 40k-OMP alone greatly diminished coaggregation and hemagglutination by *P. gingivalis*. These interesting results could be explained by the route of immunization. After its application, the vaccine antigen diffuses through the epidermal layers to the basal layer, where Langerhans cells reside. Langerhans cells are highly efficient at taking up antigens locally, followed by migration to regional lymph nodes and activation of naïve T cells. Thus, TCI may not require adjuvant for the induction of antigen-specific antibody

responses. However, several studies have shown that when protein antigens were administered transcutaneously without adjuvant, only low or undetectable levels of antibody responses were induced (2, 3, 12, 14, 15, 17), indicating that strong adjuvants, such as CT, are required for TCI.

However, the 40k-OMP, which is a key factor for *P. gingivalis* coaggregation (18, 19, 34), may also possess especially strong immunogenicity, eliminating the need for an adjuvant. Indeed, our previous study has shown that this outer membrane protein is one of the hemin-binding proteins *of P. gingivalis* (35). In another previous study, however, we found that CT is required as an adjuvant for effective antibody responses when 40k-OMP is nasally administered (31). Therefore, the combination of TCI with 40k-OMP seems to be a particularly effective vaccine regimen. This finding is consistent with observations demonstrating adjuvant activity of the recombinant protective antigen (rPA) of *Bacillus anthracis* for TCI (29). Such a regimen, which might not require a toxic adjuvant such as CT for induction of protective immune responses, would be a most attractive candidate for a human vaccine. This interesting possibility is currently under investigation in our laboratories.

Previous studies have demonstrated that TCI is capable of inducing mucosal IgA responses in addition to serum IgG (13, 14). However, our results indicated that only 40k-OMP-specific IgG, not IgA, responses were induced in saliva after TCI with 40k-OMP. Importantly, no mucosal IgA responses were induced by TCI with the 40k-OMP vaccine. Thus, it is likely that the salivary IgG AFCs detected by ELISPOT assay are from blood capillaries. Collectively, these results suggest that TCI with 40k-OMP elicits systemic, but not mucosal, Ab responses and that 40k-OMP-specific salivary IgG Abs are exudates from serum but not the products of local synthesis. Similar earlier results also indicated that TCI induced systemic antibody responses to CT and the co-administered antigen tetanus toxoid (TT), whereas no evidence was obtained for mucosal IgA responses following TCI (6). The recombinant protective antigen of *B. anthracis* and the heat-labile toxin of *E. coli* also only induced systemic immunity and protection by TCI (23). Therefore, induction of mucosal immunity by TCI may depend on whether the point of antigen entry is close to the mucosal sites.

Although the oral cavity is an important and characteristic compartment of the mucosal immune system, it differs from other mucosal compartments in that its local immune responses are both mucosal and systemic. The salivary glands, a part of the mucosal immune system, are known to produce salivary IgA antibodies. However, the IgG antibody-rich crevicular fluid, which continuously flows from the gingival capillaries, is part of the systemic immune system and is biologically active in the oral cavity (4). Indeed, our results suggest that salivary IgG induced by TCI with 40k-OMP is derived from systemic immunity. Furthermore, because *P. gingivalis* colonizes both subgingival and supragingival biofilms (10, 25, 39), generation of IgG responses in crevicular fluid could be a more effective and practical way to reduce *P. gingivalis* colonization.

The coaggregation activity of *P. gingivalis* is known to contribute to the formation and maturation of the biofilm (9, 16, 25, 39), which shelters secondary colonizing bacteria and protects them from being washed away by saliva flow (26). The importance of biofilm for human periodontal disease is supported by the evidence that most bacteria do not exist as discrete entities but as mixed populations (36). Taken together, these findings suggest that coaggregation may play an important role in the colonization of *P. gingivalis* and the formation of biofilms, thereby contributing to the development of periodontal disease. In this regard, *S. gordonii* is known to be one of the first bacteria to colonize newly cleaned teeth and is assumed to promote the colonization of other bacteria (39, 43, 44). Furthermore, *P. gingivalis* adheres readily to the cell surface of *S. gordonii* (38). Therefore, it can be concluded that *S. gordonii* supports the colonization of *P. gingivalis*. Blocking the interbacterial adhesion between *S. gordonii* and *P. gingivalis* could perhaps reduce the colonization of *P. gingivalis* in the oral cavity. Our results have shown that IgG antibodies induced by TCI with 40k-OMP alone as well as with 40k-OMP plus CT significantly inhibited coaggregation of *P. gingivalis* with *S. gordonii*. Other studies have demonstrated that 40k-OMP-specific mAbs significantly diminish the coaggregation of *P. gingivalis* vesicles with *Actinomyces viscosus* or *Actinomyces naeslundii* (1, 18, 34). Another potential virulence factor, hemagglutinin, may mediate bacterial attachment and penetration into host cells, as well as agglutinate and lyse erythrocytes to enhance heme uptake, an absolute requirement for growth. Although r40k-OMP itself does not show hemagglutinating activity, its polymeric form, constructed with a cross-linking reagent, significantly expresses this activity. Our results indicated that IgG antibodies induced by TCI with 40k-OMP alone as well as with 40k-OMP plus CT significantly inhibited the hemagglutinating activity of *P. gingivalis* vesicles in a dose-dependent manner. Moreover, a recent study has shown that the 40k-OMP binds hemin, which is a known factor for the growth and virulence of *P. gingivalis* (35). Taken together with these studies, our current results suggest that a transcutaneous 40k-OMP vaccine may reduce *P. gingivalis* infection and thereby inhibit the development of chronic periodontitis.

In summary, our current study provides evidence that 40k-OMP-specific IgG antibodies generated by TCI with 40k-OMP alone inhibit the coaggregation of *P. gingivalis* with *S. gordonii* and the hemagglutinating activity of *P. gingivalis*. In addition, the present study demonstrates that TCI of rats with 40k-OMP protects against challenge with *P. gingivalis* 381 in the abscess lesion model. These findings suggest that TCI with 40k-OMP effectively elicits protective levels of antibodies to *P. gingivalis* and therefore may act as an effective and safe vaccine delivery system for the attenuation of *P. gingivalis* infection.

Acknowledgments

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