Comparison of Mucosal Immune Response after Oral, Nasal or Sublingual Immunization with an Outer Membrane Protein of *Porphyromonas gingivalis*

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Abstract

Mucosal immunization would provide an easy and safe measure in preventing infectious diseases by facilitating mass immunization. In order to represent a promising strategy for mucosal vaccination, oral, nasal, or sublingual immunizations of mice with a recombinant 40-kDa outer membrane protein of *Porphyromonas gingivalis* (r40k-OMP) and cholera toxin (CT) as a mucosal adjuvant were performed, and mucosal and systemic immune responses were compared. Administration of the antigen via the mucosal routes, such as orally, nasally or sublingually, equally elicited significant levels of 40k-OMP-specific IgG in the serum and high numbers of 40k-OMP-specific antibody-forming cells (AFCs) in the spleen. Nasal and sublingual immunization of mice also induced high levels of mucosal IgA antibodies (Abs) in the saliva and nasal washes compared to oral immunization. Subsequently, these immunizations confirmed the Ab titers by revealing significant numbers of 40k-OMP-specific AFCs in the submandibular glands, nasopharyngeal-associated lymphoreticular tissue, and nasal passages. In contrast, mice that were orally immunized with r40k-OMP plus CT exhibited significantly increased antibody secreting cells in Peyer's patches and intestinal lamina propria, although lower level of cells were detected in nasal or sublingual immunization. These results have suggested that both nasal and sublingual immunization are more effective for prevention of periodontal disease compared to oral immunization.

Introduction

Induction of mucosal immune responses is achieved by the deposition of antigen (Ag) via the mucosa (e.g., oral route) but not the systemic route (1). Furthermore, mucosal immunization has been shown to induce Ag-specific immune responses in both mucosal and systemic compartments (1-3). Although systemic vaccination (e.g., intramuscular injection) can induce effective immune responses in the systemic compartment, it does not result in the generation of Ag-specific mucosal immune responses. Considering bacterial infection, mucosal vaccination can offer two layers of immunity (e.g., mucosal and systemic immune response), which can provide an effective barrier against invasion of pathogenic bacteria. It has been shown that delivery of soluble Ag alone is insufficient for the induction of maximum levels of Ag-specific immune responses by the mucosal vaccine. Thus, it is necessary to co-administer it with a mucosal adjuvant for inducing mucosal immune responses including those of the IgA isotype antibody (Ab) (1-3).

Cholera toxin (CT), an exotoxin produced by *Vibrio cholerae*, is the most widely known immunogen and adjuvant for studying mucosal immunity (1-3). It is a strong mucosal adjuvant for enhancing of Ag-specific mucosal IgA and systemic IgG responses to mucosally coadministered protein Ag (4, 5). Previous studies have shown that CT elicits adjuvant responses by inducing Ag-specific Th2-type $CD4^+$T cells producing IL-4, IL-5, and IL-6, which are responsible for supporting Ag-specific IgA and IgG Ab productions (4, 5). It has also been shown that CT can modulate a co-stimulatory molecule such as B-7 (6). For the development of an effective mucosal

vaccine, it is essential to consider the use of the adjuvant properties of CT.

Periodontal disease is an inflammatory disease that affects the soft and hard structures that support the teeth. Recent studies have suggested that periodontal disease influences systemic conditions such as cardiovascular disease, diabetes, preterm labor and osteoporosis (7). Therefore, the prevention of periodontitis by oral care and vaccine development is important for both oral and systemic health.

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, was previously shown to be one of the major pathogens in chronic periodontitis (8). The bacterial surface components may provide suitable targets for the Ag vaccine. For example, the 40-kDa outer membrane protein of *P. gingivalis* (40k-OMP) is a key virulence factor for coaggregation (9) and hemagglutination (10), and is conserved among many strains (11). Previous studies have shown that IgG Abs induced by the nasal administration of recombinant (r) 40k-OMP with an adjuvant inhibited co-aggregation by *P. gingivalis* (12). Furthermore, either oral, nasal or sublingual immunization with r40k-OMP prevented alveolar bone loss in response to a *P. gingivalis* challenge (13, 14, 15). Therefore, the use of r40k-OMP as a mucosal Ag vaccine may be beneficial in the development of a periodontal vaccine and also for the preventive therapy of systemic diseases. However, we cannot rule out which vaccine administration route is more suitable for prevention of periodontal disease. Therefore, in this study, we evaluated the efficacy of mucosal administration of 40k-OMP produced by *P. gingivalis*.

Materials and Methods

Mice

BALB/c mice were purchased from Sankyo Lab Services (Tokyo, Japan). These mice were maintained under pathogen-free conditions at the experimental facility of the Nihon University School of Dentistry at Matsudo experimental facility. All mice were randomly assigned to control or experimental groups (n=5 per group), and were provided *ad libitum* access to sterile food and water. They were used at 8-12 weeks of age in accordance with the Guidelines for the Care and Use of Laboratory Animals, Nihon University School of Dentistry at Matsudo (AP11MD016).

Antigen and adjuvant

The recombinant pasmid containing the 40k-OMP gene (pMD125) was kindly provided by Dr.Yoshimitsu Abiko (Nihon University). The r40k-OMP was purified to homogeneity from a cell suspension of *Escherichia coli* K-12 harboring pMD 125, as described previously (16). The purity of the r40k-OMP was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and no contaminating protein bands were noted. Furthermore, an LAL Pyrochrome kit (Associates of Cape Cod, Inc., Woods Hole, MA) was used to determine the level of residual endotoxin. A 1-mg portion of the r40k-OMP preparation contained <0.4 pg of endotoxin. CT was obtained from List Biologic Laboratories (Campbell, CA).

Mucosal Immunization and sample collection

Immunization methods were described as follows. For oral immunization, mice were deprived of food for 2 h and then given a solution of sodium bicarbonate to neutralize stomach acidity before oral immunization (17). Thirty minutes later, these mice were orally immunized by gastric intubation with 250 μl of phosphate-buffered saline (PBS) containing 200 μg of r40k-OMP in presence of 10 μg of CT as a mucosal adjuvant (13). For sublingual immunization, mice were anesthetized with pentobarbital, and immunized with 30 μl of PBS containing 20 μg of r40k-OMP, and 2 μg of CT was delivered via a micropipette that was applied against the ventral side of the tongue while directed toward the floor of the mouth. Mice were immunized with 7.5 μl of r40k-OMP four times (total volume = 30 μl). Ten minute intervals were set between each administration (18). For nasal immunization, mice were anesthetized with pentobarbital and immunized with a 10 μl aliquot (5 μl per nostril) of PBS containing 10 μg of r40k-OMP with 1 μg of CT (14). The entire immunization procedure was conducted on days 0, 7, and 14. Serum, saliva, nasal washes and fecal extracts were collected from each group to examine the 40k-OMP-specific Ab responses.

Detection of Ag-specific Ab responses

Ab titers in serum, saliva, nasal washes and fecal extract samples were determined by an enzyme-linked immunosorbent assay (ELISA) (14). Briefly, plates were coated with the r40k-OMP (5 μg/ml) and blocked with a 1% bovine serum albumin (BSA), and analyses were performed in duplicate. After the plates were blocked, serial dilutions of serum, saliva, nasal washes and fecal extracts were $1:2^8$, $1:2^2$, $1:2^2$ and $1:2^2$, respectively.

Following incubation, plates were washed and peroxidase-labeled goat anti-mouse γ- or α-heavy-chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to the appropriate wells. Finally, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) with H_2O_2 (Moss, Inc, Pasadena, MD) was added for color development. End point titers were expressed as the $\log_2$ reciprocal of the last dilution giving an optical density at 414 nm of 0.1 greater than the back ground level after 15 min of incubation.

Measurement of antibody-forming cells

Mononuclear cells from the spleen were isolated aseptically by gentle teasing through stainless steel screens as described elsewhere (19). Nasal passages (NPs) and nasopharyngeal-associated lymphoreticular tissue (NALT) were isolated using a previously described protocol with some modification (19). Mononuclear cells from the submandibular gland (SMG) were isolated by an enzymatic dissociation procedure with collagenase (0.3 mg/ml; Nitta Gelatin Co. Ltd, Osaka, Japan) followed by a discontinuous Percoll gradient centrifugation (GE Healthcare UK, Ltd, Little Chalfont, Buckinghamshire, UK) (19). Mononuclear cells from the Peyer's patches (PP) were isolated excising the PP from the small intestine's wall. Cells were dissociated with collagenase, described above (17). Lamina propria (LP) was isolated after removal of the PP and mesenteric lymph nodes from the small intestine. Cells were prepared according to the previously described method (17). To determine the numbers of 40kDa-OMP-specific Ab-forming cells (AFCs), an enzyme-linked immunospot

(ELISPOT) assay was performed as previously described (20). Briefly, 96-well nitrocellulose plates (Millititier HA; Millipore, Bedford, MA) were coated with r40k-OMP (5 μg/ml), incubated for 20h at 4°C, and then washed extensively and blocked with complete RPMI 1640 containing 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 μU/ml penicillin, and 100 μg/ml streptomycin. The blocking solution was discarded, and lymphoid cell suspensions at various dilutions were added to the wells and then incubated for 4h at 37°C in 5% CO_2 in moist air. Then, the bound Abs were detected with horseradish peroxidase-conjugated goat anti-mouse μ-, γ- or α-heavy chain-specific Abs (Southern Biotechnology Associates). Following overnight incubation, the plates were washed with PBS and developed by addition of 3-amino-9-ethylcarbazole dissolved in a 0.1 M sodium acetate buffer containing H_2O_2 (Moss, Inc, Pasadena, MD) in each well. Plates were incubated at room temperature for 25 min and were washed with water, and AFCs were counted with the aid of a stereomicroscope (SZH-ILLLB; Olympus, Tokyo, Japan)(19).

Statistical analysis

The data are presented as means±standard error (SE). Differences between the groups were considered significant at $P<0.05$ using an unpaired Student's *t* test.

Results

Induction of 40k-OMP-specific antibody responses by mucosal immunization

In our initial experiment, mice were orally, nasally, or sublingually immunized with r40k-OMP (200, 10, or 20 μg) in the presence of mucosal adjuvant CT (10, 1, or 2 μg) for the analysis of Ag-specific Abs in serum. When the antigen-specific immune responses were analyzed, comparable levels of Ag-specific IgM, IgG, and IgA Abs were induced in any group (Fig. 1A). There were no significant differences in the level of 40k-OMP specific antibody titers among the 3 groups. Next, we investigated whether Ag-specific Ig-producing cells were effectively induced in the spleen by different mucosal immunizations. The numbers of Ag-specific IgA, IgG, and IgM AFC in the mononuclear cells isolated from the spleens of mice immunized orally, nasally, or sublingually with r40k-OMP plus CT were enumerated by the ELISPOT assay. AFC responses also showed significantly higher numbers of Ag-specific IgG-producing cells in the spleen of every mucosally vaccinated mouse (Fig. 1B).

In addition, nasal and sublingual administration of r40k-OMP plus CT induced high levels of 40k-OMP-specific IgA Ab titers in saliva and nasal washes (Fig. 2). In contrast, lower levels of 40k-OMP-specific IgA responses were seen in mice immunized with the oral vaccine. In fecal extracts, the levels of 40k-OMP-specific IgAs in nasally or sublingually immunized mice were much weaker than those detected in the orally vaccinated group. These findings suggest that nasal or sublingual vaccines containing 40k-OMP plus CT are effective immunization regimens for the induction of Ag-specific IgA Abs in saliva and nasal washes, but not intestinal secretions (Fig. 2).

Effect of route of immunization on development of antibody-forming cells

Next, we investigated whether Ag-specific Ig-producing cells were effectively induced in different mucosal compartments by mucosal immunization. The numbers of Ag-specific IgA AFCs in the mononuclear cells isolated from SMGs, NALT, NPs, PP, and intestinal LP of mice immunized orally, nasally, or sublingually with r40k-OMP plus CT were enumerated by the ELISPOT assay. Nasal immunization induced high numbers of Ab-specific IgA-producing cells in SMGs, NALT, and NPs (Fig. 3). Furthermore, the mice receiving the sublingual vaccine also had elevated numbers of 40k-OMP-specific IgA AFCs in SMGs and NPs. In contrast, oral vaccination induced low numbers of Ag-specific IgA in SMGs, NALT, and NPs.

In the mucosal compartments, oral immunization induced high numbers of Ag-specific IgA-producing cells in PP and intestinal LP (Fig. 4). However, nasal or sublingual vaccination induced low numbers of Ag-specific IgA in PP and intestinal LP. These findings demonstrate that nasal or sublingual immunization with r40k-OMP plus CT is effective for the induction of localized Ag-specific IgA responses in nasal and oral compartments, while oral immunization with r40k-OMP plus CT results in compartmentalized Ag-specific IgA responses in intestine-associated tissues.

Discussion

In the present study, nasal or sublingual immunization with 10 or 20 μg of r40k-OMP plus 1 or 2 μg of CT resulted in the high titers of Ag-specific IgA Abs in saliva and nasal wash. In contrast, oral immunization with high doses of r40k-OMP (200 μg) and CT (10 μg) resulted in the induction of Ag-specific IgA Abs predominantly in intestinal secretions. These findings suggest that a compartmentalized mucosal immune system is involved for such a distinctively separated dominant IgA response induced by nasal, sublingual and oral immunization. Thus, nasal or sublingual immunization is most effective for the induction of Ag-specific Ab response in the mucosal compartment of the upper part of the body including the nasal passage and oral cavity, while oral immunization is more effective in inducing intestinal immune responses. To this end, our and other studies have shown that sublingual immunization was comparable to intranasal immunization and was superior to oral immunization for the induction of Ag-specific immune responses in the upper respiratory tract (21).

For the induction of the most optimal Ag-specific immune response in the intestinal tract or nasal-oral cavity which is the initial invasion site of different pathogenic microorganisms, our present findings provide an important implication where one could select from three immunization regimens (e.g., oral, nasal or sublingual). Oral vaccine might be more suitable for various enteric infectious diseases caused by *V. cholera*, *Salmonella*, pathogenic *Escherichia coli*, *Shigella*, etc. In contrast, nasal vaccine would be beneficial for respiratory infectious diseases caused by the influenza virus, adenovirus, respiratory syncytial virus, *Streptococcus pneumoniae*, etc. To this end, it

has been shown that nasal immunization with influenza Ag and mucosal adjuvant CT induced protective immunity (22). Sublingual administration is also a convenient means to deliver drugs and low-molecular-weight molecules to the blood stream, and it avoids enterohepatic circulation and the partial first-pass effects of hepatic metabolism, as well as the immediate destruction of ingested molecules by gastric acid. Furthermore, recent studies have demonstrated that sublingual immunization with an antigenic macromolecules plus CT as an adjuvant or inactivated influenza virus induces Ag-specific immune responses in both the mucosal and systemic compartments (21, 23, 24), indicating that the sublingual route is useful for delivery of vaccines targeting infectious diseases. In addition to these respiratory infections, our present findings provided supportive evidence that nasal or sublingual vaccination is also an effective immunization regimen for the induction of Ag-specific immune responses in the oral region. The oral cavity is immunologically considered to be a unique organ in which the mucosal and systemic origin of S-IgA and IgG Abs simultaneously provide two layers of protection via saliva and gingival crevicular fluids, respectively. Since high levels of 40k-OMP-specific S-IgA and systemic IgG are induced in the oral cavity by nasal or sublingual immunization, nasal or sublingual vaccine can be applied toward the development of a new immunoprophylaxis therapy in order to control infectious diseases in the oral cavity.

As a mucosal adjuvant, CT given via the oral routes induces Th2 cells that secrete high levels of IL-4 (4, 25, 26); however, the IL-4 cytokine provides a helper signal for the induction of IgE Abs that may cause anaphylactic reactions (27, 28). In this regard,

previous studies have shown that nasal or sublingual immunization resulted in the induction of both Th1- and Th2-type cytokines in mucosal effector sites such as NP, SMG or submandibular lymph nodes, for the production of Ag-specific S-IgA Abs (15, 29).

Although nasal immunization has been widely used for mucosal immunization because Ags are not subjected to the degradation usually caused by oral administration, several studies have reported that nasally administered Ags, such as CT and adenovirus vectors, diffuse through the perineural space as a result of retrograde passage through the olfactory epithelium (30, 31). A clinical study also suggested a strong association between the nasal influenza vaccine and Bell's palsy (32). These findings raise concerns with regard to nasal administration and the potential threat posed by vaccine trafficking in neural tissues, including the central nervous system. In this regard, it has been shown that after sublingual administration, a live or inactivated influenza virus does not migrate into the central nervous system (24). Thus, sublingual immunization may be a more promising vaccination route than the nasal route.

In summary, this study has provided evidence that the nasal or sublingual administration of the r40k-OMP together with CT was superior to oral immunization in inducing a 40k-OMP-specific serum IgG as well as salivaly IgA response associated with the increase in the antigen specific AFCs in SMG. These findings further emphasize the importance of the generation of Ag-specific IgA Ab, which possesses the ability to block the bacterial attachment to the epithelial cells. Since there are no significant differences between nasal and sublingual immunization in the

titer of serum IgG and salivary IgA, sublingual immunization may be a more suitable method to control *P. gingivalis* infections.

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Figure legends

Figure 1. Detection of 40k-OMP-specific serum IgM, IgG, and IgA Ab responses in serum (A) and numbers of IgM, IgG, and IgA AFCs in the spleen (B). Mice were orally, nasally or sublingually immunized with r40k-OMP together with CT on days 0, 7 and 14. Serum samples were collected 7 days after the last immunization and were assessed for 40k-OMP-specific IgM, IgG, and IgA titers. Mononuclear cells from the spleen were assessed for IgM, IgG, and IgA AFCs. Results are expressed as means±S.E. obtained from five mice per group. *, $P< 0.05$.

Figure 2. Detection of 40k-OMP-specific IgA Ab responses in fecal extracts, saliva, and nasal washes. Mice were orally, nasally or sublingually immunized with r40k-OMP together with CT on days 0, 7 and 14. Samples were collected 7 days after the last immunization. The levels of 40k-OMP-specific IgA Abs in fecal extracts, saliva, and nasal washes of orally, nasally, and sublingually vaccinated mice were analyzed by Ag-specific ELISA. Results are expressed as means±S.E. obtained from five mice per group. *, $P< 0.05$.

Figure 3. Detection of 40k-OMP-specific AFC in SMG, NALT, and NPs. Mice were orally, nasally or sublingually immunized with r40k-OMP together with CT on days 0, 7 and 14. After 7 days of the last immunization, mononuclear cells were isolated from SMG, NALT, and NPs of mice and examined for 40k-OMP-specific IgA AFCs. Results are expressed as means±S.E. obtained from five mice per group. *, $P< 0.05$.

Figure 4. Detection of 40k-OMP-specific AFC in PP and LP. Mice were orally, nasally or sublingually immunized with r40k-OMP together with CT on days 0, 7 and 14. After 7 days of the last immunization, mononuclear cells were isolated from the PP and LP of the mice and examined for 40k-OMP-specific IgA AFCs. Results are expressed as means±S.E. obtained from five mice per group. *, $P < 0.05$.

Figure 1

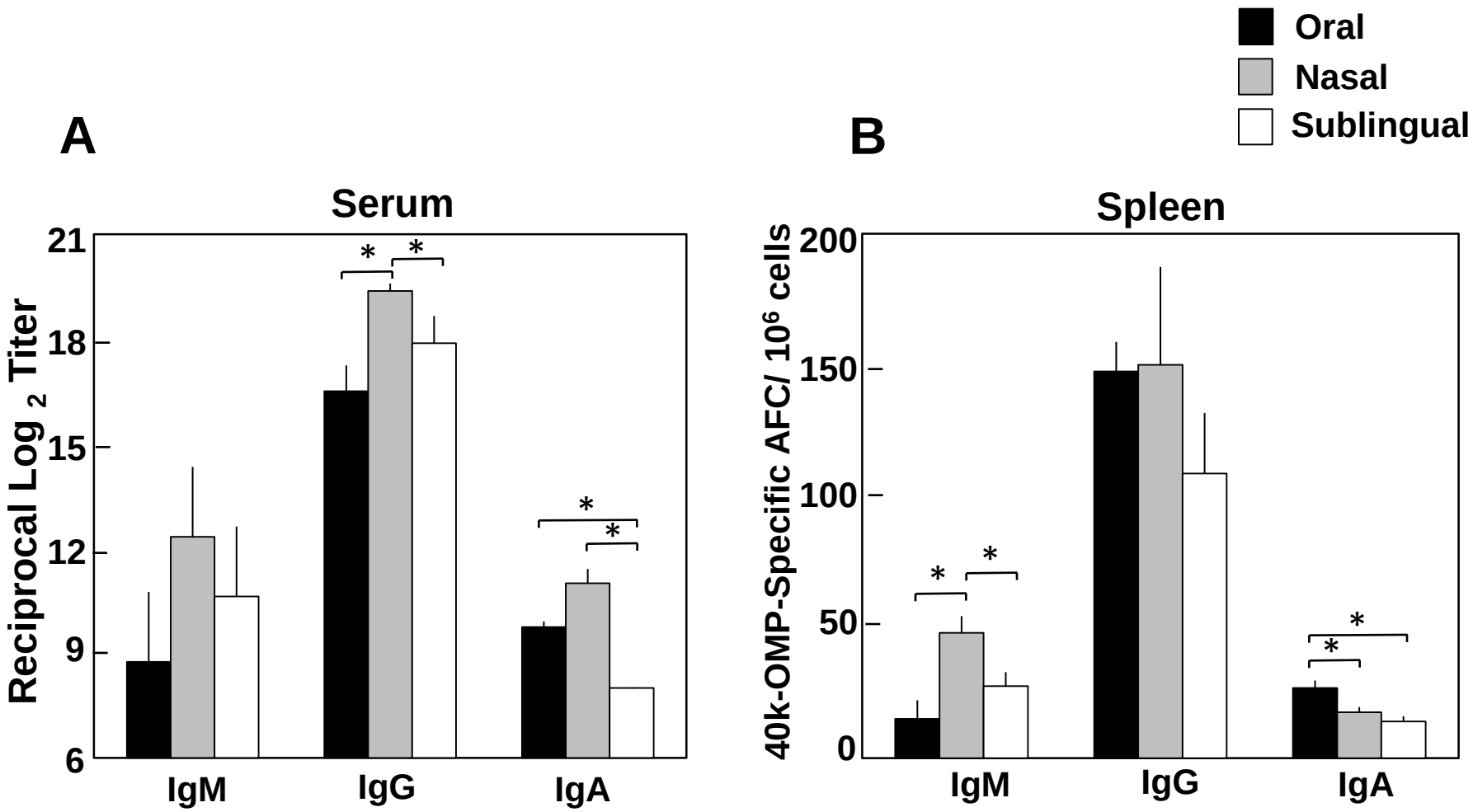
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Figure 2

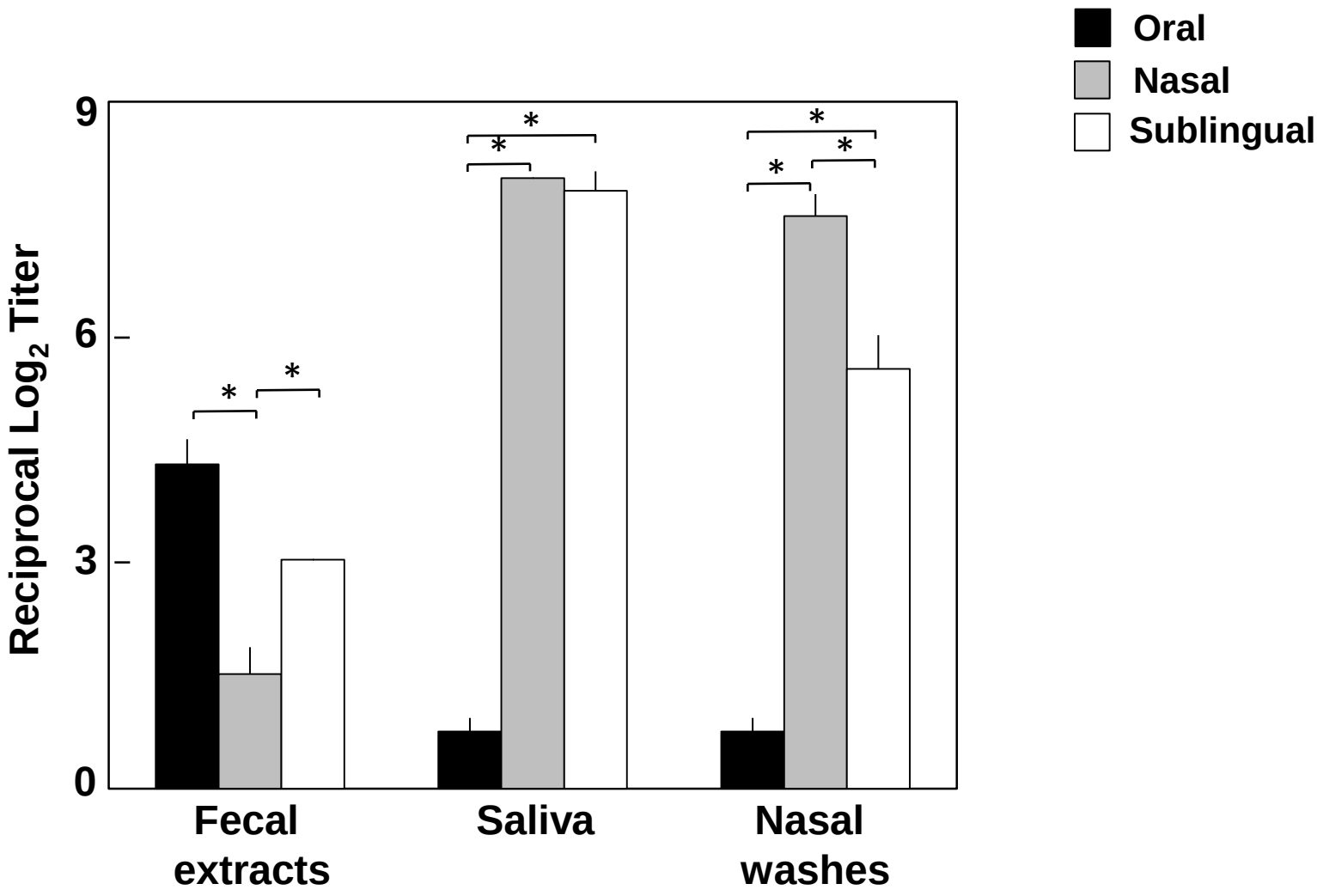
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Figure 3

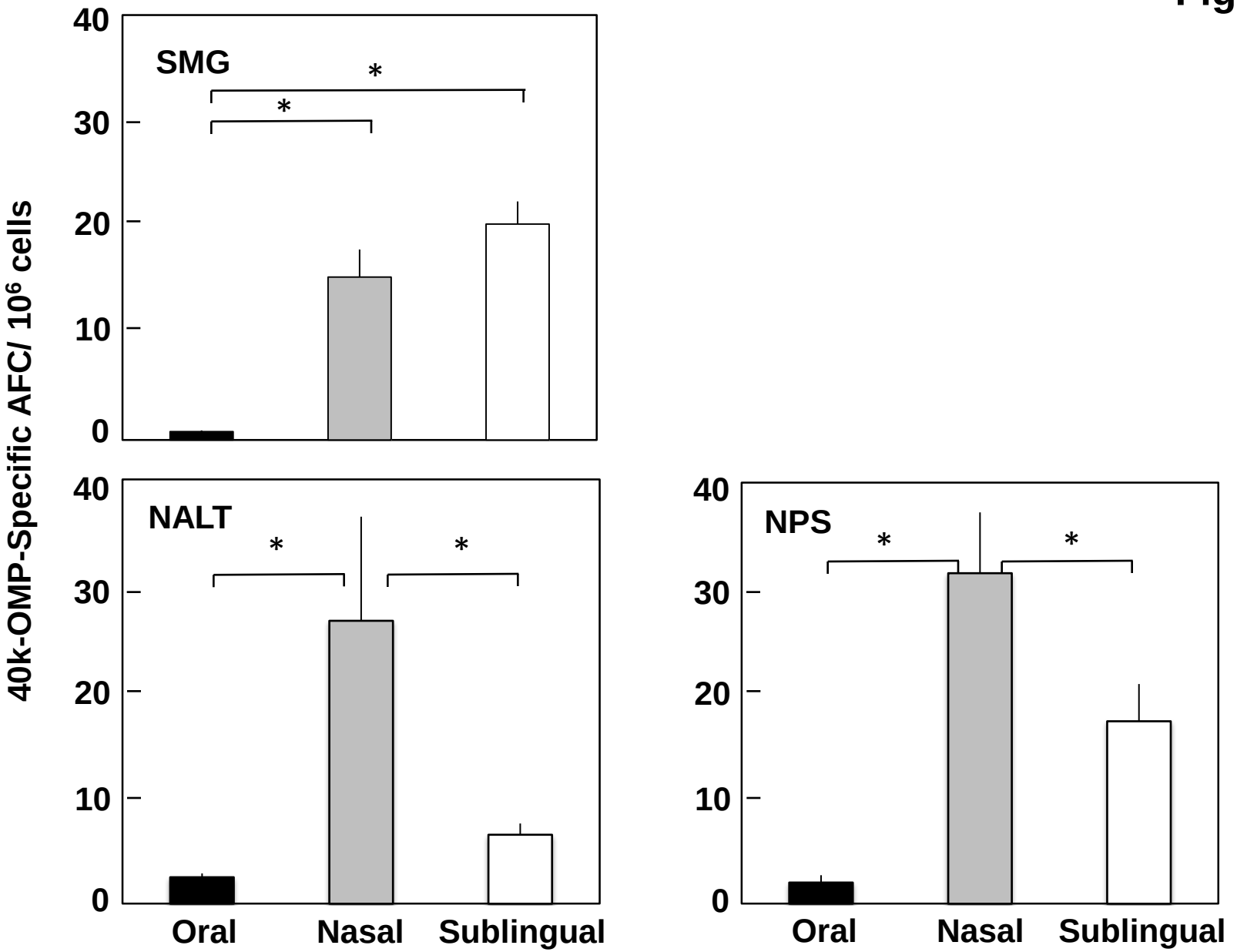
40k-OMP-Specific AFC/ 10^6 cells
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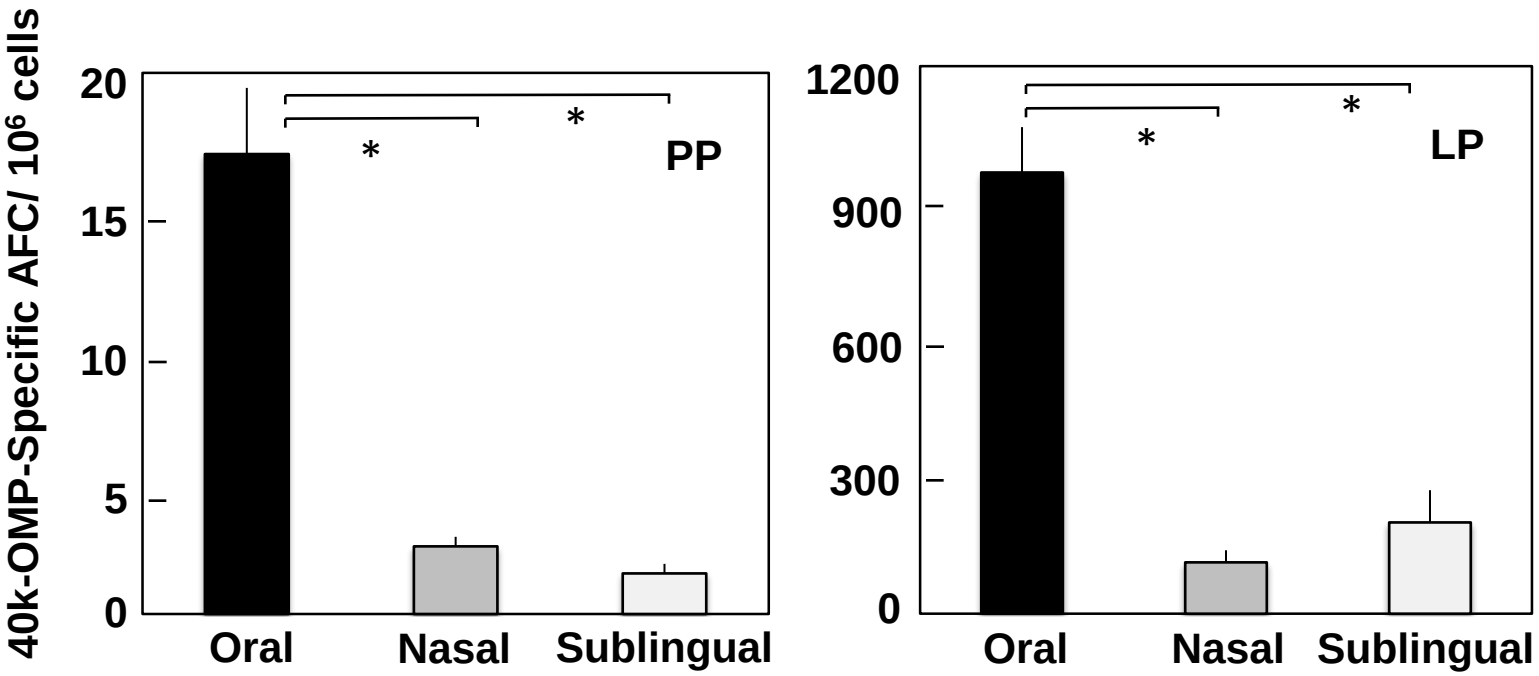
Figure 4


40k-OMP-Specific AFC/ 10⁶ cells
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