Nasal Vaccination with an Outer Membrane Protein of *Porphyromonas gingivalis* and Nontoxic Chimeric Enterotoxin Adjuvant Induces Both T Helper 1 and T Helper 2 Responses

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Abstract

Our previous study has demonstrated that the 40-kDa outer membrane protein of *Porphyromonas gingivalis* (40k-OMP) nasally administered with a nontoxic chimeric adjuvant that combines the A subunit of mutant cholera toxin E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB) elicited a long-term protective immune response. Although using adjuvant mCTA/LTB gave comparable Ab responses in the saliva as using native cholera toxin as adjuvant, the total IgE and 40k-OMP-specific IgE antibodies, as well as IL-4 levels induced by mCTA/LTB were lower than those induced by native cholera toxin. In this study, we further elucidated the nature of 40k-OMP-specific CD4⁺ T helper cells in immunized mice. When CD4⁺ T cells isolated from cervical lymph nodes and spleens of mice immunized with 40k-OMP plus mCTA/LTB as adjuvant were restimulated with 40k-OMP *in vitro*, significant levels of proliferation occurred in cervical lymph nodes and spleens. Furthermore, mCTA/LTB as adjuvant induced 40k-OMP-specific CD4⁺ T cells secreting INF- γ and IL-4-independent IL-5, IL-6, and IL-10, with IgG2a antibody responses. Analysis of IL-12 receptor expression showed that native cholera toxin, but not the mCTA/LTB, suppressed IL-12 receptor expression by activated T cells. These results suggest that mCTA/LTB as an adjuvant regulates IL-12 receptor expression and subsequent T helper cytokine responses in oral mucosal compartments and that nasal administration of 40k-OMP plus mCTA/LTB is an effective mucosal vaccine against oral infection by *P. gingivalis*.

Keywords :

Porphyromonas gingivalis, outer membrane protein, nasal vaccine, T helper cells

Introduction

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, has been shown to be one of the major pathogens of chronic periodontitis. Colonization of this bacterium in gingival tissues is considered to be the first step in the pathogenic process of periodontal disease resulting in tissue destruction (1, 2). Fimbriae and potential adhesive molecules such as hemagglutinins, aggregation factors, and lipopolysaccharides responsible for colonization have been identified as virulence factors (1, 2). An

outer membrane protein with a molecular mass of 40-kDa produced by *P. gingivalis* (40k-OMP) is a key virulence factor for the coaggregation activity of *P. gingivalis* (3). Furthermore, this outer membrane protein has been shown to be one of the hemin-binding proteins of *P. gingivalis* (4). The 40k-OMP resides both on the cell surface and in extracellular vesicles and is found on many stains of *P. gingivalis* (3, 5-8).

Previous studies have demonstrated that nasal administration of 40k-OMP plus cholera toxin (CT)

elicited 40k-OMP-specific secretory IgA antibodies in the saliva as well as serum IgG antibody responses that inhibit the coaggregation activity of *P. gingivalis* (9-11). However, although the combination of 40k-OMP with the adjuvant CT was shown to be effective, CT causes severe diarrhea and thus is unsuitable for use in humans (12). To solve this problem, we have constructed a novel nontoxic chimeric mucosal adjuvant that combines the nontoxic A subunit of mutant CT E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB) (13). Our previous study has demonstrated that immunization with 40k-OMP and mCTA/LTB induced long-term protective antibody responses against oral infection by *P. gingivalis*. While mCTA/LTB as an adjuvant induced antibody responses that were comparable to the native form of CT (nCT), 40k-OMP-specific IgE antibodies and IL-4 responses were significantly lower than those induced by nCT (14).

In this study, we further elucidated the nature of 40k-OMP-specific CD4⁺ T helper (Th) cell responses in the mucosal compartment. The results indicated that nasal immunization with 40k-OMP plus mCTA/LTB induced 40k-OMP-specific CD4⁺ T cells producing Th1 and IL-4-independent Th2 cytokine responses for induction of Ab responses in the oral cavity.

Materials and Methods

Mice

Female BALB/c mice were purchased from San-kyo Laboratories (Tokyo, Japan) and were maintained in the experimental facility under pathogen-free conditions in the Nihon University School of Dentistry at Matsudo. Mice received sterile food and water, and they were 8 to 12 weeks old when used for these experiments. All animals were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals (Nihon University School of Dentistry at Matsudo).

Antigen and adjuvants

Plasmid pMD125 expressing 40k-OMP was kindly

provided by Dr. Yoshimitsu Abiko (Nihon University). The 40k-OMP was purified to homogeneity from a cell suspension prepared by *Escherichia coli* K-12 harboring pMD125, as described previously (15). The purity of the 40k-OMP was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and no contaminating protein bands were noted. Furthermore, possible residual endotoxin was assessed in the preparation with a Limulus amoebocyte lysate pyrochrome kit (Associates of Cape Cod Inc., Woods Hole, MA). The 40k-OMP contained as little as 0.4 pg of endotoxin. The mCTA/LTB chimera protein was kindly provided by Dr. Hiroshi Kiyono (University of Tokyo). Native cholera toxin was obtained from List Biologic Laboratories (Campbell, CA).

Immunization and sample collection

Mice were immunized nasally on days 0, 7, and 14 with a 20- μ l aliquot (10 μ l per nostril) of phosphate-buffered saline (PBS) containing 10 μ g of 40k-OMP or combined with 10 μ g of mCTA/LTB. Single cell suspensions were obtained from cervical lymph nodes and spleens 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 (17). 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 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 into the culture 18 h before harvesting, and incorporated radioactivity

was measured by scintillation counting.

Analysis of cytokine profiles

Cytokine levels in culture supernatants were determined by cytokine-specific enzyme-linked immunosorbent assay (ELISA) as described previously (18). Briefly, 96-well plates (NUNC, Inc., Naperville, IL) were coated with monoclonal anti-IFN- γ , anti-IL-5, anti-IL-6, anti-IL-10 antibodies (BD Biosciences, Mountain View, CA). After blocking with PBS containing 1% bovine serum albumin, samples were added to duplicate wells and incubated overnight at 4 °C. The wells were washed and incubated with biotinylated monoclonal anti-IFN- γ , anti-IL-5, anti-IL-6, and anti-IL-10 antibodies (BD Biosciences). After incubation, horseradish peroxidase-labeled anti-biotin antibody (Vector Laboratories Burlingame, CA) was added, developed with 3, 3', 5, 5'-tetramethyl-benzidine (Moss, Inc., Pasadena, CA), and stopped with 0.5 N HCl. Standard curves were generated using mouse recombinant (r) IFN- γ , rIL-5, rIL-6, and rIL-10.

Detection of antigen-specific IgG subclass responses

The antibody titers in serum, saliva, and nasal washes were determined by an ELISA as previously described (17, 19). Briefly, plates were coated with 40k-OMP (5 μ g/ml) and blocked with 1% bovine serum albumin and analyses were performed in duplicate. After blocking, serial dilutions of serum samples were added in duplicate. Starting dilutions of serum samples were 1: 2⁵. Following incubation, the plates were washed and peroxidase-labeled goat anti-mouse γ 1, γ 2a, and γ 2b heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to appropriate wells. Finally, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) with H₂O₂ (Moss, Inc.) was added for color development. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density at 414 nm of 0.1 greater than background after 15 min of incubation.

Flow cytometric analysis of IL-12 receptor (IL-12R) β 1 expression

T cell IL-12R β 1 expression was determined by flow cytometric analysis as previously described (20). Splenic T cells were isolated from naive mice and seeded into culture plates at a density of 5 $\times$ 10⁶ cells/ml. To investigate the effects of nCT and mCTA/LTB on IL-12R β 1 expression, T cells were incubated for 1 h with 100 ng/ml of nCT or mCTA/LTB. Anti-mouse CD3 ϵ chain (1 μ g/ml; clone 145-2C11; BD Biosciences) was then added. After 3 days of incubation, the T cells were incubated for 30 min at 4 °C with PE-labeled anti-mouse IL-12R β 1 (clone 114) and FITC-labeled anti-mouse CD3 (clone 145-2C11) or anti-CD4 (clone GK1.5). Cells were then washed and fixed in 1% paraformaldehyde in PBS and analyzed by flow cytometry using the FACS Calibur system equipped with CellQuest software (BD Biosciences, Mountain View, CA).

Statistics

The data are expressed as the mean $\pm$ S.E. and were compared using the unpaired Student's *t* test.

Results

40k-OMP-specific cell proliferative responses

In the initial study, we determined whether lymphocytes in mucosal tissues as well as systemic tissues became antigen-specific cells following nasal immunization with 40k-OMP. Thus, CD4⁺ T cells isolated from the spleen or CLN of immunized mice were restimulated *in vitro* with 40k-OMP in the presence of splenic feeder cells. The results indicated that CD4⁺ T cells from the spleen and CLN of mice nasally immunized with 40k-OMP plus mCTA/LTB showed significant levels of proliferation that were comparable to those induced by 40k-OMP plus nCT. In contrast, essentially no increased cell proliferation occurred in spleen and CLN taken from mice immunized nasally with 40k-OMP alone (Fig. 1). These results showed that nasal immunization with 40k-OMP plus mCTA/LTB could elicit 40k-OMP-specific CD4⁺ T cells in both mucosal and systemic compartments.

Fig. 1. 40k-OMP-specific cell proliferative responses in mice immunized nasally with 40k-OMP plus mCTA/LTB. Group of BALB/c mice were nasally immunized with 40k-OMP alone, 40k-OMP plus mCTA/LTB, or 40k-OMP plus nCT on days 0, 7, and 14. CD4⁺ T cells were isolated from CLN and spleens 7 days after the last immunization and cultured with 2 μ g of 40k-OMP in the presence of splenic feeder cells for 4 days. To measure of 40k-OMP-specific cell proliferation, 1.0 μ Ci of [³H]thymidine was added into culture 18 h before harvesting and incorporated radioactivity was measured by scintillation counting. The results are expressed as the mean $\pm$ S.E. obtained for five mice per group. * p < 0.05 compare with results for mice given 40k-OMP alone.

Analysis of 40k-OMP-specific Th cytokine profiles

Because our results clearly indicated that CD4⁺ T cells in both mucosal and systemic lymphoid tissues were primed after nasal immunization with 40k-OMP plus mCTA/LTB, we next determined the nature of 40k-OMP-specific Th cytokine responses. Thus, CD4⁺ T cells from CLN of mice immunized with 40K-OMP plus mCTA/LTB or 40k-OMP plus nCT were restimulated with 40k-OMP *in vitro* and culture supernatants were examined for Th1- and Th2-type cytokine production. Analysis of Th1-type cytokine responses revealed that a significant level of IFN- γ production was detected in the supernatant harvested from wells containing CD4⁺ T cells from CLN of mice immunized with 40k-OMP plus mCTA/LTB that was higher than that found in mice immunized with 40k-OMP plus nCT (Fig. 2).

Furthermore, significant IL-5 and IL-10 productions were seen in culture supernatants obtained from 40k-OMP-activated CD4⁺ T cells from mice immunized with 40k-OMP plus mCTA/LTB and these levels were identical to those of mice immunized with 40k-OMP plus nCT (Fig. 2). Moreover, a higher IL-6 response was detected in CLN CD4⁺ T cells from mice nasally immunized with 40k-OMP plus mCTA/LTB compared with that from mice

Fig. 2. The production of IFN- γ , IL-5, IL-6, and IL-10 by 40k-OMP-specific cells from mice nasally immunized with 40k-OMP plus mCTA/LTB. Group of BALB/c mice were immunized with 40k-OMP alone, 40k-OMP plus mCTA/LTB or 40k-OMP plus nCT as described in the legend of Fig. 1. CD4⁺ T cells were isolated from CLN 7 days after the last immunization and were cultured with 40k-OMP in the presence of splenic feeder cells for 4 days. After restimulation with 40k-OMP, culture supernatants were subjected to IFN- γ , IL-5, IL-6, and IL-10-specific ELISA. The results are expressed as the mean $\pm$ S.E. obtained for five mice per group. * p < 0.05 compare with results for mice given 40k-OMP alone.

immunized with 40k-OMP plus nCT (Fig. 2). As expected, CD4⁺ T cells isolated from CLN of mice given 40k-OMP alone produced only low levels of Th1 and Th2 cytokines (Fig. 2). These results indicated that nasal immunization with 40k-OMP plus mCTA/LTB as a mucosal adjuvant elicited both Th1- and Th2-type cytokine responses in maxillofacial mucosal compartments.

Detection of antigen-specific IgG subclass responses

When serum IgG subclasses to 40k-OMP were assessed, nasal immunization with 40k-OMP plus mCTA/LTB induced high IgG1 levels, with IgG2a and IgG2b antibody responses. On the other hand, mice that received 40k-OMP plus nCT showed high levels of IgG1, followed by IgG2b, and only low levels of IgG2a anti-40k-OMP antibodies. As expected, when 40k-OMP was given alone via the nasal route,

Fig. 3. 40k-OMP-specific IgG subclass responses. Group of BALB/c mice were immunized with 40k-OMP alone, 40k-OMP plus mCTA/LTB or 40k-OMP plus nCT as described in the legend of Fig. 1. Serum samples were collected 7 days after the last immunization and were assessed for 40k-OMP-specific IgG subclass antibodies. The results are expressed as the mean $\pm$ S.E. obtained for five mice per group. * $p < 0.05$ compare with results for mice given 40k-OMP alone.

Fig. 4. Effect of mCTA/LTB or nCT on IL-12R β 1 expression by anti-CD3 monoclonal antibody-stimulated T cells. Naive splenic T cells were treated for 1 h with nCT or mCTA/LTB and then were cultured for 3 days in the presence of anti-CD3 monoclonal antibody. Flow cytometry was performed after staining with PE-labeled anti-mouse IL-12R β 1 and FITC-labeled anti-mouse CD3 or anti-CD4 monoclonal antibodies. Data are representative of four separate experiments.

low levels of antigen-specific serum IgG responses were detected (Fig. 3). These results suggest that antigen-specific cytokine responses were induced by mCTA/LTB as an adjuvant.

Flow cytometric analysis of IL-12R β 1 expression

We next addressed the potential mechanism involved in the induction of biased Th cell cytokine responses by analyzing the effects of mCTA/LTB on IL-12R β 1 expression on activated T cells. The addition of anti-CD3 monoclonal antibody to splenic T

cell cultures enhanced IL-12R β 1 expression (Fig. 4). Interestingly, pretreatment of splenic T cells with nCT, but not mCTA/LTB, abolished the anti-CD3 monoclonal antibody-induced IL-12R β 1 expression (Fig. 4).

Discussion

In this study, we determined the Th cytokine profile of 40k-OMP-specific CD4⁺ T cells when mice were nasally immunized with 40k-OMP plus mCTA/LTB as adjuvant. 40k-OMP is highly immunogenic surface protein of *P. gingivalis* that has been receiving attention as a periodontal disease vaccine. Our previous study has demonstrated that the nasal administration of 40k-OMP with mCTA/LTB induces 40k-OMP-specific IgG and IgA in sera, as well as salivary IgA antibodies, that persist for more than 1 year. Furthermore, those mucosally induced IgG antibodies inhibited the coaggregation and hemagglutinin activities of *P. gingivalis*. Importantly, mice given the 40k-OMP plus mCTA/LTB were significantly protected against alveolar bone loss caused by oral infection with *P. gingivalis* (14). These findings suggest that 40k-OMP is an effective antigen for a periodontal disease vaccine. In this study, we thus used 40k-OMP plus mCTA/LTB as a model system to study the nature of antigen-specific immune responses by nasal immunization. Our results indicated that CD4⁺ T cells from CLN and spleens of mice nasally immunized with 40k-OMP plus mCTA/LTB showed significant proliferative responses when restimulated with 40k-OMP *in vitro*. Furthermore, those Th cells produced significant levels of 40k-OMP-specific IFN- γ with IL-5, IL-6, and IL-10. Mechanisms in the development of Th1- and Th2-type cytokine responses were further supported by the finding that nCT inhibited IL-12R β 1 expression, while this effect was not seen after treatment with mCTA/LTB.

In the present study, we have used a chimera molecule consisting of the nontoxic A subunit of mutant CT E112K and the B subunit of heat-labile enterotoxin from *Escherichia coli* as a new generation of safe and effective mucosal adjuvants. It is

well known that CT and heat-labile enterotoxin are effective adjuvants and that they are capable of enhancing both mucosal IgA and systemic IgG antibody responses to co-administered protein antigen; however, both enterotoxins cause severe diarrhea and are thus unsuitable for use in humans (12). Therefore, several groups including ours have developed nontoxic derivatives of cholera toxin or heat-labile enterotoxin that may be suitable for use in humans (13, 21-29). The newly created mCTA/LTB chimera molecule did not induce any increases in intracellular cAMP and failed to elicit fluid accumulation in ligated ileal loops (13). Furthermore, our previous study demonstrated that nasal administration of 40k-OMP with mCTA/LTB as an adjuvant could elicit 40k-OMP-specific serum IgG and IgA as well as salivary IgA antibodies with minimal induction of IgE antibody responses. These mucosally induced antibody responses provided significant protection against oral infection with *P. gingivalis* even 1 year after immunization (14). These studies indicate that this novel chimeric mCTA/LTB molecule is a potent nontoxic mucosal adjuvant for the induction of protective immunity in the oral cavity.

As a mucosal adjuvant, CT given via mucosal routes induces Th2 cells to secrete high levels of IL-4 (30-32); however, the IL-4 cytokine provides a helper signal for the induction of IgE antibodies that may cause anaphylactic reactions (19, 33, 34). Therefore, an important criterion for the development of mucosal adjuvant should be to reduce or diminish the potential for IgE antibody production without losing the ability to generate antigen-specific mucosal IgA and serum IgG antibody responses. In this regard, our previous study has demonstrated that although using the adjuvant mCTA/LTB gave antibody responses comparable to those obtained using nCT as a mucosal adjuvant, the levels of total IgE and 40k-OMP-specific IgE antibodies as well as IL-4 levels induced by the immunization with mCTA/LTB were lower than those induced by the immunization with nCT (14). The present study also showed that nasal administration of 40k-OMP plus mCTA/LTB induced high IFN- γ with IL-4-independent Th2-type

cytokines, e.g., IL-5, IL-6, and IL-10. Furthermore, while nCT inhibited IL-12R β 1 expression on activated T cells, mCTA/LTB did not affect the expression of this cytokine receptor. In this regard, it has been shown that the B subunits of enterotoxin adjuvants regulate IL-12R with subsequent Th cell subset responses. To this end, predominant Th2-type responses are induced by the CT-B subunit, whereas the heat-labile enterotoxin B subunit induces Th1-type with low IL-4 Th2-type responses (20).

Our results showed that the mucosal adjuvant activity of mCTA/LTB resulted in plasma antibody responses characterized by the presence of antigen-specific IgG2a with IgG1 and IgG2b antibodies. This pattern of IgG subclass response is similar to that induced by native heat-labile enterotoxin and strongly suggests a major involvement of the heat-labile enterotoxin B subunit in this response profile. Interestingly, a completely different pattern of IgG subclass responses, characterized by IgG1 and IgG2b antibody responses, was seen after nasal immunization with nCT as an adjuvant. These results support a major role for the CT-B subunit in Th2-type adjuvanticity. Taken together, these studies and our results indicate that the major changes associated with substitution of the B subunit of mutant CT with the reciprocal molecule from heat-labile enterotoxin involves Th cytokine responses and the associated pattern of IgG subclass and IgE antibody responses.

Our results demonstrated that nasal immunization with 40k-OMP plus mCTA/LTB induced high IFN- γ with IL-4-independent Th2-type cytokines, e.g., IL-5, IL-6, and IL-10. In this regard, two Th2-derived cytokines, IL-5 and IL-6, are of particular importance for inducing IgA-committed B cells to differentiate into IgA-plasma cells (35, 36). Furthermore, in IL-6 gene-disrupted mice, mucosal IgA responses were markedly reduced (37). These studies together with our results suggest that induction of 40k-OMP-specific Th2 cytokine responses in mucosa-associated lymphoid tissues (e.g., CLN) effectively induce mucosal IgA antibodies in the saliva.

In summary, we showed that nasal immunization with 40k-OMP plus mCTA/LTB elicited Th1 and IL

-4-independent Th2 cytokine responses. These cytokine responses can be elicited by mCTA/LTB chimeras and depend upon the presence of the heat-labile enterotoxin B subunit. The combination of 40k-OMP plus mCTA/LTB provides a very effective means of eliciting Th1 and Th2 cytokine responses and subsequent serum IgG, IgA and mucosal IgA antibody responses with low IgE antibodies, and therefore should be considered as a candidate vaccine against oral *P. gingivalis* infection.

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