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Vaccination with recombinant RgpA peptide protects against *Porphyromonas gingivalis* induced-bone loss

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Abstract

Objective—Following *P. gingivalis* infection in mice, the efficacy of vaccination by recombinant and native RgpA in modulating the early local anti-inflammatory and immune responses and periodontal bone loss were examined.

Methods—Using the subcutaneous chamber model, exudates were analyzed for cytokines after treatment with native RgpA and adjuvant (test), or adjuvant and saline alone (controls). Mice were also immunized with recombinant RgpA after being orally infected with *P. gingivalis*. After 6 weeks, serum was examined for anti-*P. gingivalis* IgG1 and IgG2a titers and for alveolar bone resorption.

Results—Immunization with native RgpA shifted the immune response toward an anti-inflammatory response as demonstrated by decreased pro-inflammatory cytokine IL-1 β production and greater anti-inflammatory cytokine IL-4 in chamber exudates. Systemically, immunization with recombinant RgpA peptide prevented alveolar bone loss by 50%, similar to immunization with heat-killed whole bacteria. Furthermore, recombinant RgpA shifted the humoral response toward high IgG1 and low IgG2a titers, representing an in vivo anti-inflammatory response.

Conclusions—The present study demonstrates the potential of RgpA to shift the early local immune response toward an anti-inflammatory response while vaccination with recRgpA protected against *P. gingivalis*-induced periodontitis.

Keywords

Porphyromonas gingivalis; Vaccine; Experimental Periodontitis; Gingipains

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Introduction

Periodontitis is a bacterially-induced chronic inflammatory disease which results in the destruction of the dental attachment apparatus, leading to tooth loss (1). Evidence suggests that *P. gingivalis*, a Gram-negative anaerobic bacterium, is closely associated with periodontal disease in humans (2, 3). The subgingival infection stimulates various innate and adaptive immune responses which include the secretion of pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6, IL-11, IL-17, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α (4). In contrast, the dominant anti-inflammatory response, which was thought to protect the periodontal tissues from destruction by bacterial infection (5), is characterized by IL-4, IL-10, IL-12, IL-13, and IL-18 secretion (4).

In order to evade the host's immune response, *P. gingivalis* has developed a variety of virulence factors, which include gingipains. Gingipains, a group of cysteine proteases common to all *P. gingivalis* strains, consist of two arginine-specific proteases [Arg-gingipains, (Rgps)] and lysine-specific protease [Lys-gingipain, (Kgp)].

Gingipains alter intrinsic immunity by degrading defensins (6), complement components (7), and cytokines (8, 9) thereby disrupting bacterial phagocytosis (10). They inhibit adaptive immunity by cleaving various T-cell receptors (11, 12), and stimulate the expression of protease-activated receptors in T cells (13). These activities play a critical role in the induction of cytokine responses and the establishment of chronic inflammation in periodontitis and stimulating the expression of protease-activated receptors critical for inducing cytokine responses responsible for chronic inflammation (14). Gingipains also have the ability to proteolytically inactivate cytokines (8, 9), and stimulate IL-6 production by oral epithelial cells. Gingipains also stimulate pro-inflammatory cytokines from oral epithelial cells (15), and gingival fibroblasts (16).

The Rgps are encoded by two genes, *rgpA* and *rgpB*, and Kgp is encoded by a single gene, *kgp* (17). The mature forms of RgpA and Kgp proteins possess both a catalytic and a hemagglutinin domain, while RgpB is composed of only a catalytic domain (18). The hemagglutinin domain of RgpA and Kgp is further subdivided into four individual adhesion domains (A1-A4) (19, 20). The adhesin A1 encompasses several sequential motives interacting with the host systems while adhesin A2 constitutes the Hb binding domain playing a role in hem acquisition by *P. gingivalis* (21).

As major virulent factors and important housekeeping enzymes that are expressed on the cell surfaces of all *P. gingivalis* strains with more than 98% identity (21), gingipains have been suggested as candidate antigens for a vaccine against *P. gingivalis*. This contention was strongly supported by animal studies showing that immunization with purified or recombinant gingipains protects against subcutaneous challenge, significantly reduces alveolar bone loss and *P. gingivalis* colonization, and induces an anti-inflammatory immune response following oral infection (22-24). Thus, the next steps in vaccine development ought to involve the identification and testing of peptides derived from gingipains as antigens for immunization. Although it was inferred that the adhesion/hemagglutinin domain may be a functionally important target for the host-specific immune response to *P. gingivalis* in

periodontal disease, only one study systematically examined the efficiency of immunization with individual recombinant adhesion domains of gingipains to protect against *P. gingivalis* infection in the murine lesion model (25). However, there is no investigation assessing immunization with individual domains in experimental periodontitis model.

The aims of this study were:

1. First, to determine the efficacy of native RgpA-based vaccine (with adhesion domains A1, A2, A3 and A4) in directing the early local immune response (in a chamber model) toward an anti-inflammatory response to an infection triggered by *P. gingivalis*.
2. If the results obtained in the first part of the study were sufficiently encouraging, we planned to proceed to a second stage, and examine the ability of a novel recombinant RgpA (recRgpA), with A1 and A2 adhesins only, to act as a vaccine that attenuates alveolar bone loss resulting from experimental periodontitis in mice.

Materials and methods

Bacteria

P. gingivalis 53977 was grown on blood agar plates in an anaerobic chamber with 85% N₂, 5% H₂, and 10% CO₂. After incubation at 37°C for 2-3 days, the bacterial cells were inoculated into Wilkins media for 2 days of incubation under the same conditions. The bacteria were washed 3 times with sterile phosphate-buffered saline (PBS) before use, and the bacterial concentrations were standardized to an optical density of 0.1 at 650 nm, which corresponds to 10¹⁰ CFU/ml (26). Heat-killed *P. gingivalis* was prepared by incubating the bacteria at 80°C for 10 minutes (27).

Purification of gingipains

High molecular mass Arginine-specific gingipain A – 95 kDa, (native RgpA) containing adhesion domains A1-A4 was purified from *P. gingivalis* HG66 after cultivation for 48h. Briefly, proteins in the culture supernatant were precipitated with acetone, the precipitated was dissolved in a gel filtration buffer (20 mM Bis-Tris, 150 mM NaCl, 5 mM CaCl₂, 0.02% NaN₃, pH 6.8), solution clarified by ultracentrifugation (100,000 × g, 60 min, 4°C) and applied on the on Sephadex G-150. HRgpA was eluted from this column at a protein peak corresponding to 150-200 kDa. The fractions containing HRgpA activity were pooled and subjected to affinity chromatography on Arg-Sepharose as described earlier (18, 28). Protein concentration was determined using the bicinchoninic acid-based BCA Protein Assay Kit (Bio-Rad) with bovine albumin as a standard and purity of the enzyme was checked by SDS-PAGE in a 10% Tricine gel. Obtained in this manner RgpA is a non-covalent complex of a catalytic 50-kDa domain with haemagglutinin-adhesin domains (18). The complex is stable for days at room temperature, months at +4°C and years if frozen and neither decrease of activity nor proteolytic degradation of any component of the complex was evident after storage. The amount of the active enzyme was determined by active-site titration using Phe-Pro-Arg-chlormethyl ketone (FPR-cmk), a highly effective irreversible inhibitor reacting

with Rgp with the equimolar stoichiometry (29). The concentration of fully activated gingipain with cysteine was calculated from the amount of inhibitor needed for complete inactivation of the proteinase. Thus, the concentration of RgpA indicated in this paper is represented as those of active gingipain.

Recombinant RgpA peptide synthesis

The RgpA adhesin domain, G⁷²¹-R¹²⁶² (rec 70 kD RgpA) containing the adhesion domains A1 and A2 was synthesized and expressed as described previously (19). The identities of the recombinant proteins were confirmed by Western blotting, with the use of a specific antibody to *P. gingivalis* proteins, and by automated N-terminal sequencing.

Bacterial procedures

Immunization and early local infection model (intra-chamber model)—All experimental protocols were approved by the Institutional Animal Care and Ethics Committee.

In order to evaluate the effect of immunization with native RgpA on the early local immune response, we used the mouse subcutaneous chamber model (10). Briefly, two titanium coil chambers were inserted subcutaneously into the dorsolumbar region of each of the anesthetized BALB/c female mice (n=8 for each group), aged 5-6 weeks. One week before and one week after chamber implantation, the animals were immunized subcutaneously with 100 µl of either native RgpA (15µg) (test group), heat-killed *P. gingivalis* strain 53977 (2.5×10^8 CFU) (positive control), adjuvant alone or saline (negative controls). Each antigen was emulsified in alum. Two weeks following chamber implantation, all four groups were infected by means of intra-chamber injection of *P. gingivalis* (5×10^8 CFU in 100 µl of PBS). Exudates from both chambers of each mouse were collected at baseline (immediately before the intra-chamber infection), at 2 hours, 48 hours, and 4 days post-infection. Each chamber was sampled once at each time point and the individual mouse was used as the unit of analysis. The exudates were centrifuged for 10 minutes at 290 g. The supernatants were collected and stored at -70°C until they were analyzed.

Immunization and experimental periodontitis model—Female BALB/c mice, aged 5-6 weeks, were immunized subcutaneously with 100 µl of either 50 µg of the 70 kD recombinant RgpA peptide (recRgpA) in alum (test group), or 2.5×10^8 CFU of heat-killed *P. gingivalis* strain 53977 (in alum) (positive control), at 3, 2, and 1 weeks prior to oral infection. The infection was carried out as described previously (30). In brief, all animals were given sulfamethoxazole/trimethoprim (0.08% and 0.016% respectively) in drinking water, *ad libitum* for 10 days. Three days following the withdrawal of antibiotics, the animals were infected with *P. gingivalis* 53977 or vehicle only (5×10^{10} CFU/ml in 0.2 ml of PBS and 2% carboxymethylcellulose). The process was repeated 3 times, once every other day. Serum samples were obtained pre-infection and post-infection for analysis of specific anti-*P. gingivalis* IgG1 and IgG2a. Forty-six days after the first infection, the mice were sacrificed (using CO₂), and hemi-maxillae were collected and prepared for bone loss measurements using the micro-computerized tomography (µCT) technique to allow quantification of residual supportive bone volume (RSBV) as previously described (31).

Analysis of cytokines

The presence of TNF α , IL-1 β , IL-4, and IL-10 in the chamber exudates was determined using ELISA, as previously described (30). The assays were based on antibody pairs matched for ELISA.

Analysis of serum anti-*P. gingivalis* IgG titers

Six weeks after immunization, blood was drawn from the mice and the sera were stored at -80°C. Ninety-six-well plates were coated overnight at 4°C with 1 μ g of *P. gingivalis* lysate / well in 0.1 M bicarbonate buffer (pH 9). The plates were washed twice with PBS-0.02% Tween 20 and blocked with PBS 10% FCS (2 hr at RT). Subsequently, mouse serum samples diluted serially in PBS were added to the wells for 3 hours incubation at RT. This was followed by four washes in PBS-0.02% Tween 20 and the addition of anti-mouse peroxidases-conjugated IgG1 and IgG2a antibodies. After incubation for 2 hours at RT, the plates were washed five times and 100 μ l / well of TMB solution was added for 5 min followed by the addition of 100 μ l TMB stop solution. Absorption was read at 450 nm using an iMARK microplate reader.

Data analysis

Data analysis was performed using a statistical software package. One-Way Analysis of Variance (ANOVA) was used to test the significance of the differences between the treated groups. When significance was established, the inter-group differences were tested for significance by *t*-test with the Student-Newman-Keuls Method correction for multiple testing. The level of significance was determined at $p < 0.05$. All results are presented as mean values $\pm$ the standard error of the mean.

Results

Local inflammation (intra-chamber model)

Intra-chamber cytokine levels following immunization with whole bacteria or native RgpA—Two hours post-infection, IL-1 β levels peaked in the *P. gingivalis*, RgpA and adjuvant groups. At 4 days post-infection, a significant decrease in IL-1 β levels was observed only in the RgpA-immunized group after 4 days (Fig 1A).

TNF- α levels were minimal and peaked at 2 hours post-infection in all four infected groups without significant differences between groups. At 48 hours and 4 days, cytokine levels dropped, with no significant differences between all groups (Fig. 1B).

IL-4 levels peaked at 2 hours post-infection only in the RgpA-immunized group, while decreased levels were observed in the heat-killed *P. gingivalis*-, adjuvant-, and saline-immunized groups. At 48 hours and 4 days, IL-4 levels were significantly higher only in the RgpA-immunized group (Fig 1C).

IL-10 levels peaked at 2 and 48 hours without significant differences between the immunized groups (Fig 1D).

Experimental periodontitis model

Alveolar bone loss following immunization with recRgpA—Due to the observed ability of immunization with purified native RgpA to modulate the host response we were interested in examining the efficacy of vaccine containing recRgpA peptide in preventing the clinical outcome of oral infection with *P. gingivalis* in mice. Our data show that mice which were immunized with the recRgpA peptide (test group) displayed significantly lower bone loss compared to the non-immunized infected group (negative control) and the level of protection was similar, with no significant differences compared to the heat-killed *P. gingivalis*-immunized group (positive control) (Fig. 2).

Antibody response

Since the type of T-cell response is reflected in IgG specificity response (IgG1 for Th2, IgG2a for Th1) (32, 33), we examined whether the antibody response against *P. gingivalis* was different following immunization with the recRgpA. Immunization with whole-cell *P. gingivalis* induced significantly higher levels of both IgG subtypes compared to recRgpA vaccine or sham vaccine. Oral infection did not affect serum IgG1 or IgG2a titers in this group (Fig 3). Immunization with recRgpA induced serum titers of IgG1, but not IgG2a (Fig. 3A and 3C). The IgG1 titers in this group were significantly higher compared to the sham-infected group, and significantly lower compared to the *P. gingivalis*-immunized mice. Following infection (Fig. 3B and 3D), the IgG1 and IgG2a titers increased significantly in the non-immunized mice.

Discussion

Gingipains are major virulent factors that expressed on the cell surfaces of all *P. gingivalis* strains with more than 98% identity in the amino acid sequence (21). As such, gingipains have been suggested as candidate antigens for vaccines. In the first part of the present study, we examined the ability of immunization with purified native RgpA to modulate the early local immune response. The results demonstrate that immunization of mice with native RgpA has the ability to shift the early local immune response toward an anti-inflammatory response (Fig. 1). As noted previously (23), we found that IL-4 is the prominent cytokine which protects from both inflammation and alveolar bone loss. We have observed that immunization with native RgpA reduces IL- β levels. This may explain why both the present study (Fig. 2) and previous studies have demonstrated the ability of immunization with RgpA or RgpA-Kgp adhesion complex to protect against alveolar bone loss following oral infection (24, 34). IL- β has the ability to stimulate degrading proteinases and induce osteoclastogenesis leading to bone loss (35). These results are reinforced by those from previous studies, which demonstrated that RgpA plays a central role in *P. gingivalis* pathogenicity (6, 7, 10) and can be used as an antigen in immunization against experimental periodontitis (22, 23). In light of all these observations, we decided to focus on the ability of RgpA to act as an immunogen against *P. gingivalis*-induced infection in the context of experimental periodontitis in mice. Previous studies have shown that immunization with purified gingipains reduces *P. gingivalis* colonization (23, 24). It also has the ability to affect the host immune response (10, 22, 23) and protect against a *P. gingivalis* challenge in the chamber infection model (36); the murine lesion model (25); and experimental periodontitis

following oral infection (23, 24, 34, 36). Unlike these previous studies, we decided to test the effect of immunization with a recombinant peptide derived from the *rgpA* gene. Although recombinant peptides are different from purified proteins due to post-translational changes, the use of recombinant proteins as antigens in vaccines has several advantages. These include the elimination of the need to cultivate the pathogen, and the ability to avoid vaccines containing live attenuated and inactivated or killed microorganisms (37). Of note, the RgpA-Kgp complexes contain a large glucan moiety thought to be an anionic-LPS (A-LPS) covalently decorating gingipains (38-40). Nevertheless, the immune response elicited by complexes was similar to that elicited by polypeptide chain alone of domains A1 and A2 of RgpA. This argues against contribution of A-LPS to induction of protective antibodies by immunization with whole *P. gingivalis* cells.

Using the oral infection model (30), we tested the efficiency of immunization with a 70 kD recombinant peptide, containing the A1 and A2 domains of the adhesion part of RgpA (G⁷²¹-R¹²⁶²) in protecting mice from experimental periodontitis following oral infection with *P. gingivalis*. Our results demonstrate that this immunization protocol partially prevented the induced bone loss, similarly to the protection achieved with a whole-bacteria vaccine (Fig 2). To the best of our knowledge, this is the first work demonstrating the efficiency of immunization with a recombinant RgpA peptide in preventing bone loss in a murine model of periodontitis. These results are in accordance with the results of previous studies (23, 24, 34).

The importance of the humoral response to the severity of human periodontal disease has been investigated in many studies (41, 42). Some studies have shown that elevated serum levels of anti-*P. gingivalis* IgG have been directly linked to disease severity. However, others have found that the levels of antibodies in the gingival crevicular fluid are inversely related to the number of organisms at the site of sampling (43, 44). In a clinical study, anti-*P. gingivalis* IgG levels at periodontitis sites were found to be lower than at gingivitis sites in the same subjects, suggesting that a failure of local antibody production may contribute to the transition from gingivitis to periodontitis (45). Gibson *et al.* showed a correlation between IgG titer to *P. gingivalis* RgpA and opsonic uptake of *P. gingivalis* by PMNs in patients with aggressive periodontitis. These results demonstrate that RgpA specific antibodies are critical to providing immunological support for opsonization of *P. gingivalis* by the host (46). O'Brien-Simpson *et al.* showed that the sera of patients with periodontal disease possess gingipain-specific IgG predominantly of the subtype IgG4, and a negative correlation between serum IgG4 levels and oral bone loss was shown to exist (47). In light of the above, the ability of vaccines to induce a direct humoral response against *P. gingivalis* and *P. gingivalis* gingipains together with the manner in which this response affects the severity of experimental periodontitis in the murine model has been investigated extensively (23, 25, 34, 48). Klausen *et al.* (48) demonstrated that rats immunized with *P. gingivalis* produced elevated levels of serum antibodies, and that these animals were protected against *P. gingivalis*-induced bone loss. Gibson and co-workers (34) showed that immunization of mice with purified RgpA can stimulate production of both *P. gingivalis* and RgpA-specific IgG primarily directed at the adhesion domain of RgpA. The elevated levels of this antibody coincide with protection against oral bone loss elicited by *P. gingivalis*. In another study, Yasaki-Inagaki and co-workers (49) examined the ability of different recombinant peptides

derived from RgpA and Kgp to induce protective antibodies against various strains of *P. gingivalis*. The results showed that immunization with recombinant A1 domain of RgpA not only resulted in the highest titer and the strongest reactivity to *P. gingivalis* whole cells, but also in high opsonization against both non-invasive and invasive strains of *P. gingivalis*. In addition, antibodies against the recombinant A1 domain of RgpA were found to strongly induce the killing of *P. gingivalis* by neutrophils. These data suggest that an antibody directed at the A1 domain of RgpA is capable of inducing a protective immune response against infection by strains of *P. gingivalis*. In the present study, we examined how immunization with the 70kD recombinant peptide of RgpA (domains A1 and A2) influences the serum titers of anti-*P. gingivalis* IgG1 and IgG2a before and after oral infection with *P. gingivalis*. Our results showed that anti-*P. gingivalis* IgG1 titers, but not IgG2a titers, increased significantly following immunization and pre-infection (Fig 3A and 3C). Since IgG1 is representative of a Th2 cytokine response (32, 33), the high IgG1 titers found in the immunized group suggest a protective Th2 response. In contrast, IgG2a titers are significantly higher only in the heat-killed *P. gingivalis*-immunized mice (Fig 3C), with no significant differences in comparison to the non-immunized group. These results confirm those of O'Brien-Simpson *et al.* (23), who showed that immunization with RgpA-Kgp complexes induces high IgG1 and low IgG2a titers.

In summary, we are suggesting that immunization of mice with native RgpA shifts the early local immune response toward an anti-inflammatory response, which may in turn modulate the humoral response to a protective IgG1 response. The anti-inflammatory early local response, along with an elevated IgG1 response, coincides with protection against *P. gingivalis*-induced alveolar bone loss. Taken together, these data suggest that the 70 kD recombinant RgpA peptide may be a good candidate for a vaccine for the prevention of *P. gingivalis*- related periodontitis.

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Figure 1.

The impact of immunization on the early local immune response. Mice were immunized twice with heat-killed *P. gingivalis* P.g HK (positive control), RgpA (test), adjuvant (negative control), or saline (negative control), and intra-chamber infection with *P. gingivalis* was carried out one week following the last immunization. (A.) IL-1 β , (B.) TNF- α , (C.) IL-4 and (D.) IL-10 levels in the exudates obtained from the subcutaneous chambers were analyzed at 2 hours, 48 hours and 4 days after infection using ELISA. Bars represent mean values $\pm$ standard error of the mean. *- Significantly different compared to the baseline group, \$- Significantly different compared to the RgpA, and baseline groups, ^- Significantly different compared to all other groups. ANOVA, $p < 0.05$.

Figure 2.

Impact of infection and immunization on residual supportive bone volume (RSBV). Mice were not infected (RSBV control), or immunized with the recombinant 70kD peptide (test group), heat killed *P. gingivalis* (P.g HK) (positive control), or infected without prior immunization (negative control). Forty-six days following the oral infection, hemi-maxillae were evaluated for RSBV (see Methods). Bars represent mean values $\pm$ standard error of the mean. * - Significantly different compared to the infected, non-immune group. # - Significantly different compared to the non-immune and non-infected group. ANOVA, $P < 0.01$.

Figure 3.

The effect of immunization with the recombinant 70kD peptide derived from RgpA (70kD) on serum anti-*P. gingivalis* IgG1 and IgG 2a titers. Serum samples were obtained after immunization pre-infection (A. and C.) and post-infection (B. and D.). Bars represent mean values $\pm$ standard error of the mean. * - The differences between all groups are significantly different, # - Significantly different compared to all other groups. ANOVA, $p < 0.05$.