Induction of antibody response in the oral cavity of dogs following intraocular (eye drop) immunization with *Porphyromonas gingivalis* cell lysate incorporated in pH-sensitive fusogenic polymer-modified liposomes

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ABSTRACT. Induction of mucosal immune responses against *Porphyromonas gingivalis* within the oral cavity of dogs was studied by immunizing with pH-sensitive fusogenic polymer (MGluPG)-modified liposome-associated cell lysate. Dogs immunized with *P. gingivalis* cell lysate-containing MGluPG-modified liposomes by intraocular (eye drop) route displayed significant levels of *P. gingivalis* cell lysate-specific serum IgG and IgA as well as mucosal IgA antibodies in saliva secretion. Serum and salivary antibodies generated by intraocularly immunized with MGluPG-modified liposome-associated *P. gingivalis* cell lysate revealed a significant aggregation activity against *P. gingivalis,* whereas serum and saliva from dogs receiving MGluPG-modified liposomes unentrapping *P. gingivalis* cell lysate did not show the aggregation activity against *P. gingivalis*. Furthermore, *P. gingivalis*-specific antibodies in saliva of immunized dogs inhibited the adherence of *P. gingivalis* to cultured HeLa cells. More importantly, salivary antibodies induced by intraocular immunization with *P. gingivalis* cell lysate-containing MGluPG-modified liposomes significantly inhibited the coaggregation of *P. gingivalis* with *Actinomyces naeslundii* and the cell damage activity of *P. gingivalis* against FaDu cells, an oral epithelial cell. These results suggest that intraocularly administered *P. gingivalis* cell lysate-containing MGluPG-modified liposomes should be an effective mucosal vaccine against *P. gingivalis* infection in dogs and may be an important tool for the prevention of periodontitis.

KEY WORDS: intraocular immunization, liposome, mucosal immunity, periodontitis, *Porphyromonas gingivalis*

Periodontitis is probably the most common infectious disease in veterinary medicine, especially in small animal practice [11]. The disease is caused by a group of black-pigmented anaerobic bacteria. Among them, *Porphyromonas gingivalis* has been considered to be a major periodontal pathogen, because the bacterium is more frequently detected in active lesions of periodontitis in humans [40] and its subgingival implantation in mice [2], rats [19] and non-human primates [35] is associated with initiation and progression of the disease. Many pathogens cause disease by first colonizing or penetrating through the mucosal surface of the body [3, 8, 21, 22]. Also, in periodontitis, adhesion of *P. gingivalis* to the surface of the periodontal epithelium is a necessary first step in the infection. So, an effective strategy for the protection against *P. gingivalis* infection would be to induce anti-*P. gingivalis* local (mucosal) immunity in the oral cavity in addition to systemic immune responses following immunization.

The mucosal immune system plays a central role in the primary defense against pathogens by preventing binding of the microbes or their toxins to the epithelium [7, 42, 43]. Induction of mucosal immune responses is achieved by the deposition of antigen *via* the mucosa, but not the systemic route [27]. Further, mucosal immunization has been shown to induce antigen-specific immune responses in both mucosal and systemic compartments [26, 27]. Although systemic vaccination (e.g., intramuscular

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injection) can induce effective immune responses in the systemic compartment, it does not result in the generation of antigen-specific mucosal immune responses. Considering infection of pathogens, mucosal vaccination that can offer two layers of immunity (e.g., mucosal and systemic immune responses) would provide an effective barrier against invasion of pathogens. Externally secreted IgA and local IgG antibodies produced in response to the mucosal invasion or administration of antigens perform important functions in this system [4]. It has been reported that these local antibodies are effective in inhibiting the binding of pathogen to the mucosal cells [4]. However, it has been shown that delivery of antigen alone is insufficient for the induction of maximum levels of antigen-specific immune response by mucosal vaccine [26, 27]. Thus, it is necessary to co-administer with new adjuvants and carriers for the induction of mucosal immune responses.

The potential usefulness of liposomes as adjuvants for developing vaccines has led to considerable interests during the last few years, because the materials encapsulated within the liposomes are protected from degradation until they reach the target sites [39]. Several studies have demonstrated that, depending on the liposomal composition, charge and size, liposomes can have different pharmacokinetics and be formulated to obtain optimal retention and presentation of the vaccine antigens and are avidly taken up by the dendritic cells (DCs) owing to their particulate nature [5, 12, 13, 16, 18, 20, 24, 31, 38]. To establish more effective vaccine, therefore, we have developed pH-sensitive liposomes, which generate fusion ability under weakly acidic conditions, by surface modification of liposomes with pH-sensitive fusogenic polymer having carboxyl groups, such as succinylated poly (glycidol) (SucPG) and 3-methylglutarylated poly (glycidol) (MGluPG) [45]. Until now, the study of vaccination to prevent periodontal disease has been extensively done [33, 34]. Especially, there is no available information on the effect of liposome mucosal vaccine against periodontal diseases in companion animals, such as dogs.

To know the usefulness of pH-sensitive fusogenic polymer-modified liposomes as mucosal vaccine, *P. gingivalis* cell lysate-containing MGluPG-modified liposomes were inoculated to dogs by intraocular (eye drop) route, and immune responses were evaluated. In addition, a possibility of the control of *P. gingivalis* infections in dogs following intraocular immunization with *P. gingivalis* cell lysate-containing MGluPG-modified liposomes was examined *in vitro*.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylethanol- amine (DOPE) and monophosphoryl lipid A (MPL) (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) were commercial products. MGluPG polymer was prepared as previously reported [45].

Dogs

Ten-month-old female beagle dogs were obtained from Kitayama Labes Co., Ltd., Ina, Japan, and were maintained at the experimental facility, Education and Research Center for Experimental Animal Science, of Osaka Prefecture University. Animal experiments were conducted in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

Bacteria

P. gingivalis (ATCC 33277) and *Actinomyces naeslundii* (ATCC 12104) were obtained from the American Type Culture Collection. *P. gingivalis* was grown in brain heart infusion broth (Nissui Phamaceutical Co., Ltd., Tokyo, Japan) supplemented with hemin (4 μg/ml) and menadion (0.4 μg/ml). *A. naeslundii* was grown in partial arranged ATCC medium 1490. All strains were maintained at 37°C in an anaerobic chamber containing 80% N_2, 10% H and 10% CO_2 without shaking in screw-capped test tubes or bottles.

Cells

HeLa (human cervical cancer cell line) and FaDu cells (human oral epithelial cells) were kindly provided by Dr. Y. Kodama (Immunology Research Institute, Ghen Co., Gifu, Japan). HeLa cells were grown in Dulbecco's Modified Eagle medium (Nissui) supplemented with 10% fetal bovine serum (FBS). FaDu cells were maintained in Eagle's minimum essential medium (Nissui) supplemented with 10% FBS.

Preparation of P. gingivalis cell lysate-containing MGluPG-modified liposomes

P. gingivalis cell lysate was prepared as follows. The bacteria were cultivated anaerobically in *brain heart infusion broth* for 72 hr at 37°C without shaking in bottles. Formaldehyde solution was then added up to a concentration of 0.5%. The suspension was incubated overnight to deactivate the bacteria. The formalin was removed by centrifuging the cells 3 times with phosphate buffered saline (PBS; 150 mM, pH 7.4). Cell lysate of the bacteria was then prepared by ultrasound irradiation of 0.5% bacterial cell suspension for 15 min three times (BRANSON Sonifier 250, Emerson Japan, Atsugi, Japan). This mainly contains disrupted cell wall of the bacteria containing fimbriae, lipopolysaccharides, capsules, proteases (gingipains), hemagglutinins, major outer membrane proteins, etc. [23].

MGluPG-modified liposomes that entrap *P. gingivalis* cell lysate were prepared by the following method. DPPC (15 μmol), DOPE (15 μmol), MPL (60 μg) and MGluPG polymer (lipids/polymer=7/3, w/w), each dissolved in an organic solvent (DPPC and DOPE, chloroform-methanol=2:1, v/v; MPL, chloroform-methanol=1:2, v/v; MGluPG polymer, methanol), were mixed in a conical flask. The lipids were dried on a rotary evaporator and left to stand for 30 min in a high vacuum in a desiccator. After addition of 1 ml of *P. gingivalis* cell lysate solution (5 mg/ml) and incubation at an appropriate temperature for 3 min, the lipid

film was dispersed by vigorous vortexing. Any unencapsulated *P. gingivalis* cell lysate was removed by repeated centrifuging at 14,000 ×*g* for 20 min at 4°C in PBS, and the resulting liposome suspension was used for immunization as MGluPG-modified liposomes entrapping *P. gingivalis* cell lysate.

The amount of *P. gingivalis* cell lysate entrapped in liposomes was determined by the following method. Sixty *μl* of isopropyl alcohol was added to a 60 *μl* suspension of liposome-entrapped *P. gingivalis* cell lysate (at 2-fold dilution in PBS), followed by vortex mixing. The protein concentration of the resulting solutions was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.), with bovine plasma gamma globulin used as a standard.

MGluPG-modified liposomes that entrap PBS were also prepared according to the above procedure using dry membrane of a lipid mixture with polymers (lipids/polymer=7/3, w/w) and used for immunization as MGluPG-modified liposomes unentrapping *P. gingivalis* cell lysate.

Immunization of dogs

Dogs were divided into 2 groups (4 dogs per a group). Both were intraocularly immunized as follows: group I, MGluPG-modified liposomes that unentrap *P. gingivalis* antigen (100 *μl*/eye) and group II, MGluPG-modified liposomes that entrap *P. gingivalis* antigen (1 mg protein/100 *μl*/eye). Immunization was repeated three times at 2-week intervals. Peripheral blood and saliva samples were collected from each dog on weeks 0, 2, 4 and 6 after the first immunization for titration of antibodies.

Antibody titration

Ultrasound irradiated *P. gingivalis* cell lysate (20 *μg* protein/m*l*) diluted with PBS was dispensed in 50 *μl*/well into a 96-well microtiter plate (AGC Techno Glass Co., Ltd., Tokyo, Japan), followed by leaving overnight at 4°C. The plates were washed 3 times with PBS containing 0.05% Tween 20 (washing solution). The wells were treated with 100 *μl* of PBS containing 5% skim milk, incubated at 37°C for 1 hr to block nonspecific binding and then washed 3 times with the washing solution. Serum or saliva (50 *μl*), diluted in PBS, was added to each well and was incubated overnight at 4°C. After the plates were washed 3 times with the washing solution, 50 *μl* of horseradish peroxidase-labeled anti-dog IgG (1: 10,000 dilution in PBS; Bethyl Laboratories, Montgomery, TX, U.S.A.) or IgA (1: 10,000 dilution in PBS; Bethyl Laboratories) solution was added as the second antibody. Following incubation for 60 min at 37°C, the plates were washed 3 times with washing solution, and a substrate solution (100 *μl*) was added containing *o*-phenylenediamine (Sumitomo ELISA Color Reagent Kit, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The plates were allowed to stand for 20 min at room temperature, and the reaction was stopped by adding 100 *μl* of stopping solution (Sumitomo ELISA Color Reagent Kit). The optical density of each well was read at 490 nm on a microplate reader (Model 450, Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Antibody titers are represented as the reciprocal of endpoint dilution exhibiting an optical density more than 2.5 times that of background.

Agglutination test

The agglutinating activity of serum and saliva from immunized dogs against *P. gingivalis* was assayed. Briefly, *P. gingivalis* was washed twice with PBS and adjusted to OD_{600}=1, which is equivalent to 1×10^9 CFU/m*l*. Fifty microliters of a *P. gingivalis* cell suspension in PBS was transferred into v-bottom microtiter wells (AGC Techno Glass Co., Ltd.), and serial dilution of serum or saliva (50 *μl*) was added. Starting final dilution of serum and saliva samples was 1: 2. After incubation for 1 hr at 37°C with humidity, agglutinating activity of serum and saliva was observed.

Inhibition of cell adherence of P. gingivalis by specific antibodies

Bacterial adhesion assay was performed as described by Nakagawa *et al.* [29], with some modification. HeLa cells were cultivated in 24-well tissue culture plates (1×10^4 cells/well) (AGC Techno Glass Co., Ltd.) at 37°C overnight using an incubator equilibrated with 5% CO_2 and 95% air. The cells were washed 3 times with PBS, and *P. gingivalis* cells mixed with saliva samples in a final dilution of 1: 2, 1: 4, 1: 8 and 1: 16 were added to a monolayer of HeLa cells at a multiplicity of infection of 500. *P. gingivalis* cells treated with PBS instead of saliva samples were used as a control. After incubation of the cells for 90 min at 37°C in the presence of 5% CO_2, any non-adhering bacteria were removed by washing with PBS for 3 times. They were stained with Giemsa solution. The total number of the bacteria adhering to HeLa cells was determined by counting the bacteria adhering to 10^3 cells chosen at random under a microscope. The inhibition rate of the adherence of bacteria was calculated. Calculation formula is as follows: Inhibition%=(1−(the number of bacteria adhering in test culture/the number of bacteria adhering in control culture)) ×100.

Inhibition of coaggregation activity of P. gingivalis by specific antibodies

Coaggregation inhibition was determined by the visual assay method as described previously [6] with minor modifications. Briefly, *P. gingivali*s and *A. naeslundii* cells were washed 3 times with PBS and adjust to OD_{600}=1, respectively. *P. gingivalis* cell suspension (50 *μl*) was added to the wells of v-bottomed microtiter plates. Simultaneously, equal volumes of serially diluted saliva samples were added to the wells in duplicate at starting dilutions of 1: 2. This mixture was incubated at 37°C for 1 hr. Fifty microliters of *A. naeslundii* suspension was then transferred into microtiter wells. The plates were incubated at 37°C for 1 hr. The inhibition of coaggregation activity induced by adding saliva antibodies was observed (Fig. 5A). The coaggregation inhibition titer was calculated as the reciprocal of the highest saliva dilution that inhibited coaggregation between *P. gingivalis* and *A. naeslundii*.
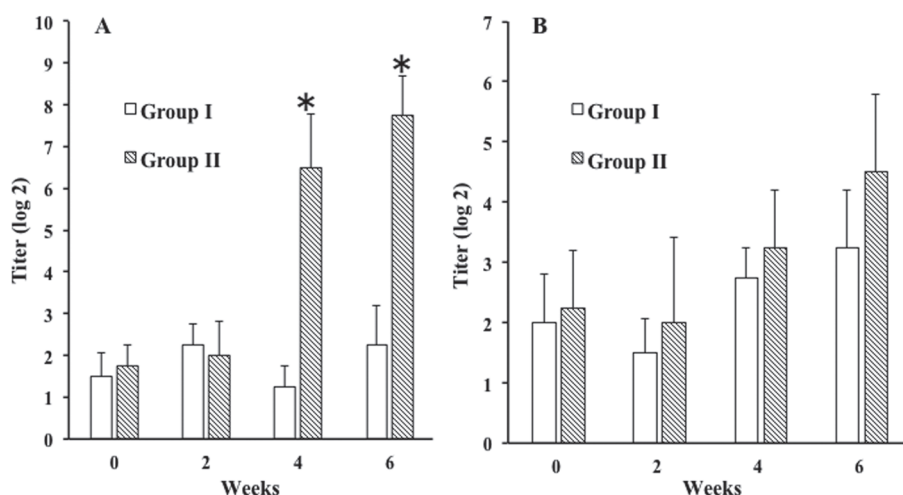
Fig. 1. Serum antibody responses in dogs immunized with liposome-associated *P. gingivalis* antigen by intraocular route. Sera of dogs immunized intraocularly were titrated for IgG (A) or IgA (B) levels by ELISA. Data are expressed as mean ± SD of 4 dogs. *$P<0.05$ compared to group I.

Cytotoxicity assay

Cytotoxicity (cell damage) assay was based on the protocol [9] as described previously with some modification. FaDu cells were plated in 24-well culture plates (AGC Techno Glass Co., Ltd.) at a concentration of 5×10^3 cells/well 24 hr before the experiment. The plates were charged with medium containing *P. gingivalis* (1×10^4 cells/well) or *P. gingivalis* treated with saliva samples (group I or II) (1×10^4 cells/well) and incubated at 37°C for 24 hr. The plates were washed 3 times with PBS to remove detached cells, and remaining attached cells were counted as live cells. FaDu cells non-treated with *P. gingivalis* served as controls. The results were expressed as percent FaDu cell-survival compared to survival of control cells. Calculation formula is as follows:% survival=(the number of cells treated with medium containing *P. gingivalis* or with medium containing *P. gingivalis* treated with saliva samples (group I or II)/the number of control cells) × 100.

Statistical analysis

All data are presented as the means ± standard deviations (SD). The statistical significance was evaluated by Welch's *t*-test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Serum antibody responses in dogs immunized with liposome-associated P. gingivalis cell lysate by intraocular route

Dogs were administered intraocularly with MGluPG-modified liposomes that unentrap *P. gingivalis* cell lysate (group I) and MGluPG-modified liposomes that entrap *P. gingivalis* cell lysate (group II) three times, and antibodies against *P. gingivalis* cell lysate were evaluated on weeks 0, 2, 4 and 6 after primary immunization. After intraocular immunization, no harmful side effects on the eye were observed (data not shown). As shown in Fig. 1, in serum from dogs receiving MGluPG-modified liposomes that unentrap *P. gingivalis* cell lysate (group I), induction of *P. gingivalis* cell lysate-specific IgG and IgA antibodies was not observed (Fig. 1A and 1B). On the other hand, serum IgG and IgA activity against *P. gingivalis* cell lysate could be seen in the group II after secondary (4 weeks after primary immunization) and tertiary immunization (6 weeks after primary immunization) (Fig. 1A and 1B). In particular, IgG antibody response against *P. gingivalis* cell lysate in the group II on weeks 4 and 6 after primary immunization was significantly higher than the group I ($P<0.05$) (Fig. 1A).

Detection of antibodies in saliva of dogs intraocularly immunized with liposome-associated P. gingivalis cell lysate

A further experiment was performed to study whether intraocular immunization can elicit antibody responses in the saliva. As shown in Fig. 2, *P. gingivalis* cell lysate-specific IgA antibody titers increased significantly 2 weeks after secondary immunization (4 weeks after primary immunization) when dogs were immunized with MGluPG-modified liposomes that entrap *P. gingivalis* cell lysate (group II). Two weeks after tertiary immunization (6 weeks after primary immunization), the highest antibody responses were observed (Fig. 2). Contrary to this, no obvious induction of antibody response was noted in dogs intraocularly with MGluPG-modified liposomes that unentrap *P. gingivalis* cell lysate (group I) (Fig. 2).

Agglutinating activity of serum and salivary antibodies induced by intraocularly immunized with liposome-associated P. gingivalis cell lysate against P. gingivalis

We examined whether antibodies induced by intraocularly immunized with liposome-associated *P. gingivalis* cell lysate were
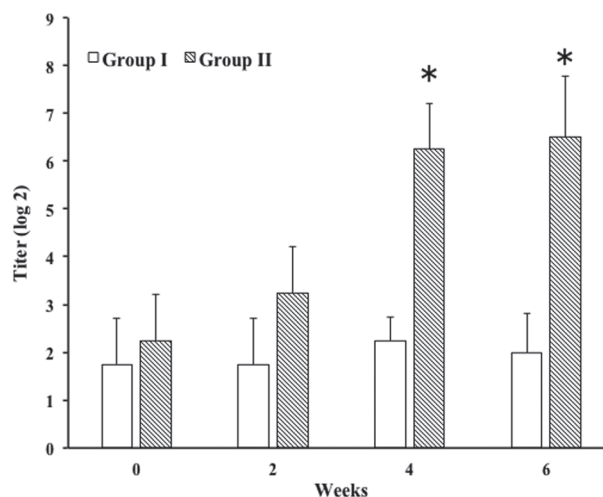
Fig. 2. Detection of antibodies in saliva of dogs intraocularly immunized with liposome-associated *P. gingivalis* antigen. Saliva samples were titrated for IgA level by ELISA. Data are expressed as mean ± SD of 4 dogs. *$P<0.05$ compared to group I.
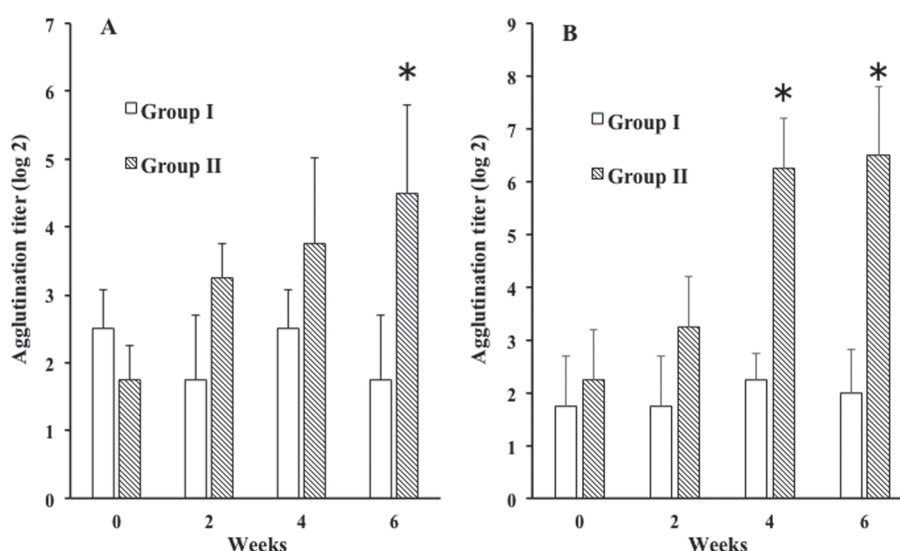

Fig. 3. Agglutinating activity of serum and salivary antibodies induced by intraocularly immunized with liposome-associated *P. gingivalis* antigen against *P. gingivalis*. Serum (A) and saliva (B) samples were titrated for agglutination level by agglutination test. Data are expressed as mean ± SD of 4 dogs. *$P<0.05$ compared to group I.

capable of showing the agglutinating activity against *P. gingivalis*. As shown in Fig. 3, the levels of agglutinating activity of serum and saliva from dogs in the group II increased depending on the times of immunization. Significant agglutination response was noted in sera after tertiary immunization (6 weeks after primary immunization) ($P<0.05$ compared to group I) (Fig. 3A). On the other hand, the agglutinating activity of saliva against *P. gingivalis* was demonstrated after secondary (4 weeks after primary immunization) ($P<0.05$ compared to group I) and tertiary immunization (6 weeks after primary immunization) ($P<0.05$ compared to group I) (Fig. 3B). As expected, serum and saliva from dogs receiving MGluPG-modified liposomes that unentrap *P. gingivalis* cell lysate (group I) failed to show the agglutinating activity against *P. gingivalis*.

Inhibition of adherence of P. gingivalis to HeLa cells by specific antibodies from saliva

The effect of *P. gingivalis*-specific salivary antibodies on inhibition of adherence of *P. gingivalis* to HeLa cells was studied *in vitro*. Figure 4 shows the dose-dependent inhibition of salivary antibodies for adherence of *P. gingivalis* to the cells. Saliva samples of group II in a final dilution of 1: 2 inhibited 35.8 ± 5.6% of bacterial adherence ($P<0.05$ compared to group I) and in a final dilution of 1: 4 also inhibited 28.0 ± 5.8% of *P. gingivalis* adherence to HeLa cells ($P<0.05$ compared to group I).
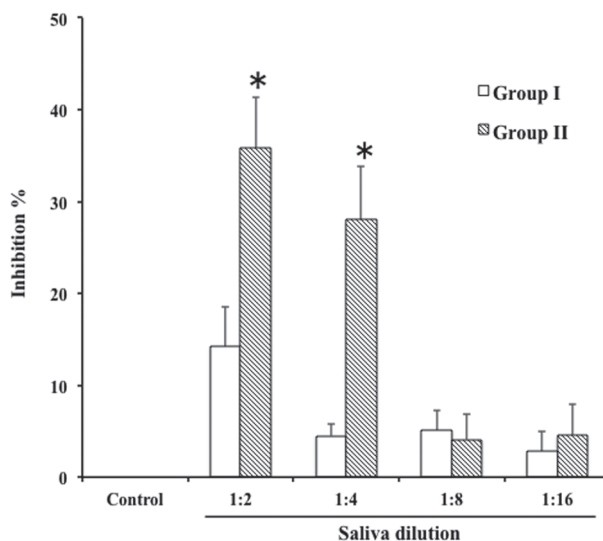
Fig. 4. Inhibition of adherence of *P. gingivalis* to HeLa cells by specific antibodies secreted in saliva. The percentage of adherence inhibition was calculated as described in "Materials and Methods". Data are expressed as mean ± SD of 4 dogs. *$P<0.05$ compared to group I.
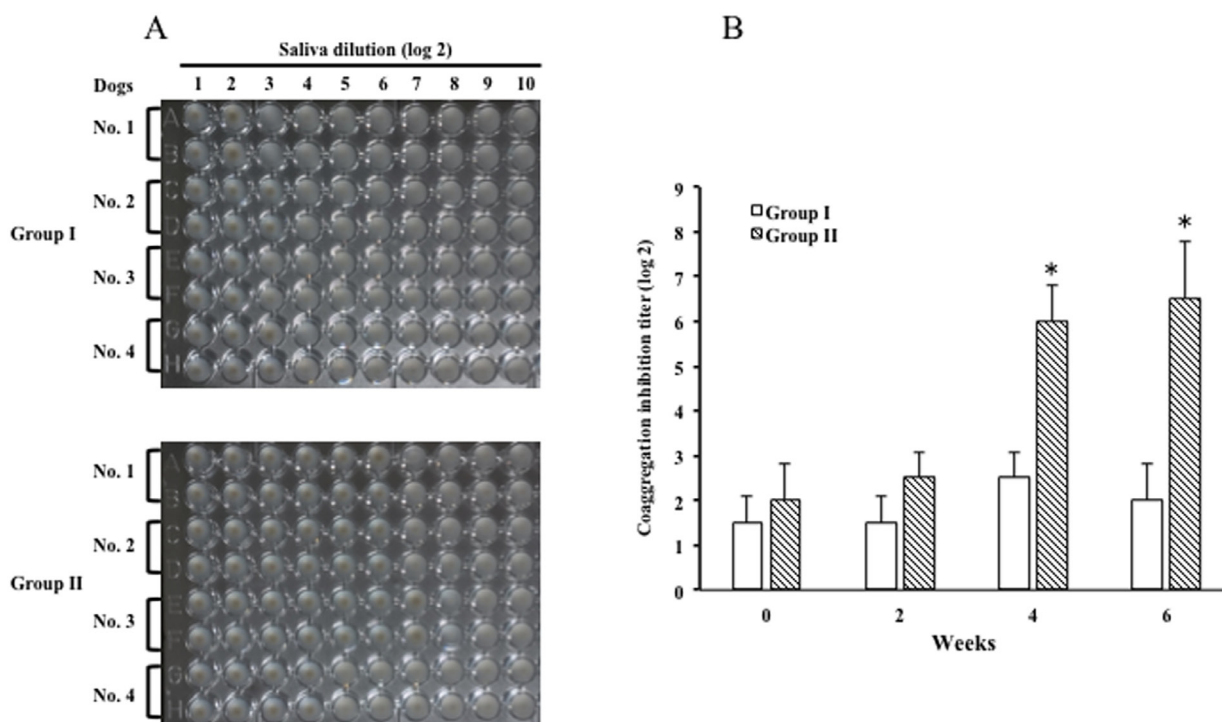

Fig. 5. Inhibition of coaggregation between *P. gingivalis* and *A. naeslundii* by specific salivary antibodies. For the coaggregation inhibition assay, *P. gingivalis* was pre-incubated with saliva samples and then mixed with *A. naeslundii* in v-bottomed microtiter plates. (A) Results of the coaggregation inhibition assay with a mixture of *A. naeslundii* and *P. gingivalis* with saliva samples at 4 weeks after primary immunization are shown. (B) The coaggregation inhibition titer of saliva antibodies was evaluated as described in "Materials and Methods". Data are expressed as mean ± SD of 4 dogs. *$P<0.05$ compared to group I.

Inhibition of coaggregation between P. gingivalis and A. naeslundii by specific salivary antibodies

In order to clarify whether salivary antibodies from the liposome-associated *P. gingivalis* cell lysate immunized dogs were capable of suppressing the coaggregation activity of *P. gingivalis*, we pre-incubated *P. gingivalis* cells with saliva samples and then incubated them with *A. naeslundii* cells. The inhibition of coaggregation between *P. gingivalis and A. naeslundii* in the
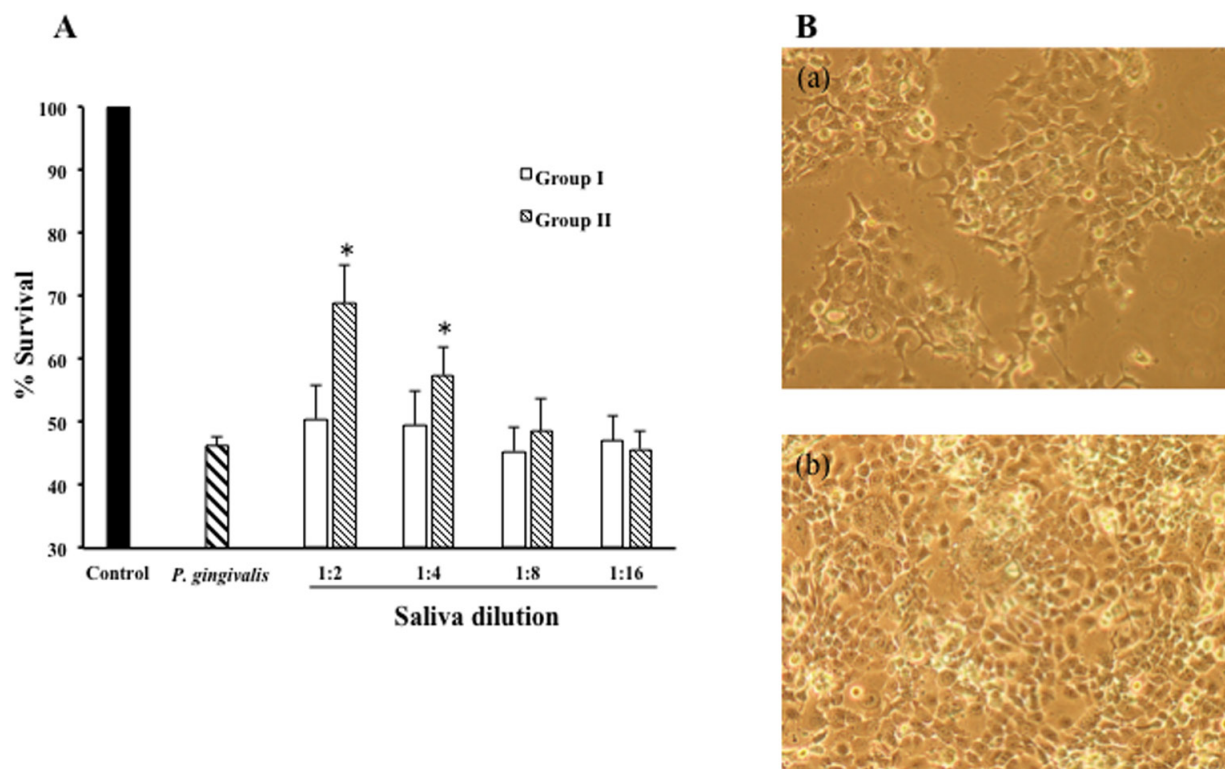
Fig. 6. The effect of saliva from dogs intraocularly immunized with liposome-associated *P. gingivalis* antigen on *P. gingivalis*-induced cytotoxicity. (A) Human oral epithelial cells (FaDu cells) were incubated with medium containing *P. gingivalis* or *P. gingivalis* treated with saliva samples (group I or II). Percentage of FaDu cell-survival was calculated as described in "Materials and Methods". Data are expressed as mean ± SD of 4 dogs. *$P<0.05$ compared to group I. (B) Influence of specific salivary antibodies against *P. gingivalis*-induced cytotoxicity. (a) FaDu cells were cultured in medium containing *P. gingivalis* treated with saliva (at final dilutions of 1:2) from dogs of group I ($\times$ 400). (b) FaDu cells were cultured in medium containing *P. gingivalis* treated with saliva (at final dilutions of 1:2) from dogs of group II ($\times$ 400).

presence of saliva antibodies was assessed. The inhibiting activity of saliva antibodies was shown as the coaggregation inhibition titer. As shown in Fig. 5, high levels of coaggregation inhibiting activity of saliva from dogs in the group II were observed after secondary (4 weeks after primary immunization) ($P<0.05$ compared to group I) and tertiary immunization (6 weeks after primary immunization) ($P<0.05$ compared to group I). On the other hand, no coaggregation inhibiting activity was detected in saliva of dogs given MGluPG-modified liposomes that unentrap *P. gingivalis* cell lysate (group I) (Fig. 5A and 5B).

Influence of specific salivary antibodies against P. gingivalis-induced cytotoxicity

To investigate the effect of saliva from dogs receiving MGluPG-modified liposomes that entrap *P. gingivalis* cell lysate against *P. gingivalis*-induced cytotoxicity (cell damage), *P. gingivalis*, with or without treatment with saliva from immunized dogs (groups I and II), was added to cultures of FaDu cells. The cells incubated with *P. gingivalis* resulted in cell death (Fig. 6). Treatment of *P. gingivalis* with saliva from dogs of group II protected the cells from damage and significantly increased cell survival compared to the *P. gingivalis*-treated cells in a final dilution of 1: 2 and 1: 4 ($P<0.05$), whereas that with saliva from dogs of group I did not show any protection effects (Fig. 6A and 6B).

DISCUSSION

Previous studies have shown that tear duct-associated lymphoid tissue, which is one of mucosa-associated lymphoid tissues, plays an important role in the induction of antigen-specific immune responses in the craniofacial mucosal immune system [28, 32]. The present study therefore examines whether intraocular (eye drop) immunization can elicit antigen-specific immune responses in dog.

In this study, none of dogs receiving MGluPG-modified liposomes that unentrap *P. gingivalis* cell lysate (group I) showed the induction of antigen-specific antibody responses in serum and saliva (Figs. 1 and 2). On the other hand, the intraocular administration of MGluPG-modified liposomes containing *P. gingivalis* cell lysate (group II) induced not only good serum IgG and IgA responses against *P. gingivalis*, but also good salivary IgA responses against *P. gingivalis* (Figs. 1 and 2). These results suggest that MGluPG-modified liposomes containing *P. gingivalis* cell lysate function as effective mucosal vaccine for increasing immune responses against the periodontal pathogen *P. gingivalis* when immunized by ocular route and that the induction of both mucosal

(anti-*P. gingivalis* saliva IgA) and systemic (anti-*P. gingivalis* serum IgG and IgA) immune responses may be useful for host protection against *P. gingivalis* infection.

In the present study, agglutination test was performed to determine whether antibodies induced by intraocularly immunized dogs with liposome-associated *P. gingivalis* cell lysate are able to recognize bacterial surface antigen. Serum and saliva antibodies from dogs in the group II showed aggregation activity against *P. gingivalis* (Fig. 3). This result indicates that intraocular immunization with MGluPG-modified liposomes containing *P. gingivalis* cell lysate can elicit *P. gingivalis* surface antigen (components of outer membrane constituents)-specific antibody responses in serum and saliva. Furthermore, this also suggests that *P. gingivalis*-specific antibody response in saliva would be effective for the prevention of *P. gingivalis* adherence to gingival epithelial cells. Indeed, adhesion of *P. gingivalis* to HeLa cells was efficiently inhibited by incubation of the bacteria and HeLa cells in the presence of antibodies from the saliva of dogs intraocularly immunized with liposome-associated *P. gingivalis* cell lysate (Fig. 4). HeLa cells were used since *P. gingivalis* is known to adhere to the cells *via* fimbriae [29].

Cytotoxicity of *P. gingivalis* to FaDu cells was efficiently inhibited by treatment of the bacteria with saliva samples from immunized dogs of group II. On the other hand, treatment with saliva from immunized dogs of group I did not show protective effects against *P. gingivalis*-induced cytotoxicity (Fig. 6). The present result indicates that antibodies secreted into the saliva can inhibit bacterial adherence to the cells, probably by preventing the adhesion of *P. gingivalis* fimbriae to the receptor moiety on the target cells [15, 23]. The precise mechanisms of the inhibition of adherence by the antibody is now under study.

The virulence of *P. gingivalis* has been attributed to a variety of potential factors associated with its cell surface, including fimbriae, lipopolysaccharides, capsules, proteases (gingipains), hemagglutinins and major outer membrane proteins [15, 23], because these cell surface components facilitate growth, nutrient acquisition, colonization, biofilm formation, periodontal tissue destruction and evasion of host defense. For example, it has been reported that *P. gingivalis* causes coaggregation with surrounding coinfecting microbial species, such as *A. naeslundii* [44]. This coaggregation contributes to the formation and maturation of biofilm, which is known to case periodontal disease [25, 41]. Furthermore, gingipains, which are major virulence factors of *P. gingivalis*, have also been shown to contribute to the tissue damage in periodontal disease caused by *P. gingivalis* [17]. Therefore, the induction of specific antibodies against cell surface components of *P. gingivalis* in the oral mucosa is a logical approach for the prevention of *P. gingivalis* infection. Indeed, a previous study has demonstrated that specific antibody against outer membrane protein of *P. gingivalis* significantly diminishes the coaggregation of *P. gingivalis* with *A. naeslundii* [1, 30]. An other study has also shown that specific antibodies against outer membrane protein of *P. gingivalis* from hen egg yolk preparations (IgY) exhibit an ability to inhibit *P. gingivalis*-associated coaggregation [14]. Further, immunization studies with gingipains have demonstrated protective effects against *P. gingivalis* infections in animal models [10, 37]. Moreover, it has also been reported that egg yolk antibody against *P. gingivalis* gingipains is useful in reduction of inflammation in oral cavity and prevention of periodontitis and gum diseases in dogs [36]. In the present study, we indicated that salivary antibodies from the liposome-associated *P. gingivalis* antigen immunized dogs inhibited the coaggregation activity of *P. gingivalis* with *A. naeslundii* (Fig. 5) and the cell damage activity of *P. gingivalis* against FaDu cells, an oral epithelial cell (Fig. 6). Therefore, present results suggest that *P. gingivalis*-specific antibodies in saliva generated by intraocular immunization may be effective in preventing *P. gingivalis* infection and thereby result in the prevention of periodontitis in dogs.

In summary, our results provide evidence that intraocular immunization with MGluPG-modified liposomes containing *P. gingivalis* cell lysate elicited *P. gingivalis*-specific IgA responses in saliva as well as IgG and IgA in serum. Furthermore, *P. gingivalis*-specific salivary antibodies were shown to inhibit the coaggregation of *P. gingivalis* with *A. naeslundii* and *P. gingivalis*-induced cell damage *in vitro*. It has been reported that effective protection against *P. gingivalis* infection requires both mucosal (saliva) and systemic (serum) antibody responses [32]. Thus, intraocularly administered *P. gingivalis*-containing MGluPG-modified liposomes should be considered as a valuable mucosal vaccine for prevention and treatment of periodontitis in dogs.

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