An Immune Response Directed to Proteinase and Adhesin Functional Epitopes Protects against Porphyromonas gingivalis-Induced Periodontal Bone Loss


*J Immunol* 2005; 175:3980-3989; doi: 10.4049/jimmunol.175.6.3980

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An Immune Response Directed to Proteinase and Adhesin Functional Epitopes Protects against Porphyromonas gingivalis-Induced Periodontal Bone Loss


Porphyromonas gingivalis, a pathogen associated with periodontitis, bound to fibrinogen, fibronectin, hemoglobin, and collagen type V with a similar profile to that of its major virulence factor, the cell surface RgpA-Kgp proteinase-adhesin complex. Using peptide-specific, purified Abs in competitive inhibition ELISAs and epitope mapping assays, we have identified potential adhesin binding motifs (ABMs) of the RgpA-Kgp complex responsible for binding to host proteins. The RgpA-Kgp complex and synthetic ABM and proteinase active site peptides conjugated to diphtheria toxoid, when used as vaccines, protected against P. gingivalis-induced periodontal bone loss in the murine periodontitis model. The most efficacious peptide and protein vaccines were found to induce a high-titer IgG1 Ab response. Furthermore, mice protected in the lesion and periodontitis models had a predominant P. gingivalis-specific IL-4 response, whereas mice with disease had a predominant IFN-γ response. The peptide-specific Abs directed to the ABM2 sequence (EGLATATTFEEDGVA) protected against periodontal bone loss and inhibited binding of the RgpA-Kgp complex to fibrinogen, fibronectin, and collagen type V. Furthermore, the peptide-specific Abs directed to the ABM3 sequence (GTPNPNPNPNPNPGT) protected against periodontal bone loss and inhibited binding to hemoglobin. However, the most protective Abs were those directed to the active sites of the RgpA and Kgp proteinases. The results suggest that when the RgpA-Kgp complex, or functional binding motif or active site peptides are used as a vaccine, they induce a Th2 response that blocks function of the RgpA-Kgp complex and protects against periodontal bone loss. The Journal of Immunology, 2005, 175: 3980–3989.

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth leading to resorption of alveolar bone and eventual tooth loss (1, 2). The disease is a major public health problem in all societies and is estimated to affect up to 15% of the adult dentate population, with severe forms affecting 5–6% (3, 4). The development and progression of chronic periodontitis has been associated with specific Gram-negative bacteria in subgingival plaque (5). The presence of three Gram-negative bacterial species, Tannerella forsythia (formally Bacteroides forsythus), Treponema denticola, and Porphyromonas gingivalis, in subgingival plaque has been strongly associated with disease (6). The persistence of P. gingivalis in subgingival plaque from periodontitis patients after treatment (scaling and root planing) has been reported to be significantly associated with progressive alveolar bone loss (7). Moreover, an increase in P. gingivalis cell numbers in subgingival plaque has been shown to correlate with disease severity as measured by attachment loss, periodontal pocket depth, and bleeding on probing (8). Furthermore, oral infection with P. gingivalis has been shown to induce periodontal bone loss in mice, rats, and nonhuman primates (9–11).

A number of virulence factors have been reported to contribute to the pathogenicity of P. gingivalis including the following: LPS, fimbriae, hemagglutinin, hemolysin, and extracellular hydrolytic enzymes (especially the Arg- and Lys-specific proteinases) (12). A virulence factor common to all strains of P. gingivalis is the cell surface Arg-specific (RgpA) and Lys-specific (Kgp) proteinases and their associated adhesins designated the RgpA-Kgp complex (13). Both outer membrane-associated and released forms of the proteinases are considered to be major virulence determinants in the onset and progression of chronic periodontitis (14). P. gingivalis wild-type cells treated with a protease inhibitor (N-α-p-tosyl-L-lysine chloromethyl ketone; TLCK)3 and spontaneous mutants with reduced Arg-specific and Lys-specific activity have been reported to be less virulent in animal models (15). We have recently shown in the murine lesion model that P. gingivalis W50 isogenic mutants lacking either RgpA, RgpB (Arg-specific proteinase without associated adhesins), or Kgp exhibited significantly reduced pathogenicity when compared with the wild-type strain (16). In these experiments, the Kgp mutant was the least virulent (16). We have also shown that immunization with the RgpA-Kgp complex induced Ag-specific, intraoral Abs and prevented subgingival colonization of P. gingivalis and periodontal bone loss in the rodent periodontitis model (11).

3 Abbreviations used in this paper: TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone; DT, diphtheria toxoid; RT, room temperature; ABM, adhesin binding motif; CMC, carboxymethylcellulose; ABC, alveolar bone crest; PDB, PBS/0.05% Tween; PVDVF, polyvinylidene difluoride; PMF, peptide mass fingerprinting; TFA, trifluoroacetic acid; PAS, proteinase active site; CI, confidence interval.

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Received for publication September 16, 2004. Accepted for publication July 7, 2005.

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1 This work was supported by the Australian National Health and Medical Research Council (Project No. 990199) and the National Institutes of Health (Grant 1R01 DE14198-01). N.M.O.-S. is a C.R. Roper Fellow.

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In this study, we show that _P. gingivalis_ W50 whole cells exhibit the same binding pattern to fibrinogen, fibronectin, hemoglobin, and collagen type V as the RgpA-Kgp complex, which binds to these proteins with nanomolar dissociation constants. We also show that the adhesins of the RgpA-Kgp complex are important in providing protection in the murine lesion and periodontitis models when the complex is used as a vaccine and that the immune response is predominately a Th2 response. Furthermore, we show that synthetic peptides corresponding to proteinase active site- and adhesin-binding sequences conjugated to diphtheria toxoid (DT) induce a Th2 Ab response that protects against _P. gingivalis_-induced bone loss in the murine periodontitis model. We also identify the minimal Ab binding epitope of the protective adhesin peptides and show that these Abs inhibit the binding of the RgpA-Kgp complex to host proteins.

**Materials and Methods**

**Bacterial strains and growth conditions**

_P. gingivalis_ W50 or ATCC 33277 were grown and harvested as described previously (8, 16).

**Purification of the complexes**

The isolation and purification of the RgpA complex and Kgp complex (from strain HG66) was conducted as described previously (17). The isolation and purification of the RgpA-Kgp complex (from strain W50) was performed as described previously (R. D. Pathirana, N. M. O’Brien-Simpson, and E. C. Reynolds, unpublished data). Briefly, _P. gingivalis_ strain W50 cells (2L) were grown to late exponential phase and harvested by centrifugation (7500 × _g_, 30 min, 4°C) and washed twice with PG buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, and 5 mM cysteine-HCl; pH 8.0) in the anaerobic workstation. Cells were resuspended in PG buffer, total volume 60 ml, containing 0.5% v/v Triton X-114 and gently mixed at room temperature (RT) for 45 min. The cell extract was centrifuged (7500 × _g_, 30 min, 4°C), and the collected supernatant was centrifuged (40,000 × _g_, 30 min, 4°C). The supernatant was filtered (0.2 μm) and then applied to an Ag-Sepharose column (Hiload XK 16/100 Q, Pharmacia), installed in a Pharmacia GP-250 PPLC system, in TC 50 buffer (50 mM Tris-HCl, 50 mM NaCl, and 5 mM CaCl₂; pH 7.4) at a flow rate of 1 ml/min. Nonspecifically bound proteins were eluted with a linear gradient of 0–40% TC 50 buffer (500 mM NaCl, 50 mM Tris-HCl, and 5 mM CaCl₂; pH 7.4) at a flow rate of 1.0 ml/min. The column was re-equilibrated with TC 50 buffer, and bound proteins were eluted with the same buffer containing 500 mM arginine (pH 7.4) at a flow rate of 1 ml/min. The eluant was monitored at 280 nm. All fractions were collected at 4°C and stored at −70°C before further processing.

Arginine-eluted fast protein liquid chromatography fractions were concentrated using Vivaspin 20 concentrator (10,000 m.w. cutoff) (Sartorius) by centrifugation at 3000 × _g_ for 15 min periods at 4°C until the eluant was reduced to a volume of −1 ml. The filter membrane of the Centriprep-10 Concentrator (Sartorius) was then rinsed with 1 ml of TC 50 buffer. Concentrated fractions were then desalted using a PD-10 column per manufacturer’s instructions (Amershams Biosciences).

**Immunization and murine lesion model**

The murine lesion model experiments were approved by the University of Melbourne Ethics Committee for Animal Experimentation and were conducted essentially as described previously (18). BALB/c mice 6–8 wk old (10 mice per group) were immunized s.c. (100 μl) with either 20 μg of the RgpA-Kgp complex, 50 μg of peptide-DT conjugate (prepared as described earlier (8)), i.e., adhesin binding motif (ABM)-1 DT conjugate (ABM1-DT), ABM2-DT, ABM3-DT, protease active site 1 from RgpA proteinase DT conjugate (PASI1-R-DT), and PASI1-K-DT from the Kgp proteinase, 50 μg of DT, or PBS (pH 7.4) emulsified in IFA. After 30 days, the mice were boosted with Ag (s.c. injection, emulsified in IFA) and then bled 12 days later from the retrobulbar plexus. After bleeding, mice received kanamycin (Sigma-Aldrich) at 1 mg/kg in deionized water ad libitum for 7 days. Before the antibiotic treatment, mice were orally inoculated four times, 2 days apart with 1 × 10⁸ viable _P. gingivalis_ W50 cells (25 μl) in PG buffer containing 2 g/100 ml carboxymethylcellulose (CMC; Sigma-Aldrich), and a control group was sham-infected with PG buffer containing 2 g/100 ml CMC alone. Two weeks later, mice received another four doses (2 days apart) of 1 × 10⁹ viable _P. gingivalis_ W50 cells (25 μl) in PG buffer containing 2 g/100 ml CMC. The number of viable cells in each inoculum was verified by enumeration on BH agar. Twenty-eight days after the second oral challenge, mice were killed, and the maxillae were removed.

Maxillae were boiled (1 min) in deionized water, mechanically de-fleshed, and immersed in 2 g/100 ml potassium hydroxide (16 h, 25°C). The maxillae were then washed (two times, deionized water) and immersed in 5% v/v hydrogen peroxide (6 h, 25–28°C). After washing, (two times, deionized water), the maxillae were stained with 0.1% aqueous methylene blue, and a digital image of the buccal aspect of each half maxilla was captured with a Scion Imaging digital camera (Scion) mounted on a dissecting microscope, using Adobe Photoshop version 4.0 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 buccal and lingual molar cusps of each left or right image were superimposed. A micrometer scale in plane with each half-maxilla was digitally imaged at the same time so that measurements could be standardized for each image. The area from the cementoenamel junction to the ABC for each tooth was measured using Scion Image Beta 4.02 (Scion) imaging software downloaded from the Scion Corporation web site (www.scioncorp.com/index.htm). Bone loss measurements were determined twice in a random and blinded protocol by two standardized examiners.

**ELISPOT assay**

ELISPOT assay was performed using the Millipore Multiscan 96-well filtration plates (MAHAS450; Millipore). Plates were coated with anti-mouse cytokine capture Abs (eBiosciences), specific for IL-4 (catalog no. 14-7041; clone 1B11) and IFN-γ (catalog no. 14-7312; clone R4-6A2), at a concentration of 4 μg/ml in 0.1 M sodium bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. Plates were then washed with PBS and blocked with T cell medium (DMEM containing 10% v/v heat-inactivated (56°C, 30 min) FCS, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM 2-ME, 30 μg/ml gentamicin, 100 IU/ml penicillin, and 100 μg/ml streptomycin) for 1 h at 37°C. Lymph node (inguinal and popliteal for lesion model and submandibular for periodontitis model) T cells from Ag-immunized and control mice were first positively isolated/sorted using CD90 beads and AutoMACS cell sorter (Miltenyi Biotech) per the manufacturer’s instructions and were incubated (1 × 10⁷ cells/well) with gamma-irradiated syngeneic spleen cells as a source of APCs (2 × 10⁵ cells/well) and 1 μg/ml of the Ag (RgpA-Kgp complex, Kgp complex, RgpA complex, or RgpB proteinase). Plates were incubated at 37°C in an atmosphere of 5% CO₂ in air for 48 h in a humidified incubator. The plates were then washed in PBS/0.05% Tween (PBST) three times and once with Milli-Q water. Cytokine-specific biotinylated Abs (eBiosciences) specific for IL-4 (catalog no. 14-7312; clone BV6D-24G2) and IFN-γ (catalog no. 13-7311; clone XM G1) were added at a concentration of 2 μg/ml in Dulbecco’s PBS cell medium (1/1, v/v) and incubated at RT for 2 h. Plates were washed six times in PBST, and Streptavidin-Alkaline Phosphatase conjugate (Roche) was added to the plates at a 1/1000 dilution in Dulbecco’s PBS/T cell medium (1/1, v/v) and incubated for 1 h at RT. The plates were washed six times in PBST and three times in PBS. One tablet of the substrate 5-bromo-4-chloro-3-indolyl phosphate/NBT (catalog no. B-565; Sigma-Aldrich) was dissolved in 10 ml of Milli-Q water, and 50 μl of this substrate buffer was added per well. Spots were allowed to develop for 20 min at RT, stopping the reaction by washing with tap water. The spots were counted using EliSpot Reader Lite (version 2.9; Autoimmun Diagnostika), and data were expressed as spot-forming cells per million.
ELISA

ELISAs were performed, as described previously (8, 18), in triplicate using a solution (1 μg/ml) of the RgpA-Kgp complex in 0.1 M PBS (pH 7.4) to coat wells of flat-bottom polystyrene microtiter plates (Microtiter; Dynatech Laboratories) overnight at 4°C.

Western blotting

Purified RgpA-Kgp complex was separated by SDS-PAGE in 12.5% acrylamide gels (1 mm) by using the method of Laemmli (19) with a minigel system (Bio-Rad). Proteins were either stained in the gel using Coomassie blue or electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane using the method of Dasher et al. (20). After sectioning the membrane, the m.w. standards were stained with 0.1 g/100 ml of CBB R250. The remaining sections were blocked for 1 h at 20°C with 5 g/100 ml of nonfat skin milk powder in TN buffer (50 mM Tris-HCl (pH 7.4) and 100 mM NaCl). Sections were subsequently incubated with sera diluted 1/25 with TN buffer. After 16 h at 20°C, the sections were washed (4 × TN buffer containing 0.05% v/v Tween 20 for 10 min) and then incubated for 1 h at 20°C with HRP-conjugated goat Ig directed against rabbit IgG (w/v) BSA in 0.1 M PBS. The percentage inhibition of the RgpA-Kgp complex binding to host proteins by each of the purified ABM peptide-specific IgG was calculated according to the following equation (22): percentage inhibition = 100 × (1 − (OD with IgG − background)/(OD without IgG − background)), where background is the absorbance from wells with no RgpA-Kgp complex added. IC50 was calculated from the inhibition curves (percentage inhibition vs IgG concentration). OD415 was measured using a Bio-Rad 450 microplate reader (Bio-Rad).

In-gel digestion and peptide mass fingerprinting (PMF)

Protein bands were excised from the Coomassie blue-stained SDS-PAGE gel, and gel pieces were washed in 100 mM NH4HCO3/ethanol 1:1, reduced and alkylated with DTT and iodoacetamide, and digested with sequencing grade-modified trypsin (Promega) overnight at 37°C as published previously (21). The peptide extract containing 20 mM NH4HCO3 was quenched with 2 mM 2-mercaptoethanol and 0.015 g/100 ml of H2O2. Color development was stopped by rinsing the membranes with Milli-Q water.

Binding of the RgpA-Kgp complex and P. gingivalis strain W50 to host proteins

ELISA was used to determine the affinity of the RgpA-Kgp proteinase adhesin complex and P. gingivalis strain W50 whole cell binding to fibrinogen, fibronectin, collagen types I, III, IV, and V, and hemoglobin. Wells of flat-bottom polystyrene microtiter plates (Microtiter; Dynatech Laboratories) were coated overnight at RT with 10 μg/ml of each fibrinogen, fibronectin, collagen types I, III, IV, and V, or hemoglobin in 0.1 M PBS (pH 7.4). After removing the coating solution, remaining uncoated plastic was blocked with 1% (w/v) BSA in 0.1 M PBS for 1 h at RT. The blocking solution was removed, and serial dilutions of a starting concentration of 2 μg/ml RgpA-Kgp complex from P. gingivalis strain W50, 10 μg/ml (total protein) of P. gingivalis W50 whole cells in 0.1 M PBS containing 1 mM TCKL was added and incubated for 3 h at RT. After washing (four times, 0.1 M PBS 0.05% v/v Tween 20, PBST), dilutions of rabbit anti-RgpA-Kgp complex antisera or P. gingivalis W50 antiserum (in 0.5% (w/v) BSA in 0.1 M PBS) was added to the plates and incubated for 2 h at RT. Plates were then washed (six times, PBST) and incubated with a 1/2000 dilution of HRP-conjugated goat Ig directed against rabbit IgG (Sigma-Aldrich) in 0.5% (w/v) BSA in 0.1 M PBS for 1 h at RT. Wells were then washed (six times, PBST), and bound Ab was detected as described above. ELISA results were used to derive the KD based on an analog of the Michaelis-Menten equation (22): OD = ODmax(protein)/KD + 1 (protein). Where OD is the OD at a given protein concentration, ODmax is the maximum OD for the ELISA plate reader, (protein) is the protein concentration of the analyte, and KD is the apparent KD for a given OD at a given protein concentration (ELISA data point). KD was calculated by Scatchard plot analysis of 1/(protein) vs OD (ELISA data point) by 1/(protein) vs OD (ELISA data point) by Gluskin et al. (22). ODmax was measured using a Bio-Rad 450 microplate reader (Bio-Rad).

Purification of peptide-specific IgG

IgG was purified from antisera obtained from mice immunized with ABM1, ABM2, and ABM3 peptide DT conjugates, or DT using the Immunopure protein (A/G) IgG purification kit per the manufacturer’s instructions (Pierce), and the protein concentrations of purified IgG samples were determined using the Bradford protein assay (Bio-Rad).

Competitive inhibition ELISA

Plates were coated overnight with 10 μg/ml fibrinogen, fibronectin, collagen V, or hemoglobin in 0.1 M PBS. After removal of the coating solution, remaining uncoated plastic was blocked with 1% (w/v) BSA in 0.1 M PBS for 1 h at RT then washed (four times, PBST). Two-fold dilutions of purified anti-ABM1, anti-ABM2, and anti-ABM3 IgG (in PBS containing 0.5% w/v BSA), starting concentration of 100 nM, were added to each plate followed by the addition of 0.5 μg/ml of the RgpA-Kgp complex (in PBS containing 0.5% BSA and 1 mM TCKL), with a final volume of 100 μl. Plates were incubated for 3 h at RT and probed with a 1/3200 dilution of rabbit anti-RgpA-Kgp complex antisera, and the plates were developed as described above. The percentage inhibition of the RgpA-Kgp complex binding to host proteins by each of the purified ABM peptide-specific IgG was calculated according to the following equation (22): percentage inhibition = 100 × (1 − (OD with IgG − background)/(OD without IgG − background)), where background is the absorbance from wells with no RgpA-Kgp complex added. IC50 was calculated from the inhibition curves (percentage inhibition vs IgG concentration). OD415 was measured using a Bio-Rad 450 microplate reader (Bio-Rad).

Epitope analysis of the ABM and proteinase active site (PAS) peptide sequences

The Ab binding site for each of the ABM and PAS peptides was determined by synthesizing overlapping eight or 10 residue peptides (offset by one) on a multitip peptide synthesis system (Chiron) using standard solid phase peptide synthesis protocols for Fmoc chemistry. Epitope mapping of the pin-bound peptides was conducted by ELISA as per Chiron Technologies’ instructions, using mouse sera at a dilution of 1/1000 in 1% w/v nonfat skim milk powder in 0.1 M PBS (pH 7.4) containing 0.1% w/v Tween 20. The bound Ab was detected by incubating the pins with 0.9 mM ABTS in 80 mM citric acid (pH 4.0) buffer containing 0.005% v/v H2O2. OD415 was measured using a Bio-Rad 450 microplate reader (Bio-Rad).

Statistical analysis

The maximum sizes of the lesions developed were statistically analyzed using the Kruskal-Wallis test and Mann-Whitney U Wilcoxon rank sum test with a Bonferroni correction for type 1 error (SPSS for Windows, Release 6.0; SPSS). The bone loss (mm2) data were statistically analyzed using one-way ANOVA and Dunnett’s T3 test (SPSS for Windows, Release 6.0; SPSS). Effect sizes, represented as Cohen’s (23) d were calculated using the effect size calculator provided on-line by Evidence-Based Education U.K. web site (http://cem.dur.ac.uk/eben/researcheffectsize/). According to Cohen (23), a small effect size is d ≥ 0.2 and <0.5, a moderate effect size is d ≥ 0.5 < 0.8, and a large effect size is d ≥ 0.8.

Results

Characterization of binding of P. gingivalis strain W50 whole cells and the RgpA-Kgp complex to fibrinogen, fibronectin, collagen types I, III, IV, and V, and hemoglobin

The binding of viable P. gingivalis whole cells of strain W50 and the RgpA-Kgp complex to a variety of host proteins is shown in Fig. 1, and the KD for each of the interactions was calculated using Scatchard plot analysis (Table I). P. gingivalis strain W50 was found to bind to all of the host proteins tested, with the strongest interaction being toward fibrinogen followed by fibronectin, hemoglobin, collagen types I, III, IV, and then collagen type V (Table I). The RgpA-Kgp complex was found to bind in the nanomolar range to the host proteins fibrinogen, fibronectin, collagen type V, and hemoglobin, with a similar pattern of binding as displayed by whole cells (Fig. 1, and Table I). However, unlike whole cells, the RgpA-Kgp complex did not bind to collagen types I, III, and IV. Peptide-specific IgG Abs against the synthetic peptides ABM1, ABM2, and ABM3 (Fig. 2) were used in a competitive inhibition
ELISA to determine the inhibition constants (IC_{50}) for any inhibition of binding of the RgpA-Kgp complex to the host proteins; fibrinogen, fibronectin, collagen type V, and hemoglobin. ABM1-specific IgG Abs did not inhibit the binding of the RgpA-Kgp complex to any of the host proteins tested. ABM2 IgG-specific Abs inhibited binding in the nanomolar range of the RgpA-Kgp complex to fibrinogen, fibronectin, and collagen type V, but not to hemoglobin (Table I). IgG-specific Abs for ABM3 inhibited binding in the nanomolar range of the RgpA-Kgp complex to fibrinogen, fibronectin, collagen type V, and hemoglobin. ABM1-specific IgG Abs did not inhibit the binding of the RgpA-Kgp complex to any of the host proteins tested (Table I).

**Effect of immunizing mice with the RgpA-Kgp complex, RgpA, or Kgp on P. gingivalis-induced lesions in the murine lesion model**

BALB/c mice were immunized with purified RgpA-Kgp complex, RgpA, Kgp, formalin-killed *P. gingivalis* cells (strain ATCC 33277), or adjuvant alone, and then challenged s.c. with viable *P. gingivalis* cells (strain ATCC 33277), or adjuvant alone, and then challenged s.c. with viable *P. gingivalis* W50. The RgpA-Kgp complex-immunized mice had significantly (*p < 0.01*) less bone loss than mice receiving injections with adjuvant alone or control (infected) groups (Fig. 4A). There was no significant difference in bone loss between the control (sham-infected) mice and the RgpA-Kgp complex-immunized mice that were orally challenged with *P. gingivalis*, indicating that immunization with the RgpA-Kgp complex completely protected against *P. gingivalis*-induced periodontal bone loss (Fig. 4A).

**Ab subclass and cytokine responses induced by immunization with the RgpA-Kgp complex, RgpA, or Kgp in the murine lesion and periodontitis models**

Before challenge with viable *P. gingivalis* cells either s.c. (lesion model) or intraorally (periodontal model), mice were bled, and the sera were collected by centrifugation. Fig. 5A shows the Ab subclass reactivity to the RgpA-Kgp complex for each immunogen (RgpA-Kgp complex, RgpA, or Kgp) in the murine lesion and periodontitis models. All of the proteins used to immunize mice in the lesion model induced a high-titer IgG and low-titer IgM and IgA (IgA ELISA titers 100–300) response to the RgpA-Kgp complex. Furthermore, the predominant Ab subclass of each immunogen induced was IgG1, with only weakly immunoreactive IgG2a, IgG2b, and IgG3 RgpA-Kgp complex-specific Abs detected (Fig. 5A). The same Ab subclass profile and titer of high IgG1 and low IgG2a, IgG2b, and IgG3 was induced by the RgpA-Kgp complex-immunized mice that were protected against *P. gingivalis*-induced bone loss in the periodontitis model (Fig. 5A). T cells isolated from popliteal and inguinal (lesion model) or submandibular (periodontitis model) lymph nodes from mice immunized and challenged with viable *P. gingivalis* cells were stimulated with the respective Ag (RgpA-Kgp complex, RgpA, or Kgp), and the cytokine response induced was determined by ELISPOT assay (Fig. 5, B and C). The predominant cytokine response in mice immunized and protected against either lesion development or periodontal bone loss was IL-4 (Fig. 5, B and C). However, in the periodontitis

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**Table I. The apparent K_{D} and inhibition constant (IC_{50}) for the binding of *P. gingivalis* W50 and the RgpA-Kgp complex to host proteins**

<table>
<thead>
<tr>
<th>Host protein</th>
<th>W50 Cells</th>
<th>RgpA-Kgp Complex</th>
<th>Anti-ABM2</th>
<th>Anti-ABM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>0.25 ± 0.09</td>
<td>1.86 ± 0.69</td>
<td>28.75 ± 5.86</td>
<td></td>
</tr>
<tr>
<td>Fibrinectin</td>
<td>0.83 ± 0.19</td>
<td>3.79 ± 1.22</td>
<td>18.73 ± 4.34</td>
<td></td>
</tr>
<tr>
<td>Collagen type I</td>
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<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Collagen type II</td>
<td>2.31 ± 0.29</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>1.50 ± 0.32</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Collagen type V</td>
<td>6.99 ± 0.63</td>
<td>3.67 ± 1.16</td>
<td>20.6 ± 3.84</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>1.28 ± 0.38</td>
<td>7.88 ± 1.23</td>
<td>5.00 ± 0.35</td>
<td></td>
</tr>
</tbody>
</table>

*No binding detected.

*No inhibition detected.*
model, control mice that exhibited disease (bone loss) had a predominant IFN-γ cytokine response to the RgpA-Kgp complex (Fig. 5C).

Western blot analysis of the murine Ab responses

Antisera from mice immunized with RgpA, Kgp, and the RgpA-Kgp complex (from the lesion and periodontitis models) were used to probe the RgpA-Kgp complex in a Western blot (Fig. 6A). The protective Abs to the RgpA-Kgp complex from both the periodontitis and lesion models were immunoreactive with the same proteins of molecular mass 44, 39, 35, and 30 kDa. These areas of the Coomassie blue-stained SDS-PAGE gel of the RgpA-Kgp complex were excised and subjected to in-gel digestion and PMF. The 48, 45, 44, and 39 kDa Coomassie blue-stained bands of the complex were identified as Kgpcat, RgpAcat, RgpAA1, and KgpA1, respectively (Figs. 2 and 6), as identified previously (24). The major band at 30 kDa was identified as HagAA1 (Figs. 2 and 6), and the faint band at 35 kDa was identified as a truncated KgpA1. Abs induced by RgpA alone and Kgp alone were also immunoreactive with complex proteins of molecular mass 44, 39, 35, and 30 kDa (Fig. 6A). A faint immunoreactive band at 45 kDa was also detected by antisera induced by RgpA, Kgp, and the RgpA-Kgp complex (Fig. 6A). To determine whether this response was directed to the catalytic domain of the RgpA proteinase, the RgpB proteinase (97% sequence identity to the RgpA proteinase) was probed with Abs raised against the RgpA-Kgp complex and the RgpB proteinase (Fig. 6B). The RgpB proteinase Abs were strongly immunoreactive with RgpB proteinase; however, the RgpA-Kgp complex Abs did not recognize the RgpB proteinase, suggesting that the faint band detected at 45 kDa was also derived from the A1 adhesins.

Effect of immunization with synthetic peptides on the development of P. gingivalis-induced bone loss in the murine periodontitis model

Mice were immunized (days 0 and 30) with synthetic peptides (Fig. 2) representing sequences from the RgpA-Kgp complex conjugated to DT and then orally challenged with viable P. gingivalis W50 cells. Fig. 4B shows the level of alveolar bone loss induced in immunized mice after challenge with P. gingivalis. Mice immunized with peptides PAS1K, PAS1R, ABM2, and ABM3 (Fig. 2), and the RgpA-Kgp complex exhibited significantly less bone loss than control DT mice. The RgpA-Kgp complex was significantly more effective than control DT (d = 3.47; 99.9% CI: 5.43, 1.27), ABM1 (d = 2.54; 99.9% CI: 0.86, 4.09), and ABM2 (d = 1.95; 99.9% CI: 0.43, 3.36) in protecting mice against P. gingivalis-induced bone loss (Fig. 4B). Mice immunized with the synthetic peptide-DT conjugates PAS1K-DT, PAS1R-DT, and ABM3-DT displayed significantly (p < 0.001) less bone loss compared with control DT-infected mice. Furthermore, immunization

**FIGURE 2.** Diagrammatic representation of RgpA, Kgp, and HagA showing the relative positions of the PAS and ABM epitopes. indicates putative LPS attachment site.
with PAS1K-DT was found to be significantly more effective at protecting against bone loss than immunization with ABM1-DT (d = 1.91; 99.9% CI: 0.59, 3.15), ABM2-DT (d = 1.12; 99% CI: 0.19, 1.99), and control DT (d = 2.59; 99.9% CI: 0.88, 4.01). The effect sizes suggested that PAS1K-DT was slightly more effective at protecting against bone loss than PAS1R-DT (d = 0.4; 99% CI: −0.73, 1.50) and moderately more effective compared with ABM3-DT (d = 0.59; 99% CI: −0.49, 1.66). PAS1R-DT and ABM3-DT immunizations were found to be significantly (p < 0.01) more effective (d = 1.43; 99.9% CI: 0.14, 2.66 and d = 1.38; 99% CI: 0.15, 2.55, respectively) at protecting against P. gingivalis-induced bone loss compared with ABM1-DT immunization and moderately more effective than ABM2-DT immunization (d = 0.63; 99.9% CI: −0.54, 1.77 and d = 0.50; 99% CI: −0.61, 1.59, respectively). Mice immunized with ABM2-DT exhibited significantly (p < 0.01) less bone loss than control DT-infected mice (d = 1.53; 99.9% CI: 0.11, 2.87); however, ABM1-DT-immunized mice were not protected from P. gingivalis-induced bone loss.

**Ab subclass responses induced by immunization with synthetic peptides in the murine periodontitis model**

Before oral challenge with viable *P. gingivalis* cells, mice were bled, and the sera were collected by centrifugation. Fig. 7 shows the Ab subclass reactivity to the RgpA-Kgp complex for each of the peptide Ags. PAS1K, PAS1R, and ABM3 induced high-titer IgG Abs (predominantly IgG1) that recognized the RgpA-Kgp complex. The PAS1K peptide induced a slightly higher IgG1 Ab titer than the PAS1R and ABM3 peptides. The Ab titers of PAS1K, PAS1R, and ABM3 were significantly (p < 0.001) higher than that induced by ABM1 and ABM2. Furthermore, the ABM2 peptide induced IgG1 Abs that displayed a higher titer (p < 0.01) of binding to the RgpA-Kgp complex than the IgG1 Abs induced by ABM1.

**Epitope mapping of PAS1K, PAS1R, ABM1, ABM2, and ABM3**

Overlapping eight residue peptides (offset by one) for PAS1K, PAS1R, ABM2, and ABM3 and overlapping 10 residue peptides (offset by one) for ABM1 were synthesized on resin pins. The minimal Ab binding epitope was then identified for each peptide, using the corresponding peptide-specific sera (Fig. 8). A 2-fold increase in OD (415 nm) above background was considered as a positive Ab response. The minimal Ab binding sites for each peptide were as follows: PAS1K, 215AHGSETAWAD224; PAS1R, 210GHGSETAWAD224; ABM1, 436PYPVSNLTATTQGQ450; ABM2, 572EGLTATFTEEEDGVAA166; and ABM3, 714GTPNPNNPNPNPNPGT177.

**Discussion**

A major virulence factor of *P. gingivalis* is the protease-adhesin complex, the RgpA-Kgp complex, that has been shown to be abundant on the cell surface by mass spectrometric analysis of outer membrane preparations separated by two-dimensional gel electrophoresis (24, 25). In this study, we demonstrate a similar pattern of binding of *P. gingivalis* W50 whole cells to the host proteins fibrinogen, fibronectin, hemoglobin, and collagen type V as the purified RgpA-Kgp complex. Furthermore, we identified ABMs common to RgpA, Kgp, and HagA that are likely to be responsible for binding to fibrinogen, fibronectin, hemoglobin, and collagen type V. This together with the abundance of RgpA, Kgp, and HagA on the cell surface suggests that these proteins may be the major mechanism of W50 whole cell binding to these host proteins. This suggestion is supported by the work of Shi et al. (26) who have shown that a mutant lacking RgpA, Kgp, and HagA was defective in hemoglobin binding.

The RgpA-Kgp complex bound with high affinities to fibrinogen, fibronectin, hemoglobin, and collagen type V; however, unlike whole cells, the complex did not bind significantly to collagen types I, II, and IV, suggesting that other cell surface adhesins are responsible for cellular binding to these host proteins. Both W50 whole cells and the RgpA-Kgp complex displayed high-affinity binding to collagen type V. Collagen type V has been shown to be an integral part of basement membrane of human aortas, arteries, atherosclerotic plaques, smooth muscle cells, interstitial connective tissue of human oral mucosa, and the placenta, and is a major component of the amniotic sac (27–31). Because RgpA has been shown to hydrolyze collagen types I, III, IV, and V (32, 33), the ability of the RgpA-Kgp complex to adhere to collagen type V may target the RgpA-Kgp complex and *P. gingivalis* cells to the endothelium of arterial walls as well as placental and connective tissues. These tissues may then be degraded by the RgpA proteinase causing tissue and vascular disruption. Recently, Lin et al. (34, 35) demonstrated that the fetuses of pregnant mice challenged s.c. with *P. gingivalis* were growth restricted and that there was an increase in the placental Th1/Th2 cytokine ratio with significant increases in proinflammatory (Th1) cytokines IFN-γ, TNF-α, IL-2, and IL-12 and decreases in the anti-inflammatory (Th2) cytokines IL-4 and IL-10. Furthermore, *P. gingivalis* DNA was detected in all of the placentas where the fetuses were growth restricted. A recent study by Jain et al. (36) showed that rabbits orally challenged with *P. gingivalis* developed periodontitis and had significantly greater lipid deposition in the aorta than control animals. In fact, there was a positive correlation (r² = 0.9501) between *P. gingivalis*-induced bone loss (disease severity) and the extent of lipid deposition. As well as enhancing colonization of host tissue, the binding to collagen type V may be an important factor in the association of periodontitis with coronary heart disease (37) and preterm births and low birth weights (38, 39).

Pike et al. (40) have previously shown that RgpA and Kgp bound to fibrinogen with Kd values of 8.5 nM and 4.0 nM, respectively. Although these data are in the nanomolar range, they are 2- to 4-fold higher than the Kd value for the whole RgpA-Kgp complex, suggesting that combining RgpA and Kgp to form a complex increases the valency of binding sites for fibrinogen and...
Peptides (PAS1K, PAS1R, ABM1, ABM2, and ABM3) conjugated to DT. Measurement of bone loss is the mean area measured in mm² from the cementoenamel junction to the ABC of the buccal aspect of each molar of the left and right maxilla. Data was normally distributed as measured by Levene’s homogeneity of variance and are presented as mean ± SD (n = 10) and were analyzed using the one-way ANOVA with Dunnett’s T3 test and Cohen’s effect size. *, **, Group significantly different (p < 0.01 and p < 0.001, respectively) from the control (infected (A) or DT (B) group.

Thus affinity, DeCarlo et al. (41) suggested that binding to hemoglobin by RgpA and Kgp is mediated by the 15-kDa adhesin domain HA2 (RgpA₂, Kgp₂, and HagA₂ (Fig. 2)), with an apparent Kᵦ of 2.1 ± 0.6 nM. In the current study, we found that Abs to ABM3 specifically inhibited the binding of the RgpA-Kgp complex to hemoglobin with an IC₅₀ of 5.00 ± 0.35 nM. This inhibitory Ab recognizes the -Asn-Pro- repeat motif in ABM3, and this sequence is found in the adhesin domains RgpA₁, Kgp₁, and HagA₁, but not in RgpA₂, Kgp₂ or HagA₂. These results therefore suggest that both the A1 and A2 adhesins may play a role in hemoglobin binding.

Abs specific for ABM2 were shown to inhibit the binding of the RgpA-Kgp complex to collagen type V, fibrinogen, and fibronectin and epitope mapping identified the binding epitope to be -EGL-TATTFF-EVGAA-. This sequence is repeated throughout the A1 and A3 adhesin domains of RgpA and Kgp and the A1’/” and A3 adhesins of HagA (Fig. 2; Ref. 13). The -FEED- sequence that appears in the epitope of ABM2 has been identified as the fibronectin binding motif for the fibronectin binding protein from Staphylococcus aureus (42, 43). As well as having a role in binding of the RgpA-Kgp complex to fibronectin, the -FEED- motif may also play a role in binding to fibrogen and collagen type V, although residues flanking the ABM2 binding sequence may also have a significant role in binding to these two proteins.

Immunization with the RgpA-Kgp complex provided complete protection against challenge with P. gingivalis in the murine lesion model. Although RgpA and Kgp when used as immunogens separately provided significant protection against P. gingivalis-induced lesions, 40 and 70% of mice, respectively, still developed small lesions. This suggests that at the Ag dose and for the immunization route trialed, fractionated RgpA and Kgp are not as efficacious as the complete RgpA-Kgp complex in providing protection against P. gingivalis challenge in the murine lesion model. The Western blot binding pattern for each of the protective Abs to the RgpA-Kgp complex (Fig. 6) was essentially the same as that against RgpA and Kgp separately, with major immunoreactive bands at 44, 39, 35, and 30 kDa representing the A1 adhesin domains of RgpA and Kgp and the A1’/” domains of HagA (Figs. 2 and 6) (13). A binding motif (ABM1; Fig. 2) found in the C-terminal segment of the proteinase catalytic domains and also in the adhesin domains has been implicated in binding to a complementary motif in the adhesins, forming the large noncovalently associated RgpA-Kgp complexes believed to be on the cell surface (44). The HagA₁,₂/” adhesin domains also contain a similar ABM1 sequence found in the RgpA and Kgp A1 adhesins (Fig. 2). A Western blot with anti-RgpA-Kgp Abs against the complex extracted from P. gingivalis W50 whole cells using the Triton X-114 procedure revealed that the complex contained the HagA₁,₂/” adhesins (Fig. 2) that also contains the ABM1, ABM2, and ABM3 motifs. These results may suggest that the large cell surface complexes on P. gingivalis W50 are composed of noncovalently associated, processed domains of all three polypeptides, RgpA, Kgp, and HagA. The superiority of the Triton X-114-extracted complex in protection may, therefore, relate to the vaccine Ag more closely resembling the form of the proteins on the cell surface. The lack of a protective effect of the anti-ABM1 antiserum may relate to the high degree of variability in the ABM1 sequence across the different adhesins (Fig. 2) while still allowing complex formation in the presence of Ab.

The RgpA-Kgp complex also provided protection against P. gingivalis-induced bone loss in the murine periodontitis model. This result confirms our recent work demonstrating protection of bone loss in the rat periodontitis model with RgpA-Kgp vaccination (11). In this study, immunization produced specific Abs detected intraorally and prevented detectable colonization of P. gingivalis in subgingival plaque. This was attributed to specific anti-RgpA-Kgp Abs present in the gingival crevicular fluid blocking binding of RgpA-Kgp to host tissue preventing colonization. In the current study, the protective Abs induced were directed to the 44 kDa (RgpA₁), 39/35 kDa (Kgp₁), and 30 kDa (HagA₁,₂/”) adhesin domains of the RgpA-Kgp-HagA complex. We have shown previously that sera from patients with no or low clinical
signs of periodontitis that have a high IgG4 and low IgG2 subclass Ab titer to the RgpA-Kgp complex recognized the RgpAA1 and KgpA1 adhesins in a Western blot (8). Patients with mild to severe disease with a low IgG4 and high IgG2 subclass Ab titer to the RgpA-Kgp complex, RgpA complex, and Kgp complex, and submandibular lymph nodes from mice (periodontitis model) immunized with the RgpA-Kgp complex, and control mice were used in an ELISPOT assay for the detection of RgpA-Kgp complex-specific IL-4 (□) and IFN-γ (■) T cell responses. Cytokine responses are expressed as spot-forming cells per million obtained minus the background, with each ELISPOT representing the mean and SD of four values.

Immunization with the peptide epitopes PAS1K, PAS1R, ABM3, and AMB2 induced protection against P. gingivalis-induced bone loss. The protective effect of immunization with PAS1K appeared to be moderately better than the protection induced by PAS1R, ABM3, and AMB2, as demonstrated by the effect sizes (Fig. 4). Immunization with PAS1R and ABM3 produced similar effects. The protection elicited by immunization with the RgpA-Kgp complex was significantly (p < 0.01) greater than all of the peptide vaccines except for PAS1K. The difference in bone loss induced by P. gingivalis in mice immunized with ABM1 compared with PAS1K, PAS1R, and ABM3 may be attributed to the difference in Ab response induced. ABM1 induced significantly less (p < 0.001) Ab compared with PAS1K, PAS1R, and ABM3. The total IgG and IgG1 Ab titers induced by PAS1K were larger than those induced by PAS1R and ABM3. This higher Ab
titer induced by PAS1K may therefore account for the better protection observed in the periodontitis model. However, we have recently shown that a W50 isogenic mutant lacking Kgp was the least virulent compared with the RgpA and RgpB isogenic mutants (16). This may also suggest, therefore, that an Ab response directed to the catalytic site of the Kgp protease may have produced a more significant effect on virulence than an Ab response directed to the RgpA protease. Each of the protective peptide and protein Ags induced a specific IgG response that was characterized by a predominant IgG1 subclass response. This Ab response was elicited by parenteral immunization, and these mice were protected against \textit{P. gingivalis}-induced bone loss after oral challenge with the bacterium. Mice parenterally vaccinated have been shown to secrete Ag-specific IgG in gingival crevicular fluid, with Ab concentration and pattern similar to that of serum (47, 48). Furthermore, oral fluid IgG has a higher avidity and affinity for a specific Ag than salivary IgA (49, 50). IgG has been shown to play an important role in protection and clearance of pathogens at mucosal surfaces (51–53). Ferrero et al. (54) have reported that mice parenterally immunized with \textit{Helicobacter pylori} GroES and urease (B-subunit) were protected against \textit{H. pylori} mucosal infection and that these mice had a predominant IgG1 Ag-specific response. Murine IgG1 is representative of a Th2 cytokine response (55, 56). Ag opsonised with murine IgG1 induces Fc receptor-dependent cellular cytotoxicity and phagocytosis via FcγRII and FcγRIII receptors (57, 58). Although murine IgG1 is reported to induce phagocytosis and endocytosis, it does not induce inflammatory cytokines compared with Ag opsonised with murine IgG2a, IgG2b, or IgG3 (57, 58). Thus, the predominant IgG1 Ab response induced by the protective peptides and complexes should neutralize and opsonise the proteinases and adhesins localized on the bacterial surface or released into the tissues, which should then stimulate phagocytosis of the bacterium or complex without the induction of proinflammatory cytokines. The predominant cytokine response in mice protected against \textit{P. gingivalis}-induced lesions or bone loss was the Th2 cytokine IL-4, whereas the predominant cytokine in mice with disease, i.e., \textit{P. gingivalis}-induced bone loss, was the proinflammatory Th1 cytokine IFN-γ. These data support the view that progression of disease is mediated by an inflammatory response and that a down-regulation of inflammation may lead to resolution and protection of disease (59).

The results presented here indicate that when the RgpA-Kgp proteinase adhesin complex or functional binding motif or active site peptides are used as a parenteral vaccine in the mouse periodontitis model, Abs of the IgG1 subclass are induced that block function of the complex and protect against \textit{P. gingivalis}-induced periodontal bone loss.

\textbf{Acknowledgments}

Rachel Rinaldi-Clarke and Carla Osinski are gratefully acknowledged for technical support.

\textbf{Disclosures}

The authors have no financial conflict of interest.

\textbf{References}


\begin{figure}[h]
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\includegraphics[width=\textwidth]{FIGURE_8.png}
\caption{PEPSSCAN analysis of peptide-specific Ab reactivity to overlapping peptides. ABM1 (A), ABM2 (B), ABM3 (C), PAS1K (D), and PAS1R (E) with 1-aa overlap. Each bar displays the Ab reactivity (OD$_{415}$).}
\end{figure}


