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Serum IgG1 and IgG2 antibody responses to *Porphyromonas gingivalis* in patients with periodontitis

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Background/aims: Protein and carbohydrate antigens of *Porphyromonas gingivalis* interact with the host to produce antibody of different subclasses. IgG1 and IgG2 antibodies frequently account for approximately 90% of the total serum IgG. This work aimed to investigate serum IgG1 and IgG2 antibody responses of periodontitis patients to protein and carbohydrate-rich antigens of *P. gingivalis*.
Methods: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blots of *P. gingivalis* antigens and proteinase K digested antigens rich in carbohydrates were used to investigate the molecular weight of antigen recognised by serum IgG1 and IgG2. Enzyme-linked immunosorbent assay was used to measure levels of IgG1 and IgG2 antibody to *P. gingivalis* and radial immunodiffusion was used to estimate the total concentration of IgG1 and IgG2 in serum.
Results: Serum IgG antibodies bound to antigens of molecular weights 47, 39 and 32 kDa. Antigen most frequently recognised by both IgG1 and IgG2 antibody had a molecular weight of 47 kDa. Serum IgG2 antibody bound to carbohydrate antigen with a molecular weight of 32 kDa but there was no recognition of carbohydrate antigens by IgG1 antibodies. There was no correlation between the titre of anti-*P. gingivalis* IgG1 or IgG2 antibody and the total concentration of serum IgG1 or IgG2 antibodies of all specificities.
Conclusions: Both IgG1 and IgG2 antibodies recognised a dominant antigen of 47 kDa, probably Arg-gingipain. Much of the response to carbohydrate antigen is of the IgG2 subclass. Neither the level of IgG1 nor the IgG2 antibody specific to *P. gingivalis* was related to the total serum concentration of that antibody.

Key words: antibody subclasses; periodontitis; *Porphyromonas gingivalis*; serum IgG

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Periodontal disease is caused by interactions between members of the oral microflora and the host. There are a number of species, including *Porphyromonas gingivalis*, which are closely associated with destructive disease. However, not all individuals with *P. gingivalis* develop periodontitis to the same extent, emphasising the importance of the host response in determining the natural progression of the disease. A number of different antigens of *P. gingivalis*, both protein and carbohydrate in nature, have been shown to provoke IgG antibody responses.

Human IgG antibody can be divided into four subclasses, of which IgG1 accounts for the greatest proportion of the total serum IgG antibody in adults (43–75%). IgG2 accounts for approximately 16–48% of IgG antibody and the relatively small proportion of remaining antibody comprises IgG3 and IgG4 antibody (10). The subclass of IgG antibody helps to determine the biological properties of the antibody, thereby potentially influencing the progression or stability of the disease process. For example, IgG1 and IgG3 are the most effective antibody subclasses at activating the complement cascade.

The race, genetic factors, diagnostic category and smoking habits of the host have been reported to influence the levels of a particular subclass of antibody in

periodontal disease (12). However, opinions vary concerning the influence of those factors in different groups of patients (6). In addition, the production of a Th2 profile of cytokines by the host's T lymphocytes is associated with production of IgG1 and a Th1 cytokine profile related to the production of IgG2 (9). However, prostaglandin E2 production could increase interferon gamma levels and promote IgG2 responses in periodontitis (25). The nature of the bacterial antigen also has a powerful influence on the subclass of antibody it elicits. In general, IgG1 is likely to be directed against protein antigens and IgG2 mainly against carbohydrate antigen, although repeated peptide sequences may also elicit IgG2 subclass antibody (23).

There is human IgG1 and IgG2 directed against antigens of *P. gingivalis,* but relatively little is known about the antigenic specificity of these antibodies. The subclass of IgG antibody against *P. gingivalis* has been related to the periodontal condition. Whitney et al. (26) reported that IgG1 and IgG4 antibody binding *P. gingivalis* was elevated in patients with early onset periodontitis compared with healthy controls. IgG4 antibody is characteristic of chronic inflammatory conditions and an elevated level of IgG4 was found in patients with periodontitis (18). Others found that progressive periodontal bone loss correlated best with the level of IgG2 antibody to *P. gingivalis* (21) and IgG2 antibody was correlated with the pocket depth in another study (16).

Aims

The aim of this work was to increase understanding of the common serum IgG1 and IgG2 subclass responses to *P. gingivalis* in patients with periodontal disease, and in particular to characterise further the IgG1 and IgG2 subclass antibody responses to protein and carbohydrate antigens of *P. gingivalis.*

Methods

Serum antibodies

Sera from 28 patients with periodontal disease were used to estimate the level of serum IgG1 and serum IgG2 antibodies specific to *P. gingivalis* using an enzyme-linked immunosorbent assay (ELISA) technique and to estimate the total concentration of serum IgG1 and serum IgG2 antibodies of all specificities using a radial immunodiffusion technique. Fourteen of these patients with chronic periodontitis were known to be colonised by *P. gingivalis* and have serum IgG antibody against *P. gingivalis* (2, 13). The IgG1 and IgG2 antibody responses of these 14 patients were further characterised by Western blotting with whole cell antigens of *P. gingivalis* and carbohydrate-rich antigens of *P. gingivalis*.

Sera from four periodontally healthy persons with very low levels of *P. gingivalis* and low serum IgG antibody responses to *P. gingivalis* (2, 13) were pooled and used throughout the study as low titre control sera. Sera from two other patients with severe periodontitis and particularly high IgG1 or IgG2 antibody titres against *P. gingivalis* were selected as high titre control sera.

All patients were free of medical conditions that could influence the periodontal status, were not taking medication affecting the periodontal condition and had received no antibiotics in the preceding 6 months. Patients provided informed consent and 10 ml of venous blood was collected. Clotted blood was centrifuged at 700 *g* for 10 min and aliquots of the serum frozen until use.

Electrophoresis and Western blot analysis

P. gingivalis (W50) was grown anaerobically at 37°C for 48 h on horse blood agar plates. *P. gingivalis* cells were washed and then resuspended in phosphate-buffered saline to an optical density of 1 at a wavelength of 525 nm. Aliquots of 1.5 ml of the suspension were centrifuged at 13,500 *g* for 5 min and the supernatant discarded. Carbohydrate-rich antigen was prepared by resuspending the cell pellet in 75 µl sodium dodecyl sulphate sample buffer and heating at 100°C for 5 min. Proteinase K 50 µg was added and the proteins were digested overnight at 50°C. Whole cell antigens were solubilised at room temperature with 135 µl of 0.2% sodium dodecyl sulphate and 15 µl of leupeptin to prevent autodegradation of whole cell antigens by *P. gingivalis* proteases. Preliminary gel analysis to optimise the conditions indicated that 15 µl of whole cell or carbohydrate antigen was added to 135 µl of sodium dodecyl sulphate sample buffer and boiled for 5 min before applying to the wells of an 11% sodium dodecyl sulphate gel. Molecular weight standards were prepared and applied to the gels according to the manufacturers' instructions. Gels were run on a 100 mm × 100 mm vertical electrophoresis system (Jencons-PLS, Leighton Buzzard, Beds, UK) apparatus at 60 mA and 150 V for approximately 1 h.

The separated antigens were blotted onto nitrocellulose sheets for 2 h at 70 V and 350 mA using a vertical electroblotting system (Jencons-PLS). Nitrocellulose sheets were cut into strips and nonspecific binding blocked by incubation with 10% skimmed milk powder in phosphate-buffered saline. Strips were incubated on a rocking table with a predetermined optimal dilution of 1 : 200 patient or control serum in phosphate-buffered saline containing 0.05% Tween 20 (PBST) with 1% skimmed milk powder and 0.5% bovine serum albumen at room temperature overnight. Strips were washed 3 times in PBST then incubated for 2 h with either a 1 : 5000 or 1 : 1000 dilution of peroxidase labelled antihuman IgG1 or antihuman IgG2 (The Binding Site Ltd, Birmingham, UK), respectively. After three more washes, blots were developed with diaminobenzidine (50 mg/100 ml phosphate-buffered saline and 66 µl hydrogen peroxide).

Enzyme-linked immunosorbent assay

An ELISA was used to measure the serum IgG1 and IgG2 antibody titres against *P. gingivalis*. *P. gingivalis* (W50) was grown in brain heart infusion broth supplemented with hemin (5 µg/ml) and vitamin K under anaerobic conditions at 37°C for 2 days. Cells were washed in phosphate buffered saline and suspended to an optical density of 0.02 (at wavelength 550 nm) in 0.05 M carbonate coating buffer. Aliquots of 100 µl of the suspension were incubated at 37°C for 4 h in 96-well microtitre plates (Dynatech, Billinghurst, UK). Plates were washed three times with PBST and nonspecific binding blocked by incubation with 100 µl of 1% bovine serum albumen in phosphate-buffered saline at room temperature for 1 h.

All serum samples, reference sera and secondary antibodies were diluted in 1% bovine serum albumen in PBST. Each patient's serum and the high or low control sera were diluted from 1 : 100 to 1 : 800. Test and control sera were omitted from certain wells to indicate whether there was nonspecific binding of the secondary antibodies. In addition, two reference sera previously shown to have a particularly high IgG1 or IgG2 titre to *P. gingivalis* were used to create a standard curve to calculate arbitrary ELISA units. Reference sera were diluted from 1 : 50 to 1 : 3200. Aliquots of 100 µl of each dilution of patient, control or reference sera were applied in duplicate to the wells and plates

were incubated overnight at 4°C. After washing three times in PBST, the wells were incubated with either a 1 : 4000 dilution of antihuman IgG1 labelled with peroxidase (The Binding Site Ltd) or a 1 : 1000 dilution of antihuman IgG2 labelled with peroxidase at 37°C for 1 h .

Plates were washed in PBST and incubated with peroxidase substrate (3,3,5,5-tetramethylbenzidine, Sigma, Poole, UK) made with one 50 mg tablet dissolved in 10 ml 0.05 M phosphate citrate buffer, pH 5, with 3 µl hydrogen peroxide added just before use, at 37°C for 30 min. The reaction was stopped by the addition of 50 µl 2 M H_2SO_4 and the optical density read at a wavelength of 450 nm using a Dynatech MR 5000 plate reader (Dynatech). A standard curve was plotted of the optical density of reference sera against the serum dilution. Individual serum IgG1 or IgG2 antibody levels were calculated from the linear portion of the IgG1 or IgG2 reference curves and expressed as relative arbitrary ELISA units.

There was negligible binding in the wells in which sera were omitted and only secondary antibody applied. All assays were repeated four times and the results presented are the mean of four assays.

Radial immuno-diffusion

The concentrations of serum IgG1 and IgG2 of all specificities were estimated by the radial immuno-diffusion method first described by Mancini et al. (15). Fresh agarose gels were prepared for each set of radial immuno-diffusion assays. Agarose 0.3 g was dissolved in 30 ml veronal buffer (0.05 M sodium barbitone and 0.01 M diethyl barbituric acid) at 60°C. Sheep antihuman IgG1 or IgG2 250 µl (5%) or 300 µl (6%) (The Binding Site Ltd), respectively, were added to 5 ml of the agarose solution, applied to 50 × 76 mm glass slides and spread to produce an even layer. After 15 min, 20 evenly spaced wells were cut into the solidified agar. Gels were stored in a humid box until required. Human IgG1 or IgG2 (1 mg/ml purified myeloma protein; The Binding Site Ltd) was diluted with normal saline (0.15 M) from 0.5 mg/ml to 0.03125 mg/ml. Preliminary assays had established that a 1 : 50 dilution of serum provided optimal gel readings. Aliquots of 5 µl of serum standards or samples diluted 1 : 50 in normal saline were applied to the wells. Gels were left undisturbed in a humid box for 24 h for diffusion to complete. Gels were dried in a hot air oven at 56°C then soaked in normal saline. They were stained with Coomassie Blue R350 stain (Pharmacia Biotech, Uppsala, Sweden) for 1 h prior to destaining in a solution of 5% methanol and 7% acetic acid. The diameter of the stained ring of insoluble antigen/antibody precipitate was read in millimetres on a calibrating viewer (Transidyne General Corporation, Ann Arbor, MI, USA) to one decimal place in three different planes. A mean diameter for each radial was calculated. The log of the concentration of the IgG1 or IgG2 standards was plotted against the square of the respective diameters to obtain a standard curve. The mean diameters of the samples were used to calculate the final concentrations of total serum IgG1 or IgG2 with respect to the standard curves.

Results

Serum IgG antibody bound most frequently to *P. gingivalis* whole cell antigens with molecular masses of approximately 32, 39, and 47 kDa (Fig. 1A). The same antigens were recognised by serum IgG1 antibodies (Fig. 1B) and by IgG2 antibodies (Fig. 1C), although the frequency of recognition of the antigens and the antibody subclasses produced varied between different patients. Antigen of molecular weight 47 kDa was most commonly recognised by both serum IgG1 and IgG2 antibody, with antigens of molecular weights 32 and 39 kDa also recognised by IgG1 and IgG2 from many patients. In three patients (Nos. 2, 6, and 7), Western blots demonstrated little IgG1 activity against *P. gingivalis*

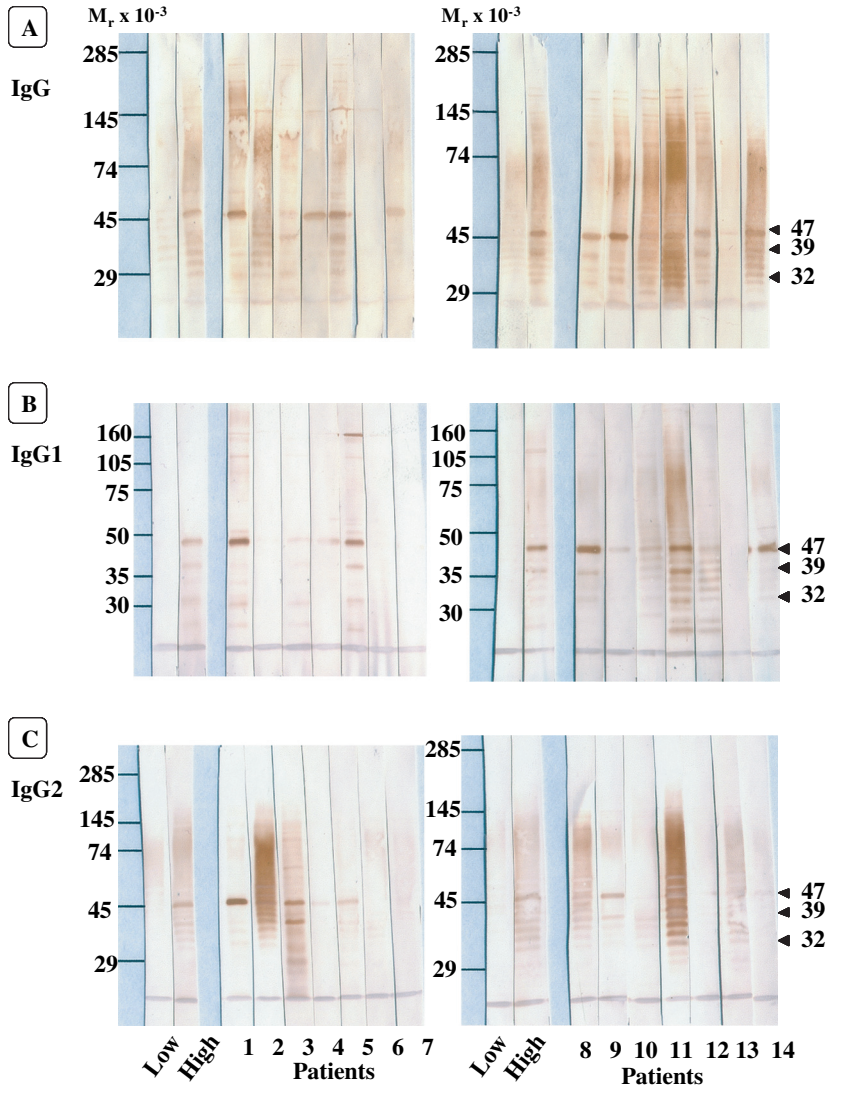

Fig. 1. Western blots of serum IgG antibody (A), IgG1 antibody (B) and IgG2 antibody (C) with whole cell antigens of *P. gingivalis* and serum from low or high human antibody controls and from periodontitis patients Nos. 1–14. Commonly recognised antigens are indicated at 47, 39, and 32 kDa.

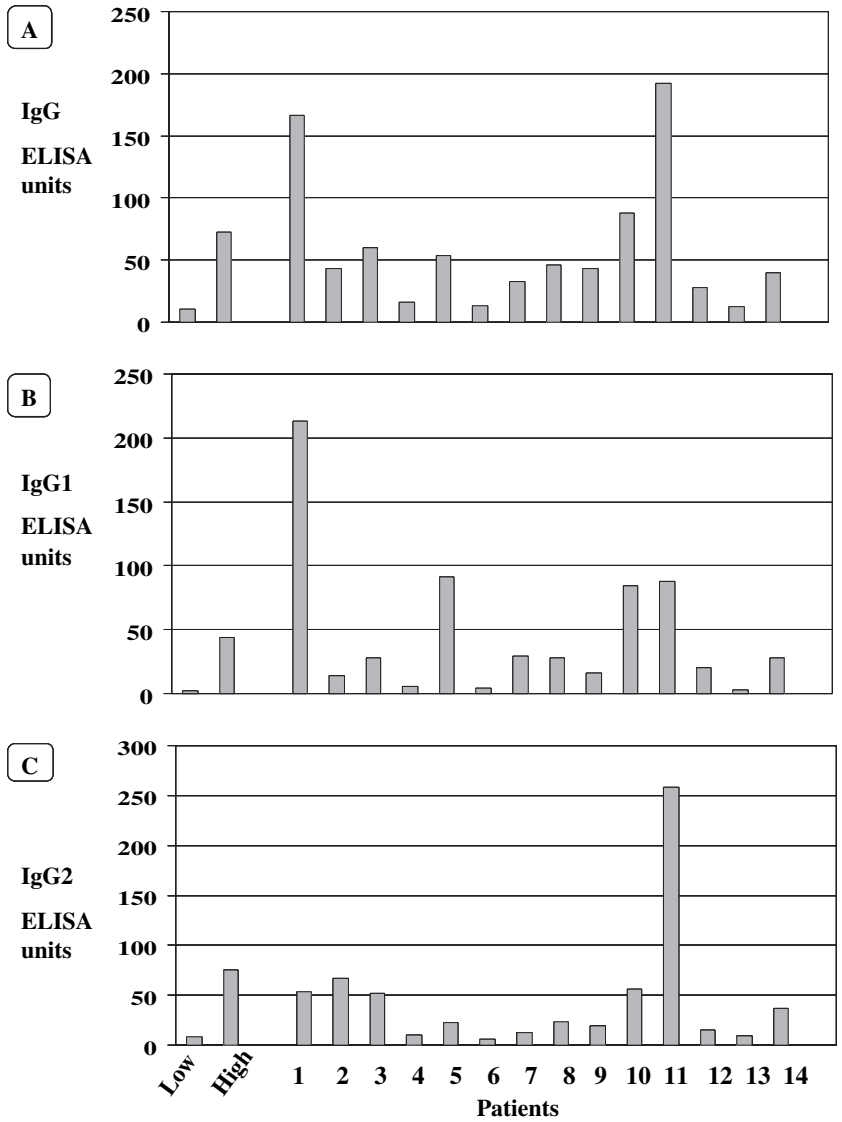

Fig. 2. ELISA of serum IgG antibody (A), IgG1 antibody (B), and IgG2 antibody (C) with whole cell antigens of *P. gingivalis* and serum from low or high human antibody controls and from periodontitis patients Nos. 1–14 with a range of antibody levels.

whole cell antigens (Fig. 1B) although there were detectable anti-*P. gingivalis* IgG and IgG2 (Fig. 1C) antibodies in these patients. 'Ladder-like' binding of IgG to *P. gingivalis* was seen with sera from several patients (Fig. 1A) and the majority of this antibody activity could be accounted for by the contribution of IgG2, particularly in patients Nos. 2 and 11 (Fig. 1C).

There were differences in the intensity of staining in the blots that tended to correspond with the levels of antibody detected in the ELISA (Fig. 2). The intense staining of the 47 kDa antigen by IgG1 antibody from patient No. 1 corresponded with a particularly high IgG1 antibody titre in this patient (Fig. 2B). The highest IgG2 antibody titres, found in patients Nos. 2 and 11 (Fig. 2C), corresponded with an intense IgG2 response to whole cell antigens binding in a ladder-like pattern in the respective blot (Fig. 1C).

When examining IgG2 responses to *P. gingivalis* carbohydrate-rich antigens, the 47 kDa antigen was not recognised; the 32 kDa antigen, however, was recognised by most patients (Fig. 3). A diffuse band of approximately 70 kDa was recognised by most patients and ladder-like patterns of antibody binding to the carbohydrate antigens were also common. There was negligible binding of serum IgG1 antibody to carbohydrate antigens of *P. gingivalis* (data not shown).

Both the levels of IgG1 antibody and IgG2 antibody specific to *P. gingivalis* and the total amount of serum IgG1 and IgG2 of all specificities were determined in 30 patients (Fig. 4), including the 14 for whom the qualitative assessment of anti-*P. gingivalis* IgG1 and IgG2 binding has been described. There was no statistically significant correlation between either the anti-*P. gingivalis* IgG1 and total serum IgG1 (Spearman's Rho $= -0.11$, $P = 0.57$) or the anti-*P. gingivalis* IgG2 and total serum IgG2 (Spearman's Rho $= -0.28$, $P = 0.14$).

Discussion

The aims of this study were to provide further information about the IgG1 and IgG2 antibody subclass responses to protein and carbohydrate antigens of *P. gingivalis*. Whole cell preparations containing both protein and carbohydrate antigens and also antigens in which the proteins had been digested by proteinase K were probed with patient sera. After establishing the IgG response to these *P. gingivalis* antigens, serum IgG1 and IgG2 antibody to *P. gingivalis* was detected; this is in agreement with other groups (16–18, 26, 27) who reported finding IgG1 and IgG2 antibody to *P. gingivalis* in the sera of patients with periodontitis. Most of these studies used quantitative ELISA techniques, which permitted them to compare the levels of the different IgG antibody subclasses in patient sera and relate them to the disease status of the patients. By also using a qualitative Western blotting technique in this study it was possible to characterise the molecular weight of the *P. gingivalis* antigens most consistently recognised by patient serum IgG1 and IgG2 antibodies. A limitation of this approach is that it does not confirm precisely which antigens are being recognised by the antibodies, but specific antigens of the molecular weights identified could be investigated in future work. In addition, carbohydrate is not necessarily charged by sodium dodecyl sulphate in the same way as protein and it is possible that carbohydrate epitopes recognised in Western blots will be found on glycosylated protein antigens (24) rather than pure carbohydrate antigens.

The possibility of some antibody to *P. gingivalis* cross-reacting with other oral and nonoral species was not ruled out in the current study. However, early work suggested that bacterial strains now classified as *P. gingivalis* had surprisingly few cross-reacting antigens in common with oral and nonoral species of the most closely related genera and species (20).

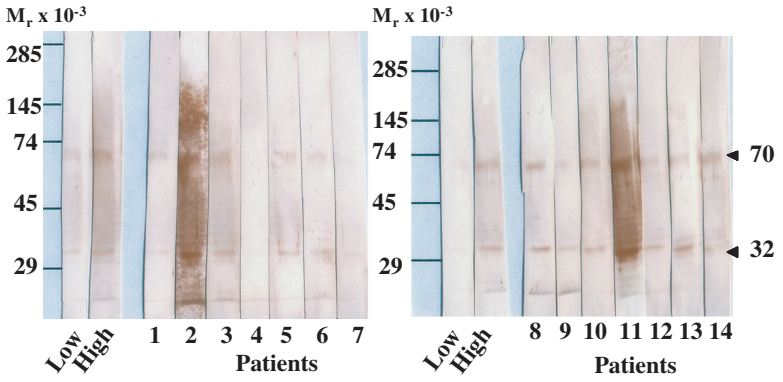

Fig. 3. Western blot of serum IgG2 responses to *P. gingivalis* carbohydrate-rich antigens with serum from low or high human antibody controls and from periodontitis patients Nos. 1–14. Commonly recognised antigens are indicated at approximately 70 and 32 kDa.

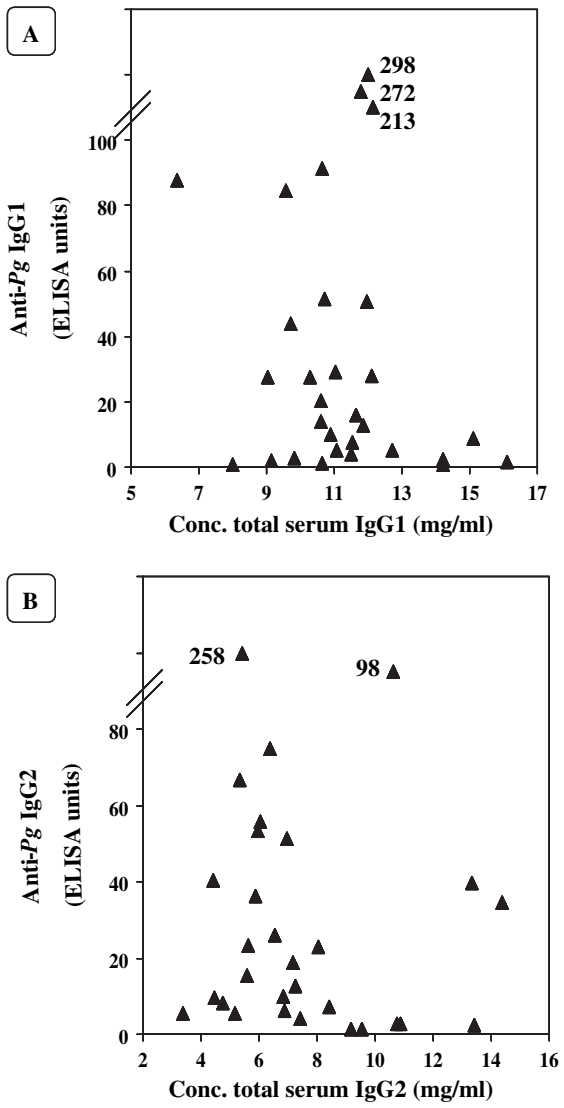

Fig. 4. The relationships between anti-*P. gingivalis* IgG1 and total serum IgG1 (A) and between anti-*P. gingivalis* IgG2 and total serum IgG2 (B) in 28 patients is not statistically significant (Spearman's Rho = − 0.11 and − 0.28, respectively).

There are oral representatives of closely related genera which cannot yet be cultured (14) and it is therefore not possible to exclude cross-reaction with those species. Cross-reactions of possible importance in the development of atheroma have been reported between antibody against oral bacteria and phosphorylcholine (11, 22). However, *P. gingivalis* was not one of the oral species named by either group.

Both serum IgG1 and IgG2 antibody recognised a characteristic 3-band pattern of approximately 32 kDa, 39 kDa, and 47 kDa. Two different mouse monoclonal antibodies bound to *P. gingivalis* antigen in a similar pattern (3, 7). The monoclonal antibodies were subsequently shown to bind to an epitope on the adhesive domain of the *rgpA* gene product, the Arg-gingipain protease (8, 13) and most of the 14 patients' sera had some IgG antibody binding to the same epitope (13). It is therefore probable that the majority of IgG1 and IgG2 antibody binding to 47 kDa antigen in the current study was recognising the adhesive domain of Arg-gingipain. The epitope of the 1A1 monoclonal antibody was common to multiple gene products including the *P. gingivalis tla* gene product, which has similarities to other bacterial receptors involved in adhesion (1). Previous and current work emphasises the importance of this epitope to the human immune system. An adhesin of the Lys-gingipain protease has a molecular mass of 39 kDa (16) and it is possible that this is the 39 kDa antigen recognised by IgG1 and IgG2 antibody in the current study.

Previously, IgG antibody has been shown to bind to *P. gingivalis* antigen of molecular weights between 84 and 104 kDa (17, 18) but the authors did not describe the subclasses of IgG involved. The current work observed multiple bands within that weight range, particularly in the IgG2 blots.

IgG antibody binding to carbohydrate-rich antigen of *P. gingivalis* was mainly of the IgG2 subclass and there was negligible IgG1 binding. IgG antibody from some patients with periodontitis has been reported to bind with a 'ladder-like' pattern to lipopolysaccharide from *P. gingivalis* (4, 17). In the current study the majority of antibody binding in a ladder-like pattern, suggestive of binding to lipopolysaccharide or another repeating unit polysaccharide, was of the IgG2 subclass. IgG antibody directed against the serotype-specific K1-6 capsular carbohydrates are described as almost exclusively IgG2

responses (5) but the molecular weights of those antigens are not described and cannot be compared with the current work. A limitation of the current work is that only the W50 strain of *P. gingivalis* was examined, but the aim of the current work was to characterise the most common IgG1 and IgG2 responses and other sero-types of *P. gingivalis* could be examined in future work. In addition, sero-type specific responses to the capsular carbohydrates were related to different forms of periodontitis (5) and in the current study only patients with chronic periodontitis were examined.

The reduction of IgG2 antibody binding to the higher molecular weight antigens after the *P. gingivalis* antigens had been digested with proteinase K suggests that the high molecular weight binding was to protein antigen or glycosylated protein antigen that had been digested. Although IgG2 antibody is commonly induced by bacterial carbohydrate antigens, repeated peptide sequences may induce IgG2 antibody (23) and such repeated sequences are found in the *rgpA* products. However, some of the high molecular weight *rgpA* products may also be post-translationally modified by the addition of carbohydrate (19).

The level of IgG1 and IgG2 antibody directed against *P. gingivalis* was not significantly correlated with the total serum IgG1 and IgG2 antibody of all specificities, indicating that the IgG antibody subclass responses specific to *P. gingivalis* could not be accounted for simply by the total production of IgG1 or IgG2 antibody, which is under strong genetic influence. This suggests that the mechanisms of specific IgG1 and IgG2 antibody production against *P. gingivalis* in patients with periodontal disease is related to the nature of the microbial antigens in addition to the exposure. Further work would be required to elucidate the way in which these interact with the host's genotype and other host-related factors.

Both the levels and the antigenic specificity of the IgG1 and IgG2 antibodies varied between patients. This might be explained by the patients' genotypes, smoking habits, or racial heritage (12), or by variations in their resident strain of *P. gingivalis*. All patients in this study were diagnosed with chronic periodontitis but no attempt was made to relate the antibody levels or specificity to the severity of disease.

In summary, this work is consistent with the importance of the *P. gingivalis rgpA* gene products as immunodominant antigens which can provoke both IgG1 and IgG2 antibody responses. The carbohydrate antigens of *P. gingivalis,* including lipopolysaccharide, provoke an IgG2 antibody response.

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