Kgp and RgpB, but Not RgpA, Are Important for Porphyromonas gingivalis Virulence in the Murine Periodontitis Model

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The contributions of three proteinase genes (rgpA, rgpB, and kgp) to the virulence of Porphyromonas gingivalis W50 were investigated in the murine periodontitis model. Mice were orally inoculated with eight doses (1 × 10⁸ cells per dose) of rgpA, rgpB, kgp, rgpA rgpB, or rgpA rgpB kgp isogenic mutants, and the level of alveolar bone loss, immune response induced, and number of bacterial cells per half maxilla were compared with those of animals inoculated with wild-type P. gingivalis. The kgp, rgpB, rgpA rgpB, and rgpA kgp isogenic mutants induced significantly (P < 0.05) less bone loss than the rgpA isogenic mutant and the wild type did, and the virulence of the rgpA isogenic mutant and the wild type were not significantly different. Mice inoculated with the wild type or the rgpA isogenic mutant exhibited significantly (P < 0.01) more P. gingivalis cells per half maxilla than mice inoculated with rgpB, kgp, rgpA rgpB, and rgpA kgp isogenic mutants or nonchallenged mice did, as determined using real-time PCR. A significant positive correlation was found between the number of P. gingivalis cells detected per half maxilla and the amount of alveolar bone loss induced. Enzyme-linked immunosorbent assay results showed that each isogenic mutant and the wild type induced a predominant antigen-specific immunoglobulin G3 (IgG3) response. Furthermore, the kgp and rgpA rgpB kgp isogenic mutants induced significantly (P < 0.05) lower IgG3 antibody responses than the responses induced by the wild type or the rgpA, rgpB, and rgpA rgpB isogenic mutants. The results suggest that the order in which the proteinases contribute to the virulence of P. gingivalis in the murine periodontitis model is Kgp ≧ RgpB ≧ RgpA.

Chronic periodontitis is an inflammatory disease of the supporting tissues of teeth involving alveolar bone resorption, which can lead to eventual tooth loss (13, 33). The presence of a consortium of gram-negative bacteria in subgingival plaque has been associated with the development of chronic periodontitis (47). In this consortium, Porphyromonas gingivalis has been identified as a major pathogen (31). A number of virulence factors have been reported to contribute to the pathogenicity of P. gingivalis (reviewed in reference 31). Among these virulence factors, the Arg- and Lys-specific cysteine proteinases and their associated adhesins are considered major virulence determinants in the onset and progression of chronic periodontitis (14, 27). The Arg-specific proteinase activity of P. gingivalis is encoded by two genes designated rgpA and rgpB, and the Lys-specific proteinase activity is encoded by one gene designated kgp (8). The rgpA gene encodes a polypeptide consisting of an N-terminal preprofragment followed by a 45-kDa Arg-specific, calcium-stabilized cysteine proteinase RgpA_{34} (catalytic domain of RgpA) and four sequence-related adhesin domains RgpA_{31}, RgpA_{32}, RgpA_{33}, and RgpA_{34} (37, 45, 50). The rgpB gene encodes an N-terminal preprofragment followed by the Arg-specific polypeptide RgpB without the C-terminal adhesin extension of RgpA (43). The kgp gene encodes a polypeptide with an N-terminal preprofragment followed by a 48-kDa Lys-specific cysteine proteinase Kgp_{34} and five C-terminal adhesin domains Kgp_{31}, Kgp_{32}, Kgp_{33}, Kgp_{34}, and Kgp_{35} (44, 50). The proteins encoded by rgpA and kgp of P. gingivalis strain W50 have been characterized as cell surface complexes of noncovalently associated proteinases and adhesins designated the RgpA-Kgp complex (27, 36).

The pathogenicity of P. gingivalis and its virulence factors has been examined using a variety of experimental animal models (reviewed in references 12 and 38). Among these experimental animal models, the murine lesion model has been widely used as a model to study the virulence of P. gingivalis (10, 24). Spontaneous P. gingivalis mutants with reduced Arg- and Lys-specific proteinase activity and wild-type P. gingivalis treated with an inhibitor of trypsin-like proteinases have been reported to be avirulent in the murine lesion model (18). Further, P. gingivalis W50 isogenic mutants lacking either rgpA, rgpB, or kgp exhibited significantly reduced pathogenicity compared to the wild-type strain in this model (29). In these experiments, the kgp isogenic mutant was the least virulent, followed by rgpB and rgpA mutants, respectively. Yoneda et al. (51) have also reported that an rgpA rgpB double mutant and kgp mutant induced significantly smaller lesions in mice than wild-type P. gingivalis did, while the rgpA rgpB kgp triple mutant did not induce lesions at all. These studies suggest that the rgpA, rgpB, and kgp gene products are important for the virulence of P. gingivalis.

Despite being widely used, the murine lesion model, where the bacteria are injected subcutaneously, is not an ideal model for the study of periodontitis because it does not assess the abilities of the bacteria to colonize intra- orally and to induce...
periodontal (alveolar) bone resorption. For these reasons, animal models of periodontitis have been developed. As the rodent molar periodontal anatomy resembles that of humans (12, 22, 35), mice and rats have been used as models for periodontal bone loss induced by pathogenic bacteria introduced into the oral cavity. The pathological processes of periodontal destruction in the rodent model, including alterations in epithelia, destruction of connective tissue of the gingiva and periodontal ligament, and resorption of alveolar bone that occurs on subgingival implantation of a periodontal pathogen, are very similar to those in humans, making the rodent a valuable model to study Porphyromonas gingivalis-induced disease (12, 30, 34). We have developed and modified the rodent periodontitis model (3, 11) to show that oral inoculation with a defined, viable inoculum of P. gingivalis produces reproducible periodontal bone loss in BALB/c mice and Sprague-Dawley rats (30, 40). Furthermore, in both the mouse and rat periodontitis models, the RgpA-Kgp complex when used as a vaccine prevented P. gingivalis-induced bone loss (30, 40). In these models, it was shown that a Th2 antibody response (immunoglobulin G2 [IgG2] and IgG1) directed towards the RgpA-Kgp complex inhibited bone loss and P. gingivalis intra-oral colonization, respectively, as analyzed by DNA probe analysis of subgingival plaque samples (30, 40). This protection was attributed to specific antibodies directed towards adhesin binding motifs of the RgpA-Kgp complex blocking binding of P. gingivalis to subgingival plaque microorganisms and host tissue, hence preventing colonization (30).

These results suggest that the Arg- and Lys-specific proteinases of P. gingivalis and their associated adhesins may play a significant role in the establishment of P. gingivalis in subgingival plaque and in the induction of alveolar bone loss.

In the present study, the contributions of RgpA, RgpB, and Kgp to intra-oral colonization, alveolar bone loss, and the immune response induced by P. gingivalis were investigated using rgpA, rgpB, kgp, rgpA rgpB, and rgpA rgpB kgp isogenic mutants in the murine periodontitis model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Lyophilized cultures of the P. gingivalis W50 wild-type strain and isogenic mutants, rgpA (W501), kgp (W50KIA), rgpB (W50D7), and rgpA rgpB (W50AB) mutants, were obtained from the culture collection of the Cooperative Research Centre for Oral Health Science, University of Melbourne, Australia, and have been described before (29, 30). The rgpA rgpB kgp triple mutant (W50ABK) was generated for this study (see below). Bacteria were maintained in an anaerobic chamber (MK3 anaerobic workstation; Don Whitley Scientific Ltd., Shipley, England) at 37°C on horse blood agar plates supplemented with 10% (vol/vol) lysed horse blood. Bacterial colonies were used to inoculate heart infusion medium containing 5 μg/mL hemin and 0.5 μg/mL cysteine; for growth of the rgpA (W501), rgpB (W50D7), and kgp (W50KIA) P. gingivalis W50 isogenic mutants, the medium also contained 10 μg/mL erythromycin (29). For the rgpA rgpB (W50AB) isogenic mutant, the medium also contained 1 μg/mL tetracycline and 10 μg/mL chloramphenicol. Batch culture growth was monitored at 650 nm using a spectrophotometer (model 295E; Perkin-Elmer). Culture purity was routinely checked by Gram staining and by colony morphology.

Generation of the rgpA rgpB kgp P. gingivalis isogenic mutant (W50ABK). The P. gingivalis W50 isogenic mutant (W50ABK) lacking RgpA, RgpB, and Kgp was generated using the rgpA rgpB isogenic mutant (W50AB) and pNS1 (1) containing kgp inserted via a kanamycin resistance cassette. The kgp::ermF4 insert of pNS1 was amplified by PCR and electroporated into P. gingivalis W50AB to generate W50ABK. Disruption of kgp in W50ABK was confirmed by Southern blot analysis whereby chromosomal DNA was probed with a 2.1-kb KpnI-BamHI ErnF/AM cassette and with a 3.3-kb BamHI fragment from pNS1 encoding the catalytic domain of Kgp (44). Whole-cell assays of W50ABK using Arg- and Lys-chromogenic substrates showed that the mutant was devoid of RgpA/B and Kgp proteolytic activity (29).

Murine periodontitis model. The mouse periodontitis experiments were performed as described previously (30) and were approved by the University of Melbourne Ethics Committee for Animal Experimentation. BALB/c mice 6 to 8 weeks old (10 mice per group) were given kanamycin (Sigma-Aldrich, New South Wales, Australia) at 1 mg/ml in deionized water ad libitum for 7 days. Three days after the antibiotic treatment, mice were orally inoculated four times 2 days apart with 1 × 1010 viable P. gingivalis W50 or P. gingivalis rgpA, rgpB, kgp, rgpA rgpB, or rgpA rgpB kgp isogenic mutants (25 μl) in PG buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, and 5 mM cysteine-HCl, pH 8.0) containing 2% (wt/vol) carboxymethyl cellulose (CMC; Sigma-Aldrich, New South Wales, Australia), and a control group was sham infected with PG buffer containing 2% (wt/vol) CMC alone. The inocula were prepared in the anaerobic chamber and then immediately applied to the gingival margin of the maxillary molar teeth. Two weeks later, mice received another four doses (2 days apart) of 1 × 1010 cells of viable P. gingivalis W50 or the isogenic mutants (25 μl) in PG buffer containing 2% (wt/vol) CMC. The number of viable bacteria in each inoculum was verified by enumeration on blood agar. Mice were fed a soft powdered diet (Barastock, Australia) and housed in cages fitted with a raised wire mesh bottom to prevent access to bedding. Four weeks after the last dose, mice were bled from the retrobulbar plexus and killed, and the maxillae were removed and cut in half (right) for use as alveolar bone loss measurement and the other half (left) used for real-time PCR.

The right half maxillae were boiled (1 min) in deionized water, mechanically defleshed, and immersed in 2% (wt/vol) potassium hydroxide (16 h, 25°C). The half maxillae were then rinsed twice with deionized water and processed in 3% (wt/vol) hydrogen peroxide (6 h, 25°C). After the half maxillae were washed (two times with deionized water), they were stained with 0.1% (wt/vol) aqueous methylene blue, and a digital image of the buccal aspect of each maxilla was captured with an Olympus DP12 digital camera mounted on a dissecting microscope, using OLYSIA BioReport software version 3.2 (Olympus Australia Pty Ltd., New South Wales, Australia) to assess horizontal bone loss. Horizontal bone loss is loss occurring in a horizontal plane, perpendicular to the alveolar bone crest (ABC) that results in a reduction of the crest height. Each half maxilla was aligned so that the molar buccal and lingual cusps of each image were superimposed, and the image was captured with a micrometer in frame, so that measurements could be standardized for each image. The area from the cementoenamel junction to the ABC for each molar tooth was measured using OLYSIA BioReport software version 3.2 imaging software. Bone loss measurements were determined twice by a single examiner using a randomized and blinded protocol.

Determination of subclass antibody by an ELISA. To determine the subclass antibody responses of mouse sera, enzyme-linked immunosorbent assays (ELISAs) were performed in triplicate using a 5-μg/ml solution of P. gingivalis W50 outer membrane protein (OMP) (see below) in phosphate-buffered saline (PBS) containing 0.1% (vol/vol) heat-inactivated fetal bovine serum (1.1 mg/ml bovine serum albumin, Sigma, New South Wales, Australia), and 0.1% (vol/vol) Tween 20 (PBST) to coat wells of flat-bottom polystyrene microtiter plates (Dynalab Laboratories, McLean, VA). After removal of the coating solution, PBST containing 2% (wt/vol) skim milk powder was added to wells to block the uncoated plastic for 1 h at room temperature. After the wells were washed four times with PBST, serial dilutions of mouse sera in PBST containing 0.5% (vol/vol) skim milk (5K-PBST) were added to each well and incubated for 16 h at room temperature. After the wells were washed six times with PBST, a 1/2,000 dilution of goat IgG to mouse IgM, IgA, IgG2a, IgG2b, or IgG3 (Sigma, New South Wales, Australia) was added in 5K-PBST and allowed to bind for 2 h at room temperature. Plates were washed six times in PBST, and a 1/5,000 dilution of horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (Sigma, New South Wales, Australia) in 5K-PBST was added to each well and incubated for 1 h at room temperature. After the wells were washed nine times with PBST, bound antibody was detected by the addition of 100 μl of ABTS substrate [0.9 mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6) sulfonic acid in 80 mM citric acid containing 0.005% (vol/vol) hydrogen peroxide, pH 4.0] to each well. The optical density at 415 nm was measured using a microplate reader (Bio-Rad microplate reader, model 450).

Western blot of P. gingivalis outer membrane protein preparation probed with P. gingivalis sera. P. gingivalis outer membrane protein preparation was probed with sera from mice inoculated with P. gingivalis W50 or with the protease isogenic mutants in a Western blot. P. gingivalis W50 OMP was extracted using Triton X-114 as described previously (36). P. gingivalis OMP was precipitated by the addition of trichloroacetic acid to a final concentration of 10% (vol/vol) and incubation for 20 min at 4°C. Precipitated protein was collected by centrifugation.
(10 min, 16,000 × g) and resuspended in 20 μl of reducing sample buffer (10% [wt/vol] SDS, 0.05% [wt/vol] bromophenol blue, 25% [vol/vol] glycerol, and 0.05% [vol/vol] 2-mercaptoethanol). The pH was adjusted to pH 8.0 with 1.5 M Tris-HCl, and then the solution was heated for 5 min at 100°C. *P. gingivalis OMP (10 μg/lane) was loaded onto Novex 12% (wt/vol) Tris-glycine precast mini gels, and electrophoresis was performed using a current of 30 to 50 mA and a potential difference of 125 V using a Novex electrophoresis system (Novex, San Diego, CA). For Western blot analysis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Problott; Applied Biosystems, New South Wales, Australia) using a transblot cell (Bio-Rad, New South Wales, Australia). The PVDF membrane was wetted in 100% methanol and soaked in transfer buffer [10 mM 3(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid–10% (vol/vol) methanol, pH 11.5] for 1 min. Transfer was performed using a potential difference of 60 V for 90 min. The PVDF membrane was blocked with 2% (wt/vol) skim milk powder in Tris-sodium chloride (TN) buffer (50 mM Tris-HCl, pH 7.4) for 2 h at room temperature. The membrane was then incubated overnight at room temperature with *P. gingivalis W50 and isogenic mutant sera at a dilution of 1:25 in TN buffer. After incubation, the membrane was washed four times in TN buffer containing 0.05% (vol/vol) Tween 20 and then incubated with a 1:200 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma, New South Wales, Australia) for 2 h at room temperature. The membrane was then washed four times with TN buffer containing 0.05% (vol/vol) Tween 20, and the bound antibodies were detected by 0.005% (wt/vol) 4-chloro-1-naphthol in TN buffer containing 16.6% (vol/vol) methanol and 0.015% (wt/vol) hydrogen peroxide. A digital image of the Western blot was captured using a FujiFilm LAS 3000 image analyzer (FujiFilm, New South Wales, Australia), and the relative intensity (densitometry) of each lane of the Western blot was measured by FujiFilm multigauge imaging software (FujiFilm, New South Wales, Australia).

**Determination of *P. gingivalis* cell numbers per mouse half maxilla using real-time PCR.** Genomic DNA was isolated from the left half maxillae (including soft and hard tissues) using a DNeasy tissue kit following the animal tissue isolation protocol as described by the manufacturer (QIAGEN Pty Ltd., New South Wales, Australia). The amount and quality of DNA present in the samples was determined using spectrophotometer readings (A_{260} and A_{280}, respectively) and the Quant-iT DNA assay kit (Molecular Probes, Invitrogen, Mt. Waverley, Victoria, Australia) according to the manufacturer’s instructions with fluorescence measured on a Wallace 1420 multilabel counter (Perkin-Elmer, Wettlesy, MA). Each half maxilla sample was diluted to contain 10 ng/μl DNA and analyzed by real-time PCR. *P. gingivalis* standard curve was created using known amounts of *P. gingivalis* DNA (serial dilutions from 1 × 10^{10} to 1 × 10^{3} cells) added to half maxilla DNA from a noninfected mouse diluted to 10 ng/μl.

The cell numbers of *P. gingivalis* per half maxilla were quantified using the *P. gingivalis*-specific 16S RNA forward and reverse primers as described by Kuboniwa et al. (19) and the platinum SYBR green supermix UDG kit (Invitrogen, New South Wales, Australia). Using these primers, only one PCR product was produced as confirmed by melt curve analysis and agarose gel electrophoresis.

Real-time PCR was carried out in triplicate using a Rotor-Gene 3000 system (Corbett Research, Australia). A reaction mixture volume of 25 μl contained half maxilla DNA (80 ng), 12.5 μl of SYBR green supermix, 400 nM concentration of each primer, and 5.0 mM MgCl₂ and was dispensed using a CAS 1200 liquid-handling robot system (Corbett Research, New South Wales, Australia). The following PCR cycling conditions were used: an initial hold at 95°C for 2 min, followed by a denaturation step at 95°C for 2 min. The following cycling conditions were repeated for 50 cycles: denaturation at 95°C for 15 s, annealing at 63°C for 30 s, and then extension at 72°C for 30 s. Fluorescence data were collected immediately following the extension step of each cycle. The specificity of the primer pairs was confirmed by melt curve analysis by heating from 72°C to 95°C in 0.2°C increments. Melting peaks were compared with the bands obtained following agarose gel electrophoresis. The detection limit for the assay using the standard curve was 5 × 10⁴ *P. gingivalis* cells.

**Statistical analysis.** The bone loss data were statistically analyzed using a one-way analysis of variance (ANOVA) and Dunnett’s T3 test and Cohen’s effect size (SPSS for Windows, release 6.0; SPSS). Effect sizes, represented as Cohen’s d (6) were calculated using the effect size calculator provided online by the United Kingdom Evidence-Based Education website (http://ecm.dur.ac.uk/ebek/research/effectsize). According to Cohen (6), a small effect size is 0.2 ≤ d < 0.5, a moderate effect size is 0.5 ≤ d < 0.8, and a large effect size is d ≥ 0.8.

The number of *P. gingivalis* cells per half maxilla as determined by real-time PCR was analyzed using a one-way ANOVA. The IgA, IgM, and IgG subclass antibody titers were statistically analyzed using Student’s t test using SPSS software (SPSS for Windows, version 12).
rgpA rrgpB kgp isogenic mutants were not significantly higher than those detected in the nonchallenged control. However, the results of effect size analysis suggested that there were more P. gingivalis cells per half maxilla detected in mice challenged with rrgpB (d = 1.63; 95% CI, 0.56–2.56) or rrgpA rrgpB (d = 1.32; 95% CI, 0.31–2.23) isogenic mutants than in mice challenged with the kgp or rrgpA rrgpB kgp isogenic mutants or the nonchallenged control. Regression analysis showed a significant positive linear relationship (P < 0.02; r² = 0.77) between the number of P. gingivalis cells detected per half maxilla and the amount of alveolar bone loss induced by P. gingivalis W50 and the proteinase isogenic mutants.

**Antibody subclass and Western blot analysis of sera from mice orally inoculated with P. gingivalis W50 (wild type) and proteinase isogenic mutants in the mouse periodontitis model.**

Mice inoculated with P. gingivalis W50 and proteinase isogenic mutant strains were bled, and serum IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM titers were determined by an ELISA using a P. gingivalis W50 outer membrane protein preparation as the adsorbed antigen (Fig. 3). Mice orally inoculated with P. gingivalis W50 and proteinase isogenic mutants induced a significant serum IgG response (Fig. 3). P. gingivalis kgp, rrgpA rrgpB, and rrgpA rrgpB kgp isogenic mutants induced a significantly (P < 0.05) lower IgG response than that induced by the P. gingivalis W50 wild-type strain. However, the serum IgG response induced by the rrgpA or rrgpB isogenic mutants was not significantly different from that induced by the wild type. Regression analysis showed a significant positive linear relationship (P < 0.007; r² = 0.863) between the serum IgG response and the level of alveolar bone loss induced by P. gingivalis W50 and the proteinase isogenic mutants. A significant exponential relationship (P < 0.05; r² = 0.790) was also observed between the serum IgG levels and the number of P. gingivalis W50 and proteinase isogenic mutant cells detected per half maxilla. The predominant serum IgG subclass induced by each P. gingivalis strain was IgG3, followed typically by IgG2a > IgG2b > IgM > IgA = IgG1 responses (Fig. 3). P. gingivalis kgp and rrgpA rrgpB kgp isogenic mutants induced a significantly (P < 0.05) lower IgG2a and IgG3 responses than that induced by the P. gingivalis W50 wild-type strain. However, the IgG2a and IgG3 responses induced by the rrgpA, rrgpB, and rrgpA rrgpB isogenic mutants were not significantly different from those induced by the wild type. No antibody response to P. gingivalis OMP was detected in sera from nonchallenged (control) mice. Regression analysis showed a significant positive linear relationship (P < 0.01; r² = 0.843) between the serum IgG3 response and the level of alveolar bone loss induced by P. gingivalis W50 (wild type) and the proteinase isogenic mutants. Furthermore, a significant exponential relationship (P < 0.05; r² = 0.78) was observed between the serum IgG3 levels and the number of P. gingivalis W50 wild-type and proteinase isogenic mutant cells detected per half maxilla.

Sera from mice orally inoculated with the P. gingivalis W50 wild-type strain and proteinase isogenic mutants were used to probe a P. gingivalis W50 OMP Western blot preparation in a Western blot (Fig. 4). Western blot densitometric analysis (relative intensity) of the individual lanes was used to compare the antibody response induced by P. gingivalis W50 (wild type) to that of each of the proteinase isogenic mutants. The highest intensity was observed for wild-type P. gingivalis W50, and this intensity was set at 100% (Fig. 4). The relative intensity observed for the rrgpA isogenic mutant was 95% of that observed for P. gingivalis W50. A decrease in the relative intensity compared to that of wild-type P. gingivalis W50 was found for the rrgpB isogenic mutant (64%), and a similar decrease was found for the rrgpA rrgpB isogenic mutant (70%). The kgp and rrgpA rrgpB kgp isogenic mutants induced the weakest antibody response with relative intensities of 37% and 31%, respectively, compared with that of the P. gingivalis W50 wild-type strain. Statistical
FIG. 4. Western blot analysis of P. gingivalis outer membrane preparation using anti-P. gingivalis sera. P. gingivalis outer membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and probed with anti-rgpA, rgpB, kgp, anti-rgpA rgpB mutant sera, anti-kgp mutant sera, anti-rgpB mutant sera, anti-rgpA mutant sera. P. gingivalis W50 (wild type [wt]) sera, and nonchallenged control sera. The positions of molecular mass markers (in kilodaltons) are shown to the left of the gel.

Analysis indicated a significant positive linear relationship ($P < 0.01; r^2 = 0.934$) between the relative intensity of the antibody response as determined by Western blot densitometry and the level of alveolar bone loss induced by the P. gingivalis W50 wild-type strain and the proteinase isogenic mutants. Furthermore, a significant exponential relationship ($P < 0.01; r^2 = 0.89$) was observed between the relative intensity of the antibody response as determined by Western blot densitometry and the number of P. gingivalis W50 wild-type and isogenic mutant cells detected per half maxilla by real-time PCR.

**DISCUSSION**

In this study, the ability of the P. gingivalis W50 wild-type strain and kgp, rgpA, rgpB, rgpA, and rgpA, rgpB kgp isogenic mutants to induce alveolar bone loss, colonize the maxilla, and induce an immune response was studied using the murine periodontitis model. The kgp and rgpB isogenic mutants induced significantly ($P < 0.05$) less alveolar bone loss than the wild type did. The results of effect size analysis suggested that the kgp isogenic mutant was less virulent than the rgpB isogenic mutant was. In contrast, inactivation of the rgpA gene did not affect the ability of P. gingivalis to induce alveolar bone loss. The results of effect size analysis suggested that the rgpA, rgpB, kgp isogenic mutant was the least effective at inducing alveolar bone loss, suggesting a possible additive effect between Kgp and RgpB in the virulence of P. gingivalis. However, no additive effect between RgpB and RgpA in inducing alveolar bone loss was observed, as mice inoculated with the rgpA, rgpB isogenic mutant were found to have levels of alveolar bone loss similar to those of the mice inoculated with the rgpB isogenic mutant. These data suggest that the order in which the proteinases contribute to the induction of alveolar bone loss in the murine periodontitis model is Kgp $\geq$ RgpB $\gg$ RgpA, which is consistent with the contribution of these proteinases to the virulence of P. gingivalis in the murine lesion model (29).

The number of P. gingivalis cells per half maxilla of each mouse was determined using real-time PCR. Previous studies have used real-time PCR to quantify periodontopathic bacteria from human subgingival plaque samples, and the technique is a more reliable, sensitive and rapid method of enumerating bacteria than culturing is (2, 25, 26, 42, 46). In this study, the number of P. gingivalis cells per half maxilla in mice inoculated with the kgp isogenic mutant or rgpB isogenic mutant were found not to be significantly different from that of nonchallenged mice, thus suggesting that Kgp and RgpB are essential for intra-oral colonization. Furthermore, the smaller number of P. gingivalis cells per half maxilla of mice inoculated with the kgp isogenic mutant compared to the number of mice inoculated with the rgpA isogenic mutant indicates that Kgp has a greater role in facilitating P. gingivalis colonization than RgpA does. A role for RgpA in colonization is also possible, as the number of rgpA isogenic mutant cells detected per half maxilla was significantly ($P < 0.05$) lower than that for wild-type cells (Fig. 2). RgpA and Kgp have been implicated in the adherence of P. gingivalis to oral epithelial cells (4, 5) and have also been reported to play a significant role in the coaggregation of P. gingivalis with other plaque bacteria, such as Prevotella intermedia and streptococcal species (15–17). This may help explain the role suggested in this study for these proteinase-adhesin complexes in the intra-oral colonization of P. gingivalis.

The greater role for RgpB over RgpA may be due in part to the role attributed to RgpB as a processing enzyme, involved in the processing of a number of P. gingivalis outer membrane virulence factors, for example, fimbriae, which may also aid intra-oral colonization (39). The significance of Kgp in colonization and pathogenicity may also be partly explained by its role in hemoglobin binding, degradation, and heme accumulation, which is essential for growth and virulence of P. gingivalis (9, 21, 23, 32). Thus, a reduction in the ability of the P. gingivalis kgp isogenic mutant to accumulate heme would result in reduced growth and virulence and consequently therefore may help explain the lower intra-oral recovery of this mutant.

In this study, we found that there was a highly significant correlation between the number of P. gingivalis cells per half maxilla and the severity of alveolar bone loss. These findings support previous human studies where the level of P. gingivalis in subgingival plaque has been associated with the severity of periodontitis as measured by periodontal pocket depth and loss of clinical attachment (20, 26, 28, 47).

Western blot densitometry of a P. gingivalis W50 OMP preparation, probed with sera from mice orally inoculated with the P. gingivalis W50 wild-type strain and proteinase isogenic mutants, showed that mice inoculated with the wild type and the rgpA isogenic mutant induced the strongest P. gingivalis-specific serum antibody responses. In contrast, compared to mice inoculated with the wild type, mice inoculated with the RgpB and rgpA, rgpB isogenic mutants were found to have a weaker P. gingivalis-specific serum antibody response, and the serum antibody response was weaker still in mice inoculated with the kgp and rgpA, rgpB, kgp isogenic mutants. Furthermore, significant positive correlations were observed between the densito-
metric values and the level of alveolar bone loss and number of
Porphyromonas gingivalis cells per half maxilla. The induction of a lower P.
gingivalis-specific antibody response and alveolar bone loss in mice inoculated with the rggB, rggA rggB, kgp, and rggA rggE kgp isogenic mutants compared to those in mice inoculated with the wild type or the rggA isogenic mutant reflects the poor intra-oral colonization by these isogenic mutants in this study. The results of ELISA analysis of mouse sera showed that challenge with P. gingivalis induced high-titer serum IgG and IgG3 responses, and this was positively correlated with the level of alveolar bone loss and the number of Porphyromonas gingivalis cells detected per half maxilla. A similar positive correlation between P. gingivalis-specific serum IgG and IgG2 levels and disease severity as measured by probing depth and loss of clinical attachment has been reported in humans with chronic periodontitis (7, 28, 41). Similar to human IgG2, mouse IgG3 is a Th1 cytokine-induced antibody, and a predominantly Th1 response is suggested to be destructive in human periodontal disease (28, 49). Furthermore, it has been shown in the murine periodontitis model that alveolar bone resorption is associated with a predominantly Th1 response (30). Thus, in these respects, the microbiology and immunopathology of the murine periodontitis model are similar to those of human disease.

In summary, the data presented here indicate that P. gingivalis kgp and rggB isogenic mutants colonize the maxilla poorly, elicit a weak P. gingivalis-specific antibody response, and induce significantly less alveolar bone loss than the wild type in the murine periodontitis model. The order in which the proteinases contributed to the virulence of P. gingivalis in this model was Kgp ≥ RgpB >> RgpA.

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