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Serum Immunoglobulin G (IgG) and IgG Subclass Responses to the RgpA-Kgp Proteinase-Adhesin Complex of *Porphyromonas gingivalis* in Adult Periodontitis

NEIL M. O'BRIEN-SIMPSON, CATHERINE L. BLACK, PETER S. BHOGAL, STEVEN M. CLEAL, NADA SLAKESKI, THOMAS J. HIGGINS, AND ERIC C. REYNOLDS*

Oral Health Sciences Unit, School of Dental Science, The University of Melbourne, Melbourne, Victoria 3000, Australia

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Serum immunoglobulin G (IgG), IgM, and IgG subclass responses to the RgpA-Kgp proteinase-adhesin complex of *Porphyromonas gingivalis* were examined by enzyme-linked immunosorbent assay using adult periodontitis patients and age- and sex-matched controls. Twenty-five sera from subjects with adult periodontitis (diseased group) and 25 sera from healthy subjects (control group) were used for the study. Sera and subgingival plaque samples from 10 sites were collected from each patient at the time of clinical examination. The level of *P. gingivalis* in the plaque samples was determined using a DNA probe. Highly significant positive associations between the percentage of sites positive for *P. gingivalis* and measures of disease severity (mean pocket depth, mean attachment loss, and percentage of sites that bled on probing) were found. The diseased group had significantly higher specific IgG responses to the RgpA-Kgp complex than did the control group, and the responses were significantly associated with mean probing depths and percentage of sites positive for *P. gingivalis*. Analysis of the IgG subclass responses to the RgpA-Kgp complex revealed that the subclass distribution for both the diseased and control groups was IgG4 > IgG2 > IgG3 = IgG1. The IgG2 response to the complex was positively correlated with mean probing depth, whereas the IgG4 response was negatively correlated with this measure of disease severity. Immunoblot analysis of the RgpA-Kgp complex showed that sera from healthy subjects and those with low levels of disease, with high IgG4 and low IgG2 responses, reacted with the RgpA27, Kgp39, and RgpA44 adhesins; however, sera from diseased subjects with low IgG4 and high IgG2 responses reacted only with the RgpA44 and/or Kgp44 adhesins. Epitope mapping of the RgpA27 adhesin localized a major epitope recognized by IgG4 antibodies in sera from subjects with high IgG4 and low IgG2 responses to the RgpA-Kgp complex which was not recognized by sera from diseased subjects with low IgG4 and high IgG2 responses.

Periodontitis is an inflammatory disease of the supporting tissue of the teeth and is a major cause of tooth loss in adults (54). The onset and progression of adult periodontitis have been associated with the subgingival emergence of a consortium of specific gram-negative bacteria. One bacterium of that consortium, *Porphyromonas gingivalis*, is now considered to be a major periodontal pathogen, as it is closely associated with disease in humans (53) and is capable of inducing disease in experimental animal models of periodontitis (10, 40).

Several studies have reported higher antibody titers (immunoglobulin G [IgG], IgM, and IgA) to *P. gingivalis* whole cells and outer membrane preparations in sera from adult periodontitis patients than in sera from healthy subjects (32–34). Furthermore, the severity of periodontitis has been associated with an increased IgG response to *P. gingivalis* (14, 16). Few studies have investigated the antibody response to purified antigens from *P. gingivalis*. Schenk and Michaelsen (46) have reported that sera from patients with periodontitis had elevated IgG titers to purified *P. gingivalis* lipopolysaccharide (LPS) with an IgG isotype distribution of IgG2 >> IgG1 > IgG3 > IgG4. An IgG subclass distribution dominated by IgG2, followed by IgG3 > IgG1 > IgG4, has also been reported; the distribution was determined by using periodontitis patient sera against a *P. gingivalis* whole-cell sonicate (59) and against a *P. gingivalis* outer membrane preparation (43). All these preparations, however, contained significant amounts of LPS, which is known to induce a dominant IgG2 subclass response (17). Ogawa et al. (37) have also reported that IgG2 is the dominant subclass response against *P. gingivalis* LPS and that the IgG subclass distribution against a purified fimbrial protein was IgG3 > IgG1 > IgG2 > IgG4. However, in an earlier report by the same group, the fimbria-specific IgG subclass distribution was found to be IgG4 dominant, followed by IgG1 > IgG3 > IgG2 (35).

The pathogenicity of *P. gingivalis* has been attributed to a number of virulence factors including LPS, fimbriae, hemagglutinin, hemolysin, and extracellular hydrolytic enzymes, especially proteinases. The most significant of these are the extracellular Arg- and Lys-specific cysteine proteinases, which have been shown to be major virulence factors and which, it has been suggested, play a major role in disease pathogenesis by dysregulation of the host immune and inflammatory responses (27). We have recently characterized the major cell-associated Arg- and Lys-specific proteinases of *P. gingivalis* W50 as a complex of noncovalently associated proteins, designated the RgpA-Kgp proteinase-adhesin complex, formerly designated the PrtR-PrtK complex (3). This complex is composed of 45-kDa Arg-specific, calcium-stabilized cysteine proteinase RgpA45 (formerly PrtR45), also referred to as Arg-gingipain (4), 48-kDa Lys-specific cysteine proteinase Kgp48 (formerly PrtK48), and seven sequence-related adhesins designated RgpA44, RgpA15, RgpA17, RgpA27, Kgp39, Kgp15, and Kgp44 (formerly PrtR44, PrtR15, PrtR17, PrtR27, PrtK39,

* Corresponding author. Mailing address: Oral Health Sciences Unit, School of Dental Science, The University of Melbourne, 711 Elizabeth St., Melbourne, Victoria 3000, Australia. Phone: 61 3 9341 0270. Fax: 61 3 9341 0236. E-mail: e.reynolds@dent.unimelb.edu.au.

PrtK15, and PrtK44, respectively) (3). These proteins are encoded by the two genes *rgpA* (39) and *kgp* (38), also known as *prtR* and *prtK*, respectively, as characterized in the *P. gingivalis* strain W50 (49–51). The adhesins bind to a range of host extracellular matrix proteins (42), and it has been proposed that they facilitate the action of the cysteine proteinases by targeting them to appropriate substrates (3, 50).

We report here the IgG antibody responses to, and the subclass distribution of, the purified RgpA-Kgp proteinase-adhesin complex from *P. gingivalis* strain W50 in sera from patients with adult periodontitis and age- and sex-matched controls.

MATERIALS AND METHODS

Human subjects. Sera were obtained from 50 age- and sex-matched adult subjects (26 males, 24 females; age (mean ± standard deviation), 51.8 ± 9.70 years; age range, 36 to 70 years). Patients with adult periodontitis were recruited from the Periodontal Clinic of the Royal Melbourne Dental Hospital, and age- and sex-matched controls were staff and relatives of staff of the School of Dental Science, The University of Melbourne, and the Royal Melbourne Dental Hospital. Ethics approval was obtained from the Human Research Ethics Committee of the University of Melbourne. Full medical and dental histories were obtained for each subject. Exclusionary criteria included recent use of nonsteriodal anti-inflammatory drugs, antibiotics, or antiplaque preparations, periodontal treatment in the last 6 months, and a history of periodontal surgery. Subjects had no history of systemic diseases affecting the periodontium directly or indirectly by interfering with the ability to perform adequate oral hygiene. Dental examinations included recording number of teeth present, restorations, carious lesions, pocket depths from the gingival margin (six sites per tooth), recession from the cementoenamel junction (six sites per tooth), mobility (Miller's index), furcation involvement, and bleeding on probing (six sites per tooth). The six sites assessed per tooth were the mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual sites. Ten subgingival plaque samples were taken from each patient. The sites sampled were diagnosed as diseased or clinically healthy on the basis of pocket probing depths, radiographs, attachment levels, bleeding on probing, and clinical appearance. After removal of supragingival plaque and calculus, subgingival plaque samples were obtained by placing sterile Gracey curettes at the bottom of the pocket and drawing in a coronal direction. Plaque samples were analyzed for the presence of *P. gingivalis* using a DNA probe method (see below). The control group contained 25 subjects (13 males and 12 females; mean age, 50.7 ± 10.0 years) with no probing depths >4 mm and no sites that bled on probing. The diseased group contained 25 subjects (13 males and 12 females; mean age, 50.0 ± 9.40 years) who exhibited moderate-to-severe periodontal attachment loss. Diseased individuals had at least one probing depth >6 mm and numerous sites that bled on probing.

To assess the relationship between severity of disease and predominant subclass response, mean pocket depth and mean attachment loss were determined for each subject by calculating from the complete periodontal charting done at the initial examination.

Bacterial strain and growth conditions. Lyophilized cultures of *P. gingivalis* W50 were grown anaerobically at 37°C on lysed horse blood agar plates (<10 passages), and after 3 to 4 days colonies were used to inoculate modified basal media containing hemin (1 μg ml^{-1}) (30). Growth of batch cultures was monitored at 650 nm using a spectrophotometer (model 295E; Perkin-Elmer). Culture purity was checked routinely by Gram staining, microscopic examination, and a variety of biochemical tests (52).

Purification of the proteinase-adhesin complex (RgpA-Kgp complex) of *P. gingivalis*. The RgpA-Kgp proteinase-adhesin complex of *P. gingivalis* strain W50 was purified from a cell sonicate and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transblotting, and N-terminal sequence analysis as described previously (3).

DNA probe analysis of subgingival plaque samples. DNA was extracted from each of the 500 subgingival plaque samples according to the method of Dix et al. (7). After extraction, the DNA was resuspended in 200 μl of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA)–200 μl of 6.9 M formaldehyde–200 μl of 9× SSC (1.35 M NaCl, 135 mM trisodium citrate). The DNA solutions were heated to 60°C for 15 min and applied to a Hybond N^+ membrane prewetted with 6× SSC using a dot blot apparatus (Schleicher and Schuell, Keene, N.H.). The immobilized DNA was denatured by soaking membranes in 1.5 M NaCl–0.5 M NaOH for 5 min and then neutralized in 1.5 M NaCl–0.5 M Tris-HCl (pH 7.2)–1 mM EDTA for 5 min. A synthetic oligonucleotide corresponding to nucleotides 178 to 207 of the *P. gingivalis* gene, *prtC* (56), was 5′-end-labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase. Membranes were hybridized with the radiolabeled oligonucleotide overnight in hybridization buffer (6× SSC, 0.5% SDS, 0.25% skim milk, 5× Denhardt's solution) at 42°C. Filters were washed extensively in a solution of 1× SSC containing 0.5% (wt/vol) SDS at 50°C. Standard protocols (45) were used for radiolabeling oligonucleotides, immobilizing DNA on nylon membranes, and screening. The radioactive counts for each plaque sample were converted to number of *P. gingivalis* cells by reference to a series of samples of DNA extracted from known numbers of *P. gingivalis* cells included on every membrane. The detection limit of the DNA probe method was 10^3 *P. gingivalis* cells.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) were performed in triplicate using a solution (1 μg/ml) of the RgpA-Kgp complex in 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% (vol/vol) Tween 20 (PBST) and 0.1% (wt/vol) sodium azide to coat wells of flat-bottom polyvinyl microtiter plates (Microtiter; Dynatech Laboratories, McLean, Va.) overnight at 4°C. After removal of the coating solution, 2% (wt/vol) skim milk powder in PBST was added to block the remaining uncoated plastic for 1 h at room temperature. After a washing (four times in PBST), a 1/500 dilution of the subject sera in PBST containing 1% (wt/vol) skim milk powder was added to each well and incubated for 3 h at 37°C. After a washing (six times in PBST), bound antibody was detected by incubation with horseradish peroxidase-conjugated goat Ig directed against human IgG (1/2,000 dilution) or human IgM (1/2,000 dilution) (Bio-Rad, Richmond, Calif.) for 1.5 h at 37°C. After a washing (six times in PBST), substrate (0.4 mM 3,3′,5,5′-tetramethylbenzidine in 0.1 M sodium acetate-citric acid buffer containing 0.004% [vol/vol] hydrogen peroxide) was added and color development was stopped by the addition of 2 M H_2SO_4. Optical density at 450 nm (OD_{450}) was measured using a Bio-Rad microplate reader, model 450.

To determine the IgG subclass antibody responses of patient sera, microtiter plates were coated with the RgpA-Kgp complex and incubated with patient sera as described above. After a washing (six times in PBST), bound IgG subclass antibody was detected by incubation with a 1/1,000 dilution of biotinylated mouse anti-human IgG subclass antibody (clones 8c/6-39, anti-IgG1; HP-6014, anti-IgG2; HP-6050, anti-IgG3; and HP-6025, anti-IgG4; Sigma Chemical Co., Sydney, New South Wales, Australia) at 37°C for 1 h. The plates were then washed (six times in PBST), and a 1/4,000 dilution of avidin peroxidase conjugate (Sigma Chemical Co.) was added to each well and incubated for 1 h at 37°C. After a washing (six times in PBST), the plates were developed as described above. The specificities of the mouse monoclonal antihuman subclass-specific antibodies used in this study have been well characterised in an International Union of Immunological Societies/World Health Organization international collaborative study (21). Each subclass-specific monoclonal antibody does not cross-react with the other IgG subclasses, and, when used at the same dilution, the antibodies have similar antigen-binding capacities.

The appropriate dilution of subject sera used in the ELISAs was determined from preliminary experiments involving serial dilutions of sera and measurement of antibody binding to adsorbed RgpA-Kgp. Second-antibody and peroxidase conjugate dilutions were optimized by constructing dilution curves using sera from a subject with a strong anti-RgpA-Kgp antibody response (positive control) and a subject with no detectable anti-RgpA-Kgp antibody response (negative control). The following controls were included (in triplicate) on each plate: positive- and negative-control subject sera and wells excluding (i) the coating antigen (RgpA-Kgp), (ii) subject sera, and (iii) horseradish peroxidase-conjugated antibody. Interplate variation was less than 15%.

Immunoblotting. Purified RgpA-Kgp complex was separated by SDS-PAGE in gels of 12.5% acrylamide (1 mm thick) by using the method of Laemmli (26) with a minigel system (Bio-Rad). Proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane using the method of Dashper et al. (5). After the membrane was sectioned, the molecular weight standards were stained with 0.1% (wt/vol) Coomassie blue R250. The remaining sections were blocked for 1 h at 20°C with 5% (wt/vol) nonfat skim milk powder in TN buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl). Sections were subsequently incubated with patient sera diluted 1:25 with TN buffer. After 5 h at 20°C the sections were washed (four times in TN buffer containing 0.05% [vol/vol] Tween 20) and then incubated for 1 h at 20°C with anti-human IgG horseradish peroxidase conjugate (Bio-Rad). After a washing (four times in TN buffer containing 0.05% [vol/vol] Tween 20), bound antibody was detected with 0.005% (wt/vol) 4-chloro-1-napthol in TN buffer containing 16.6% (vol/vol) methanol and 0.015% (vol/vol) H_2O_2. Color development was stopped by rinsing the membranes with Milli Q water.

Epitope mapping. Epitope mapping of the first 148 residues of the N terminus of the RgpA27 adhesin of the RgpA-Kgp complex (3) was accomplished using 21 overlapping 13-mer peptides (overlay by 6 residues and offset by 7 residues). Peptides were synthesized by Chiron Technologies (Melbourne, Australia) using the multipin peptide synthesis system. Epitope mapping of the pin-bound peptides was carried out by ELISA in accordance with Chiron Technologies instructions by using human sera at a dilution of 1:1,000 in 1% (wt/vol) nonfat skim milk powder in PBST. After a washing (four times in PBS), bound IgG antibody or IgG subclass antibody was detected by incubation with either horseradish peroxidase-conjugated goat Ig directed against human Ig (1/2,000 dilution) or biotinylated mouse anti-human IgG subclass antibody (IgG1, IgG2, IgG3, or IgG4; 1/1,000 dilution). For the subclass antibodies, the pins were then washed (four times in PBS) and a 1/4,000 dilution of avidin peroxidase conjugate was added and incubated for 1 h. The bound antibody was detected by incubating the peptide pins with 0.4 mM 3,3′,5,5′-tetramethylbenzidine in 0.1 M sodium acetate-citric acid buffer containing 0.004% (vol/vol) H_2O_2. Color development was stopped by the addition of 2 M H_2SO_4. Optical density was measured at 450 nm using a Bio-Rad microplate reader, model 450.

Statistical analysis. Serum IgG, IgM, and IgG subclass responses to the RgpA-Kgp complex were analyzed using the Levene test (M. J. Norusis, SPSS for Windows: base system user's guide, release 6.0, SPSS Inc., Chicago, Ill., 1993) for

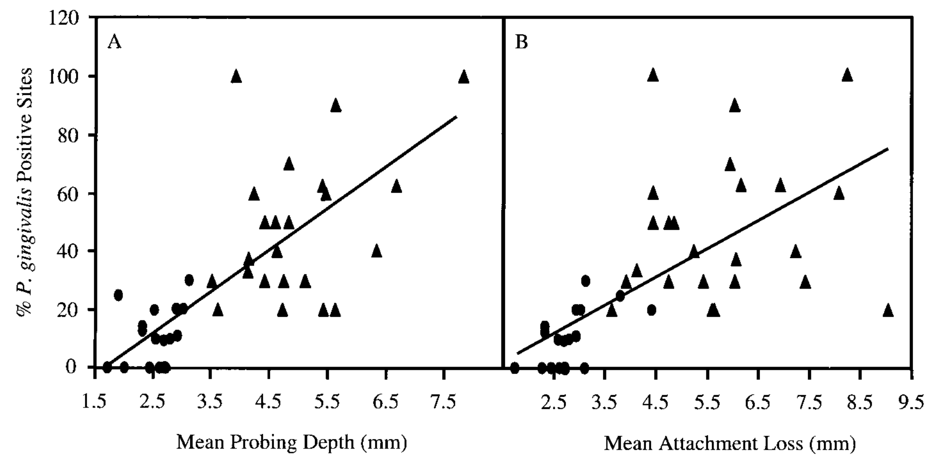

FIG. 1. Relationship between the percentage of sites positive for *P. gingivalis* and mean probing depth (A) and mean attachment loss (B). ●, control group; ▲, diseased group. Linear regression lines are shown.

homogeneity of variances and the Mann-Whitney U Wilcoxon rank sum test (M. J. Norusis, 1993). Spearman's rank correlation (M. J. Norusis, 1993) was used for all correlation analyses. The chi-square test (M. J. Norusis, 1993) was used for the association between the presence of *P. gingivalis* at a site and bleeding on probing.

RESULTS

DNA probe analysis of plaque samples for *P. gingivalis*. The mean percentage of sites positive for *P. gingivalis* in the control group (± the standard deviation) was 9.6% ± 9.1%, whereas for the diseased group the value was 45.8 ± 24.9%. There was a highly significant ($P < 0.001$) association between the presence of *P. gingivalis* at a site and bleeding on probing, with 83% of the sites positive for *P. gingivalis* bleeding on gentle probing. The number of *P. gingivalis* cells at a site was also significantly ($P < 0.001$) associated with pocket depth and attachment loss at that site, the strongest association being that the highest numbers of *P. gingivalis* cells were recovered from the deepest pockets. In order to associate specific serum antibody responses to disease severity, subject measures of disease severity were required; therefore mean pocket depth and mean attachment loss were determined for each subject. These mean measures of disease severity were also significantly associated with the presence of *P. gingivalis*, as there was a highly significant, positive correlation between the percentage of sites positive for *P. gingivalis* and mean probing depth ($r = 0.765$, $P < 0.001$) and mean attachment loss ($r = 0.786$, $P < 0.001$) (Fig. 1).

IgG and IgM response to the RgpA-Kgp proteinase complex from *P. gingivalis*. The IgG and IgM antibody responses for the control and diseased patient sera are shown in Fig. 2. Analysis of homogeneity of variances (Levene test [M. J. Norusis, 1993]) indicated that the data were not normally distributed, so the data were subjected to nonparametric analyses using the Mann-Whitney U Wilcoxon rank sum test (M. J. Norusis, 1993). No significant difference between the control and diseased group IgM responses to the RgpA-Kgp complex was found, whereas the diseased group had a significantly higher total IgG response ($P < 0.001$) to the complex than the control group. Figure 3 shows the relationship between total IgG responses to the RgpA-Kgp complex for both groups and the mean probing depth (Fig. 3A) and the percentage of sites positive for *P. gingivalis* (Fig. 3B). Statistical analysis of the data indicated that there was a highly significant ($r = 0.774$, $P < 0.001$) positive correlation between the total IgG response and mean probing depth. Furthermore a highly significant, positive correlation ($r = 0.554$, $P < 0.001$) between the total IgG response and the percentage of sites positive for *P. gingivalis* was also found (Fig. 3B).

IgG subclass response to the RgpA-Kgp proteinase complex. The specific IgG subclass responses to the RgpA-Kgp complex for the control and diseased groups are shown in Fig. 4. Analysis of homogeneity of variance indicated that the data were not normally distributed, and the data were therefore analyzed using the Mann-Whitney U Wilcoxon rank sum test (M. J. Norusis, 1993). For both the control and diseased groups the IgG2 and IgG4 subclass responses were higher, although more variable, than those for IgG1 and IgG3. The diseased-group IgG1 and IgG3 responses to the complex were significantly higher ($P < 0.02$) than the corresponding responses of the control group, whereas there were no significant differences between the IgG2 or IgG4 responses of the diseased and control groups. The subclass distribution for both the control and diseased groups was found to be IgG4 > IgG2 > IgG3 = IgG1. The subclass order was found to be significant ($P < 0.001$) for both groups, except that no significant difference between IgG1 and IgG3 for either the diseased or control group was found. In both groups IgG4 was the dominant IgG subclass response, with 80% of subjects in the control group and 72% of subjects in the diseased group responding (based on the subclass ELISA OD_{450} being greater than double the median of the total IgG ELISA OD_{450} for the control group). The second most prominent IgG subclass response was IgG2, with 36% of the diseased subjects and 20% of the control subjects responding. Only the diseased group had an IgG1 (24%) or IgG3 (24%) response.

Disease severity and subclass response. The major serum IgG subclass responses to the RgpA-Kgp complex were IgG4 and IgG2; however, the classification of subjects into diseased and control groups did not reveal any differences due to the large variation in IgG4 and IgG2 responses in both the diseased and control groups (Fig. 4). In an approach to investigate the relationship between disease severity and serum IgG2 and IgG4 responses to the RgpA-Kgp complex, we selected subjects from both the control and diseased groups to form two groups, one of IgG2 responders and the other of IgG4 responders. Responders were defined as those with serum IgG2 and IgG4 ELISA OD values that were greater than double the median value of the total IgG response to the RgpA-Kgp complex for the control group. Interestingly those subjects with a high serum IgG2 response to the complex exhibited a low

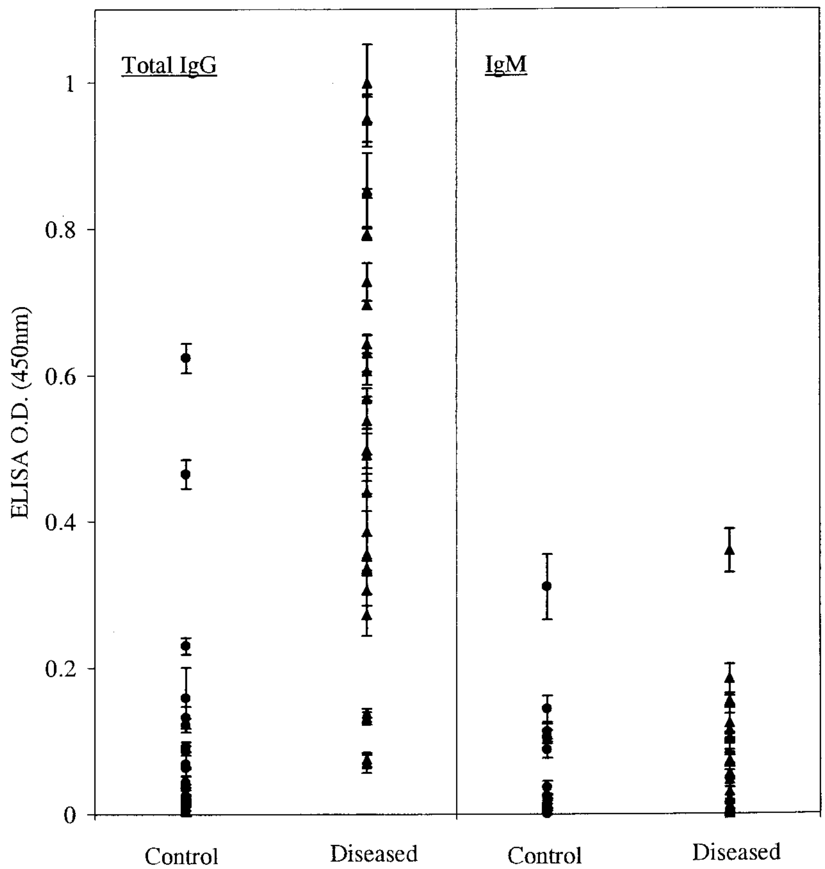

FIG. 2. Serum IgG and IgM responses to the RgpA-Kgp complex of *P. gingivalis*. Sera from control subjects (●) and diseased subjects (▲) were used in the ELISA with the RgpA-Kgp complex as the adsorbed antigen. Antibody responses are expressed as the ELISA OD_{450} obtained minus background, with each point representing the mean ± standard deviation of three values.

IgG4 response, and conversely those with a high serum IgG4 response exhibited a low IgG2 response, as demonstrated by the significant negative correlation ($r = -0.555$, $P < 0.05$) between the IgG2 and IgG4 responses to RgpA-Kgp.

The serum IgG2 responses to the RgpA-Kgp complex were found to be positively correlated ($r = 0.837$, $P < 0.001$) with mean probing depth values (Fig. 5A). Furthermore, a significant positive correlation ($r = 0.712$, $P < 0.01$) between the

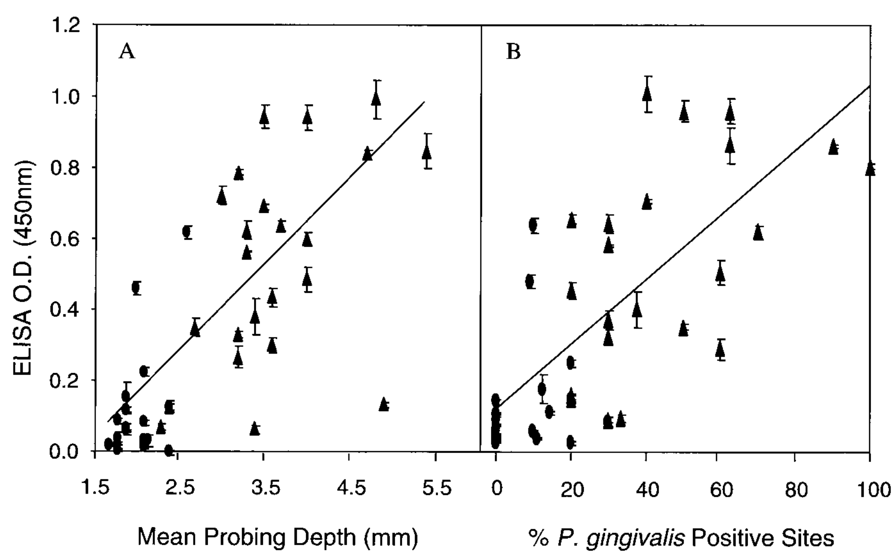

FIG. 3. Relationship between serum IgG response to the RgpA-Kgp complex and mean probing depth (A) and percentage of sites positive for *P. gingivalis* (B). ●, control subjects; ▲, diseased subjects. Antibody responses are expressed as the ELISA OD_{450} obtained minus background, with each point representing the mean ± standard deviation of three values.

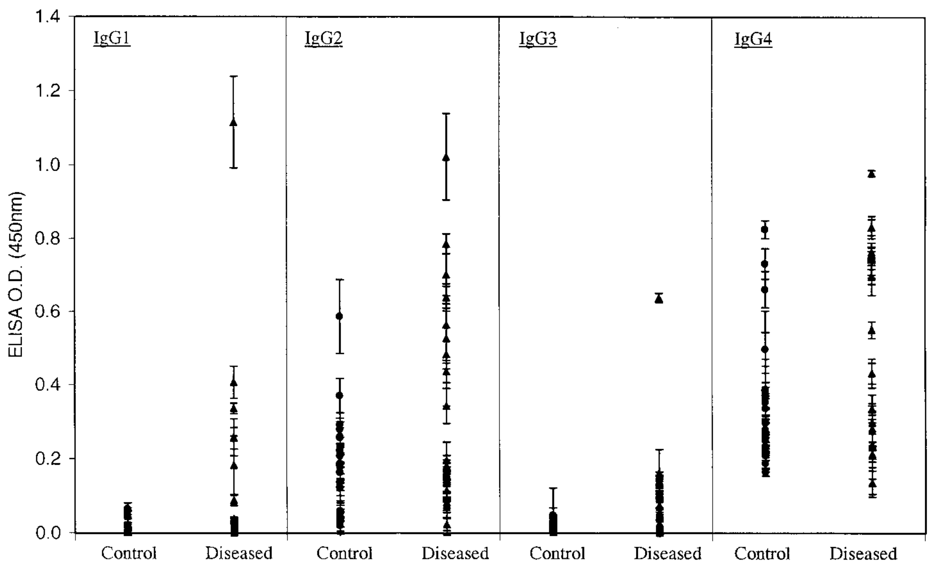

FIG. 4. Serum IgG subclass responses to the RgpA-Kgp complex of *P. gingivalis*. Sera from control subjects (●) and diseased subjects (▲) were used in the ELISA with the RgpA-Kgp complex as the adsorbed antigen. Antibody responses are expressed as the ELISA OD_{450} obtained minus background, with each point representing the mean ± standard deviation of three values.

IgG2 responses and the percentage of sites positive for *P. gingivalis* was also found (data not shown). Conversely, the serum IgG4 responses to the RgpA-Kgp complex were found to be negatively correlated ($r = -0.568$, $P < 0.005$) with mean probing depth values (Fig. 5B). These results indicate that a high disease severity was associated with a high IgG2 response and a low IgG4 response to the RgpA-Kgp complex, whereas a high IgG4 serum response to the complex was associated with low disease severity and a low IgG2 response.

Immunoblot analysis of the RgpA-Kgp complex. Immunoblot analysis of the RgpA-Kgp complex using sera from subjects C10, D20, and D24 is shown in Fig. 6. Subject C10 (from the control group) and D24 (from the diseased group) both had high IgG4 and low IgG2 responses to the complex, whereas D20 (from the diseased group) had a low IgG4 and a high IgG2 response. Subject C10 had no probing depths >4 mm, D24 had only low-to-moderate disease with only three 6-mm probing depths (2% of sites examined), and D20 exhibited advanced generalized disease with 64 probing depths ≥6 mm (44% of sites examined). All of the subject sera showed an immunoreactive response to a 44-kDa protein band that corresponded to the RgpA44 and/or Kgp44 adhesins of the complex (3). Two additional major immunoreactive bands corresponding to the RgpA27 and Kgp39 adhesins were detected by the highly IgG4-specific sera from C10 and D24, whereas no immunoreactive bands were detected below the 44-kDa pro-

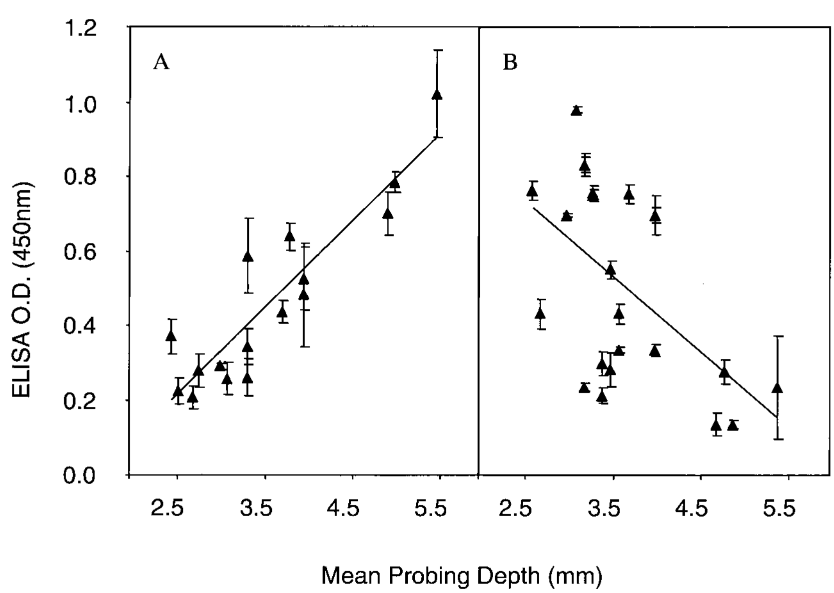

FIG. 5. Relationship between serum IgG2 (A) and IgG4 (B) responses to the RgpA-Kgp complex and disease severity (as mean probing depth). Antibody responses are expressed as the ELISA OD_{450} obtained minus the background, with each point representing the mean ± standard deviation of three values. Only those IgG2 or IgG4 ELISA OD_{450} values which were greater than double the median value of the total IgG response for the control group are shown.

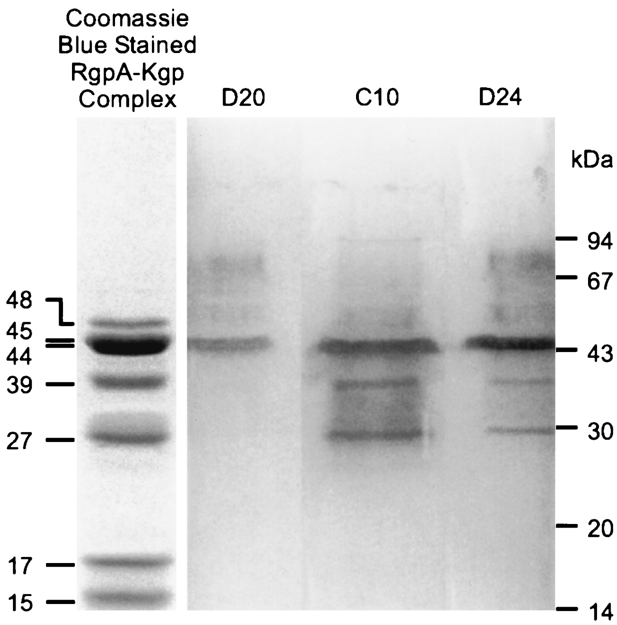

FIG. 6. Immunoblot analysis of responses by human sera from control and diseased patient groups against the RgpA-Kgp complex of *P. gingivalis*. The transblotted RgpA-Kgp complex after SDS-PAGE was probed with sera from subjects C10 and D24 (low IgG2, high IgG4 response to the RgpA-Kgp complex) and D20 (high IgG2, low IgG4 response to the RgpA-Kgp complex). Molecular mass markers are shown at the right.

tein with the highly IgG2-specific sera from subject D20. Immunoblot analysis of the RgpA-Kgp complex using the remaining subjects from the diseased and control groups with high IgG2 and IgG4 responses, respectively, to the RgpA-Kgp complex confirmed the reactivity of only the highly IgG4-specific sera with the RgpA27, Kgp39, and RgpA44-Kgp44 adhesins. Only the RgpA44-Kgp44 adhesins of the complex were reactive with sera with the high IgG2 response.

Epitope mapping of the RgpA27 adhesin protein. Twenty-one overlapping 13-mer peptides representing the N-terminal 148 residues of RgpA27 were synthesized (offset, 7 residues; overlap, 6 residues) on pins and mapped using sera from the control and diseased groups as shown in Fig. 7. Two major (EP1 and EP2) and two minor (EP3 and EP4) immunoreactive peptide epitopes were detected using the highly IgG4-specific sera. Sequences were as follows: EP1, RYDDFTFEAGKKYT FTMRRAGMGDGTD; EP2, TNPEPASGKMWIAGDGG NQP; EP3, FLLDADHNTFGSVIPATGPLFTGTASS; EP4, LYSANFEYLIPANADPVVTTQNIIVTG. Sera from patients with a high IgG2 response (e.g., D20 in Fig. 7) and a low IgG response (e.g., C4 in Fig. 7) to the complex were found to be weakly immunoreactive with EP1 to -4 of RgpA27, consistent with the negative responses of these sera to RgpA27 on immunoblotting. Subclass analysis of the antibodies bound to the pins confirmed that the major subclass binding to EP1 and EP2 was IgG4. The results of the subclass analysis with pooled subject immunoreactive sera is shown in Fig. 8. These results show that IgG4 antibodies bound to the two major epitopes (EP1 and EP2) as well as the two minor epitopes (EP3 and EP4). It was interesting to note that there was also some detectable binding of IgG2 antibodies to the minor epitopes (EP3 and EP4), although this was significantly lower ($P < 0.001$) than the binding of the IgG4 subclass antibodies to these epitopes (Fig. 8).

DISCUSSION

This study showed that there was a highly significant association between the percentage of sites positive for *P. gingivalis* and the severity of periodontitis as measured by probing depth,

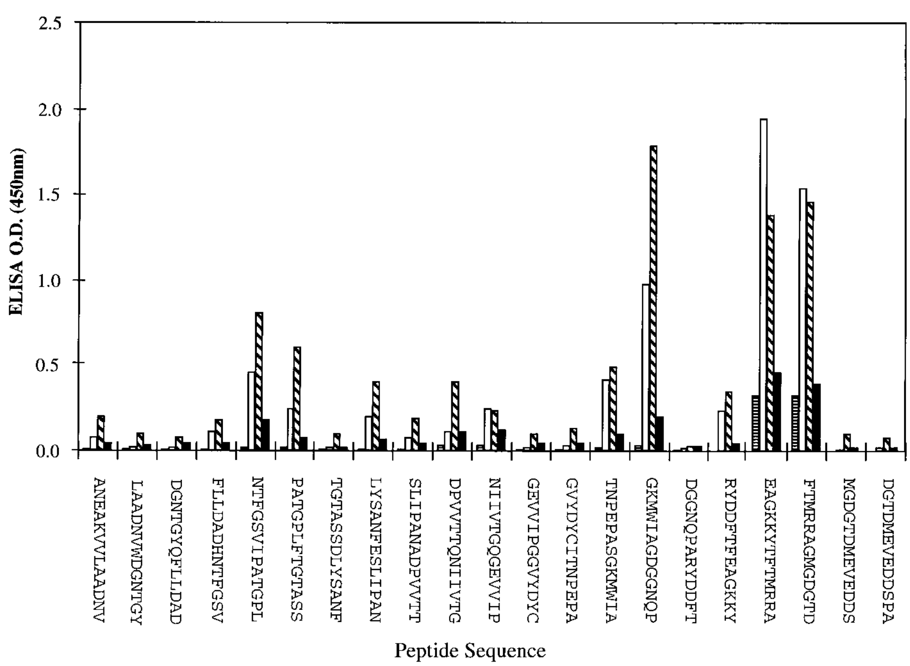

FIG. 7. Epitope mapping of the RgpA27 adhesin of the RgpA-Kgp complex. Shown are serum IgG antibody responses, assessed by ELISA, to *P. gingivalis* W50 RgpA27 overlapping peptides. Twenty-one overlapping pin-bound peptides representing the N-terminal 148 residues of the RgpA27 adhesin were probed with sera from subjects C4 (low IgG response to the RgpA-Kgp complex; ▤), D24 (low IgG2, high IgG4 response to the RgpA-Kgp complex; ▧), C10 (low IgG2, high IgG4 response to the RgpA-Kgp complex; □), and D20 (high IgG2, low IgG4 response to the RgpA-Kgp complex; ■).

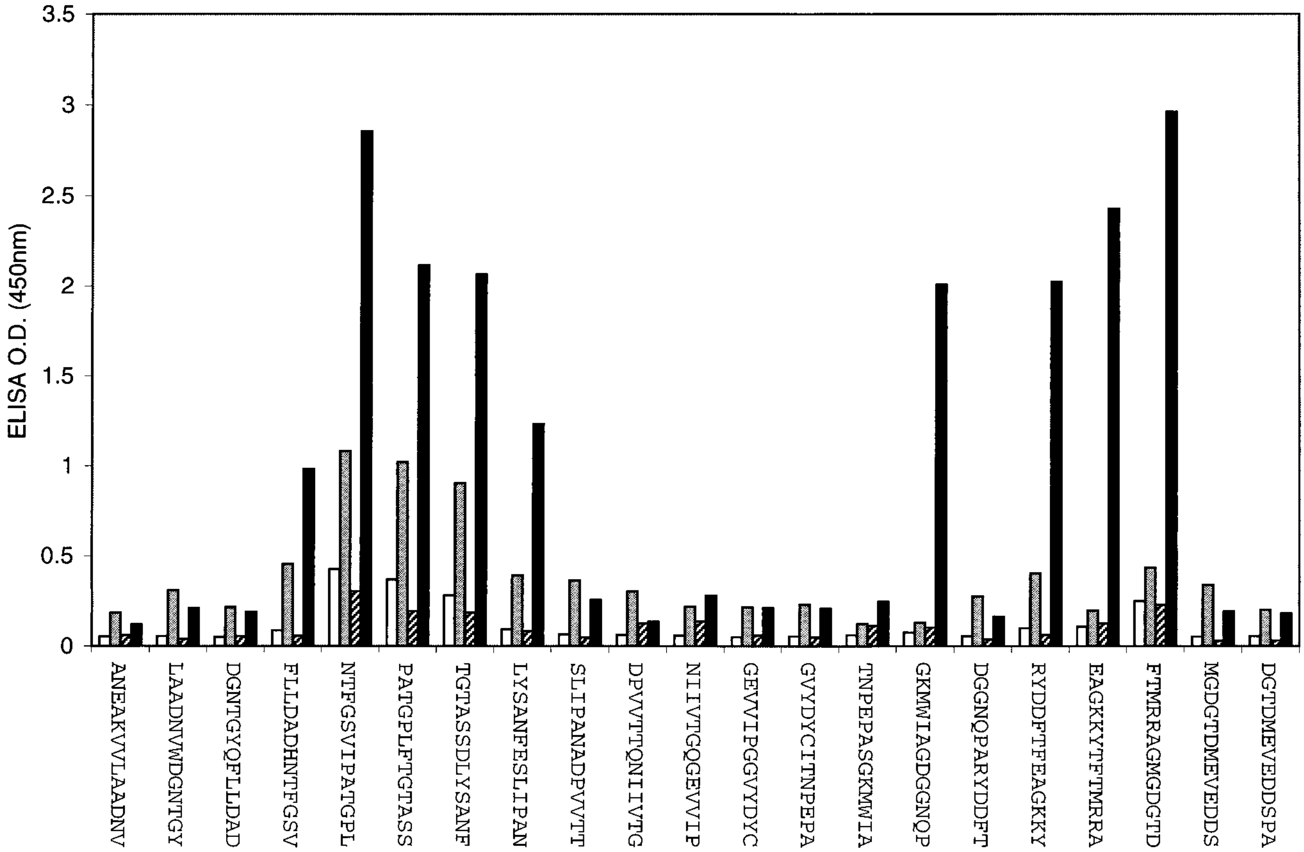

FIG. 8. IgG subclass analysis of antibody binding to the overlapping peptides of the RgpA27 adhesin. Twenty-one overlapping peptides representing the N-terminal 148 residues of the RgpA27 adhesin were probed with pooled subject immunoreactive sera. Sera from patients with a positive response to either EP1, EP2, EP3, or EP4 were pooled. The antibody subclass was determined using IgG-specific subclass antibodies IgG1 (□), IgG2 (▤), IgG3 (▨), and IgG4 (■).

attachment loss, and bleeding on probing. These findings corroborate previous reports on a proportional increase in the level of *P. gingivalis* with severity of periodontitis (27, 53).

Analysis of the specific IgM and IgG responses to the purified RgpA-Kgp complex demonstrated a significantly higher IgG response for both diseased and control groups than IgM response (Fig. 2). The diseased group had a significantly higher IgG response to the complex than the control group, although no significant difference for IgM antibodies was detected, a finding consistent with earlier reports using whole cells, cell extracts, and a purified fimbrial protein (8, 35, 41). Levels of serum IgG to the RgpA-Kgp complex were found to have a strong positive association with the percentage of sites positive for *P. gingivalis* and disease severity as measured by mean probing depth (Fig. 3). These data corroborate the findings of Kojima et al. (25), who reported that levels of serum IgG to a *P. gingivalis* whole-cell sonicate increased with the percentage of sites positive for *P. gingivalis*. Ebersole et al. (9) have also reported that the serum IgG response to *P. gingivalis* cells correlated with the presence of the bacterium in subgingival plaque.

Analysis of the IgG subclass responses to the RgpA-Kgp complex revealed that, for the antigen-specific responses, IgG4 predominated, followed by IgG2 and then IgG3 and IgG1, for both the control and the diseased groups. A number of reports have also found a dominant IgG4 response to either whole cells, cell extracts, or purified fimbrial antigens from *P. gingivalis* (12, 35, 60). The dominant IgG4 response in periodontitis may reflect the chronic nature of the disease. Chronic infection, where there is persistent antigen stimulation, has been reported to induce a predominant IgG4 response (1, 2, 58). The other major subclass response to the RgpA-Kgp complex found in this study was that of IgG2. Although, IgG2 antibodies are commonly induced by bacterial glycolipids such as LPS (17), a specific IgG2 response may be induced by the RgpA-Kgp complex, as components of the complex have been reported to be glycolipid modified (44) and as the adhesins, particularly the RgpA44 adhesin, contain repeated peptide sequences (49). Repeated peptide sequences are known to induce a specific protein IgG2 response (48).

In the present study, correlation of the RgpA-Kgp-specific IgG2 and IgG4 responses with mean probing depth demonstrated that as mean probing depth increased there was a corresponding increase in the specific IgG2 response but a decrease in the specific IgG4 response. The expression of IgG4 is reported to be interleukin 4 (IL-4) dependent and thus requires the stimulation of T-helper type 2 (Th2) cells (13, 28). The Th1 cytokine gamma interferon (IFN-γ) has been reported to be necessary for the induction of Cγ2 germ line transcripts and, thus, B-cell isotype switching to IgG2 (24). This may suggest that a high IgG4 response to the RgpA-Kgp complex is associated with a predominantly Th2-like response and that a high serum IgG2 response to the complex may be associated with a predominantly Th1-like response. Increased levels of the Th1 cytokine IFN-γ have been reported in diseased gingival tissue from adult periodontitis patients (29, 57). Also, an absence of the Th2 cytokine IL-4 in inflamed gingival tissue has been associated with the onset and progression of periodontitis (47, 61). These results may suggest that in periodontitis-susceptible individuals emergence of periodontal pathogens in subgingival plaque leads to an inflammatory Th1-like response, with the production of nonprotective IgG2 antibodies and inflammatory mediators of bone resorption, resulting in the onset and progression of disease. However, in nonsusceptible individuals, antibody switching may occur, leading to the production of specific IgG4 antibodies which may be protective against the progression of disease.

Unlike the other IgG subclass antibodies, IgG4 is considered to have a noninflammatory effector function profile (similar to secretory IgA) as it does not bind complement C1q or activate C3 or C5 and hence does not activate the classical complement pathway (22). A suggested biological function of IgG4 is a protective/defensive role in mucosal immunity (31), as there are a number of reports indicating that IgG4-committed B cells are enriched at mucosal sites (19, 36). Furthermore low levels of IgG4 antibody at mucosal surfaces have been associated with exacerbation of a number of diseases including recurrent respiratory tract infection (15, 31). IgG4 is known to bind to the Fc receptor FcγR1 and induce phagocytosis of antibody-coated antigen by monocytes, macrophages, and dendritic cells (6, 11). Furthermore, an antigen-specific IgG4 monoclonal antibody has been reported to deplete target cells in humans with little or no expression of the inflammatory cytokines tumor necrosis factor alpha and IFN-γ (20). In a recent study by Sutterwala et al. (55) it was reported that the ligation of FcγR1 can enhance the production of IL-10, reversing the proinflammatory response of macrophages to bacteria or bacterial products such as LPS. These findings may suggest that the specific IgG4 response to the RgpA-Kgp complex of *P. gingivalis* in individuals with no or low levels of periodontal attachment loss may have protected against the progression of disease by blocking the function of the proteinase-adhesin complex and promoting phagocytosis of *P. gingivalis* without inducing inflammatory cytokines.

Epitope mapping of the RgpA27 adhesin of the RgpA-Kgp complex with highly IgG4-specific sera identified epitope EP1, which is also present in the Kgp39 adhesin; a similar sequence is also present in the RgpA44 adhesin. The EP1 sequences are as follows: RgpA27 (amino acids 1542 to 1568), DDFTFEAG KKYTFTMRRAGMGDGTD; Kgp39 (amino acids 1081 to 1107), DDFTFEAGKKYTFTMRRAGMGDGTD; RgpA44 (amino acids 831 to 855), DDYVFEAGKKYHFLMKKMG SGDGTE. The presence of EP1-related epitopes in the RgpA27, Kgp39, and RgpA44 adhesins of the RgpA-Kgp complex is consistent with the highly IgG4-specific sera recognizing the 27-, 39-, and 44-kDa proteins of the RgpA-Kgp complex in the immunoblot (Fig. 6). The EP1 sequence in RgpA44 is located 50 residues N terminal to an adhesin binding motif previously identified as important in the formation and function of the RgpA-Kgp proteinase-adhesin complex (50) and to the epitope identified by Kelly et al. (23) that was recognized by a monoclonal antibody which prevented colonization of *P. gingivalis* in the human oral cavity. The epitopes identified are not present in the hemagglutinin (Hag) proteins of *P. gingivalis*, which are known to have a high degree of sequence similarity with the adhesin proteins of the RgpA-Kgp complex (18). This may indicate that a specific and protective immune response in the patients with no or low levels of disease was directed towards the RgpA-Kgp complex by the binding of the IgG4 antibodies to the RgpA27, Kgp39, and RgpA44 adhesins, preventing their binding to host proteins and restricting colonization by the bacterium and the functioning of its major virulence factor.

In conclusion, the results presented here indicate that patients with adult periodontitis have a significant serum IgG response to the RgpA-Kgp proteinase-adhesin complex of *P. gingivalis* compared with control subjects and that a high serum IgG2 subclass response against the complex was associated with increased disease severity whereas a high IgG4 subclass response was associated with low-level or no disease.

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