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<th>Arg-gingipain A DNA Vaccine Prevents Alveolar Bone Loss in Mice</th>
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INTRODUCTION

Porphyromonas gingivalis, a Gram-negative anaerobic bacterium, has emerged as a leading candidate pathogen in the development of chronic periodontitis (Socransky et al., 1998). This micro-organism possesses multiple pathogenic factors such as lipopolysaccharides, capsular polysaccharides, fimbriae, hemagglutinins, and proteases (Mayrand and Holt, 1988). Among these, gingipains, a group of cysteine proteases, are major weapons in its arsenal of attack on the periodontal region. Gingipains consist of Arg-gingipains (RgpA and RgpB) and Lys-gingipain (Curtis et al., 1999). They are believed to play a major role in the pathogenesis of periodontitis, degrading a variety of host proteins. In this way, they dysregulate the host defense mechanisms, resulting in tissue destruction and alveolar bone resorption (Grenier et al., 2003; Houle et al., 2003; Imamura, 2003; Goulet et al., 2004).

Immunization studies with P. gingivalis in animal models have produced encouraging results which suggest that vaccine-elevated IgG levels can protect against periodontal disease (Gibson and Genco, 2001; Rajapakse et al., 2002; Gonzalez et al., 2003). Mucosal immunity, in which sIgA is a key factor, is the first line of defense against pathogens, working to prevent systemic or local infection. We previously reported that immunization of mice with rgpA DNA vaccine elicited high levels of specific IgGs against P. gingivalis (Yonezawa et al., 2001). In this study, we evaluated the effect of intranasal (i.n.) immunization with a DNA vaccine.

MATERIALS & METHODS

Vaccination with Gene Gun and HVJ Envelope Vector

Approval to conduct this study was obtained from the Animal Use Committee of Tokyo Dental College. A six-week-old female BALB/c mouse (Sankyo Laboratories Service Co. Inc., Tokyo, Japan) was used for each vaccination.

Immunization with the rgpA DNA vaccine was performed as described previously (Yonezawa et al., 2001). Briefly, pVax1 (Invitrogen, Carlsbad, CA, USA) harboring the whole rgpA open reading frame from P. gingivalis ATCC33277 was used as the rgpA DNA vaccine. Gold particles (1.6-μm) were coated with the vaccine according to the manufacturer's instructions. A Helios Gene Gun (Bio-Rad Laboratories, Hercules, CA, USA) was used to give the mice a total of 2.5 μg DNA via the skin of the abdomen at wks 0, 1, 2, 3, 4, 5, 6, and 7.

Intranasal immunization with the rgpA DNA vaccine was performed with the hemagglutinating virus of Japan (HVJ) envelope vector kit (Ishihara-Sangyo Kaisha Ltd., Osaka, Japan), according to the manufacturer's instructions. A 160-μL HVJ envelope vector suspension was centrifuged at 10,000 x g for 5 min. The pellet was re-suspended in 10 μL rgpA DNA vaccine (16 μg/μL) and 80 μL bovine serum albumin (BSA) solution. After the addition of 2% Triton X-100 (8 μL), the mixture was centrifuged at 10,000 x g for 5 min. The pellet was...
finally re-suspended in 80 µL BSA solution. The mice were nasally immunized with rgpA-HVJ-E vector (20 µg/10 μL/mouse) at wks 0, 1, 2, 3, 4, 5, 6, and 7. Vaccination for investigation of the kinetics of the antibody response and evaluation of the protective effect of the vaccination described below was performed independently.

**ELISA and Immunoblot**

Serum and saliva samples were obtained from the mice on days 0, 7, 14, 21, 28, 35, 42, and 49. Anti-rgpA antibodies in the serum and saliva were detected by enzyme-linked immunosorbent assay (ELISA) as previously described (Yonezawa et al., 2001). A 20-µg sonic extract of *P. gingivalis* ATCC33277 was coated onto flat-bottomed polystyrene microplates (Corning, Corning, NY, USA), and the wells were blocked. Duplicate serial two-fold-diluted samples were applied to the wells and incubated at 37°C for 60 min. After wells were washed, peroxidase-labeled goat antibody against mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgA (ICN Biomedicals, Inc., Aurora, OH, USA) was applied. After development, absorbance at 405 nm was measured with a microplate reader (Bio-Rad Laboratories). The endpoint titer for antigen-specific immunoglobulins was defined as the last dilution giving an optical density at 405 nm of > 0.15. Specificity of salivary sIgA was confirmed by immunoblot analysis, with each recombinant fusion protein of the Rgp domain expressed by pET32Xa/LIC (Novagen, Gibbstown, NJ, USA), as described previously (Yonezawa et al., 2001). These consisted of the histidine tag protein and each domain. The deduced molecular masses of the histidine-tag (17388.5) of pET32Xa/LIC and catalytic subunits, HGRP44 and HGRP 15-27, were 71258.3, 62058.8, and 78729.4, respectively.

**P. gingivalis Challenge Studies**

To evaluate the protective effect of the vaccination against *P. gingivalis*, we infected the immunized mice with *P. gingivalis* as described previously (Baker et al., 1994), with minor modifications. The BALB/c mice were separated into 4 groups: a non-immunized group (n = 10), a group immunized with the rgpA DNA vaccine via the skin of the abdomen by a Gene Gun (n = 5), a group immunized intranasally with rgpA-HVJ-E vector (n = 5), and a group immunized with pVAX1 (Invitrogen, Carlsbad, CA, USA) alone (n = 5). The serum IgG and salivary sIgA antibody titers were evaluated at 4 to 6 wks as described above. When the antibody titers of the mice immunized by Gene Gun and intranasally with rgpA-HVJ-E vector reached a plateau, the mice were challenged with *P. gingivalis*. Following immunization, the mice were given 5 mg each of kanamycin and ampicillin by gavage, once a day for 4 days. This was followed by a three-day antibiotic-free period. They were then challenged by 1 x 10⁶ CFUs of live *P. gingivalis* ATCC33277 suspended in 100 μL of 2% carboxymethylcellulose via a feeding needle, except for the 5 non-immunized mice. These 5 non-infected and non-immunized mice were used to determine the baseline value from the cementum-enameled junction (CEJ) to the alveolar bone crest (ABC) in normal mice. The challenge was carried out 3 times at two-day intervals. Forty-two days after the last gavage, the mice were killed. We then repeated the experiment to confirm reproducibility, and performed statistical analysis using the pooled data from both experiments.

**Measurement of Alveolar Bone Loss**

Alveolar bone loss was assessed at defined landmark sites on the maxillary molars of each mouse as described previously (Gonzalez et al., 2003). We performed measurements (14 sites) of each skull from the CEJ to the ABC with a stereomicroscope. Measurements were made under a dissecting microscope fitted
with a video image-maker measurement system, MS-803 (MORITEX Co., Tokyo, Japan), standardized to give measurements in millimeters.

**Statistical Analysis**

We used a one-way ANOVA followed by a Student-Newman-Keuls test to make multiple comparisons between the relative protective effects of the DNA vaccinations against bone loss. The differences in antibody levels among the groups were also evaluated with a one-way ANOVA, followed by a Student-Newman-Keuls test.

**RESULTS**

**Responses of Immunoglobulins to P. gingivalis**

Specific IgGs were clearly induced in both groups of BALB/c mice inoculated with the rgpA DNA vaccine, either by Gene Gun or intranasally (Fig. 1A). Following immunization, the responses in both groups reached plateaus on day 42. The titers of IgGs at day 49 in both immunized groups were elevated ($p < 0.01$), with the Gene Gun group yielding slightly lower titers than the intranasal group. The specificities of serum IgGs to recombinant RgpA domains were confirmed as described previously (Yonezawa et al., 2001).

Intranasal immunization with rgpA DNA vaccine induced P. gingivalis-specific salivary IgA responses in the saliva, with this response reaching a plateau on day 42 ($p < 0.01$, Fig. 1B). In contrast, no salivary IgA production was observed in the Gene Gun group.

The specificity of salivary IgA against each recombinant RgpA domain is shown in Fig. 2. It reacted to the catalytic domains of RgpA, HGRP 44, and HGRP 15-27. The molecular masses corresponded well with the deduced molecular masses of the recombinant fusion proteins of each domain with a histidine-tag of pET32Xa/LIC.

**Immunization with rgpA DNA Vaccine Protects against Alveolar Bone Loss Incurred by Infection with P. gingivalis.**

The level of alveolar bone loss was assessed at 42 days after challenge with P. gingivalis ATCC33277. The infected mice showed significantly greater maxillary molar alveolar bone loss than did the uninfected control mice ($p < 0.01$, Fig. 4). Both the Gene Gun group and the intranasal group were protected from alveolar bone loss ($p < 0.01$), although the magnitude of protection was greater in the latter ($p < 0.01$).

**DISCUSSION**

Gingipain is a major pathogen of P. gingivalis (Potempa et al., 2003). We found that both serum IgG and salivary IgA production was induced in mice intranasally immunized with rgpA DNA vaccine by an HVJ envelope vector; in contrast, immunization via the abdominal skin by Gene Gun induced serum IgG alone. These results suggest that intranasal administration of DNA vaccine by an HVJ envelope vector can induce a stronger response in the mucosal immune system. We found that the levels of induced IgG against RgpA were almost the same in both groups. These results indicate that intranasal rgpA DNA vaccine immunization is a more effective procedure for inducing serum and salivary antibody responses in the absence of any adjuvant. Kawabata et al. (1999) demonstrated that injection of fimbriae DNA vaccine into salivary glands induced salivary IgA and serum IgG. In our results, the level of salivary
IgA was almost the same as that reported in their data; however, the serum IgG titer was lower. It is possible that this variation in IgG titers was a result of the different immunization routes used.

Analysis of each IgG subclass level against *P. gingivalis* revealed that immunization with *rgpA* DNA vaccine via either the abdominal or the intranasal route strongly induced production of IgG1 and IgG2b. This DNA vaccine offers the advantage of inducing both humoral and cell-mediated immune responses. Our results suggest that immunization with *rgpA* DNA vaccine preferentially induced antibody via Th2-type responses. Another group has also reported a protective effect resulting from serum IgG response to *P. gingivalis* (Gibson et al., 2004). Analysis of these data, taken together, indicates that the induction of antibody via Th2-type response plays an important role in protecting against infection by *P. gingivalis*.

The Th1-type response has been associated with destruction of periodontal tissue and bone resorption (Takeichi et al., 2000; Taubman and Kawai, 2001). In an earlier study, we found that *rgpA* DNA vaccine induced a reduction in Th1-type cytokines such as IFN-γ, and a protective effect in mice challenged with a lethal dose of *P. gingivalis* (Yonezawa et al., 2005). It is possible that the protection afforded by vaccination with the *rgpA* DNA vaccine resulted from a reduction in the Th1-type response. However, the Th1-type reaction plays an important role in protection against acute infection (Spellberg and Edwards, 2001). To clarify the relationship between reduction in Th1-type response and protective response to vaccination, further analysis is required to investigate the Th1/Th2 balance in vaccinated mice during the inflammation process.

Numerous clinical studies in patients with chronic periodontitis have demonstrated high antibody levels against *P. gingivalis* in serum and gingival crevicular fluid (Tew et al., 1985; Ebersole et al., 1986; Inagaki et al., 2003). However, few reports have investigated the protective effects of immunoresponses against periodontal disease in humans. Booth et al. (1996) found that passive immunization with monoclonal antibody against *P. gingivalis* inhibited recolonization. We previously reported that *rgpA* DNA vaccine induced a protective effect against *P. gingivalis* infection in mice (Yonezawa et al., 2001). Another study (Genco et al., 1998) showed that a peptide domain on R gingipain had a protective effect against *P. gingivalis* infection in mice. Mice immunized with RgpA polypeptides were protected from *P. gingivalis*-elicited oral bone loss, with this antigen also eliciting a potent IgG response (Gibson et al., 2001). In addition, IgG against purified RgpA also showed a protective effect, enhancing PMN-mediated bacterial killing of *P. gingivalis* strains (Nakagawa et al., 2001). When these findings are considered together with the present results, it seems clear that IgG can exert a preventive effect against components of *P. gingivalis*.

Intranasally immunized mice were better protected against bone loss than those immunized with the Gene Gun, suggesting that slgA offers a protective advantage over high IgG antibody levels alone. Specific slgA against *Streptococcus mutans* has a protective effect against colonization by the pathogens in the oral cavity (Saito et al., 2001). It is possible that slgA reduces colonization by *P. gingivalis*. However, in this study, it was not possible to evaluate the inhibition of oral colonization, since *P. gingivalis* is not a resident of the oral cavity in the mouse; furthermore, exposure to such high numbers of this microorganism here may have induced bone loss. Such factors must be taken into account in evaluation of the reduction of colonization. Therefore, further study is required in a suitable animal model to clarify the effect of slgA.

The results of this study suggest that intranasal immunization with an *rgpA* DNA vaccine offers strong potential in the prevention of periodontal disease. This research offers a new approach to the clinical prevention of periodontal disease using a combination of *rgpA* DNA vaccine and the HVJ envelope vector system.

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