J Periodont Res 2004; 39; 228–235
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JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00730.x

Serum antibodies against the hemoglobin-binding domain (HA2) of *Porphyromonas gingivalis*

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DeCarlo AA, Nadkarni M, Paramaesvaran M, Yun PW, Collyer CA, Hunter N. Serum antibodies against the hemoglobin-binding domain (HA2) of Porphyromonas gingivalis. *J Periodont Res 2004; 39; 228–235.*

Background: The hemoglobin-binding domain (HA2) of the *Porphyromonas gingivalis* gingipains and hemagglutinins strongly binds hemoglobin and hemin and is thought to play a key role in acquisition of this essential metabolite by the microorganism.

Methods: In this report, we partially characterized human anti-HA2 humoral antibodies and their relationship to periodontal disease in an analysis of titer and function.

Results: Overall, serum anti-HA2 antibodies were relatively low and dominated by the immunoglobulin M (IgM) isotype. Pre-therapy titers had a direct association with periodontal health. Levels of *P. gingivalis* in the plaque were directly related to pre-therapy anti-HA2 IgG levels, and were an important covariant in a significant direct relationship between pre- and post-therapy anti-HA2 titers. Post-therapy anti-HA2 IgG antibody titers were directly related to the capacity of serum IgG fractions to neutralize hemoglobin binding by Lys-gingipain (Kgp). Further, lower levels of neutralizing activity post-therapy were directly related to severe periodontitis within the patient cohort.

Conclusions: These data suggest that anti-HA2 IgG antibodies correspond directly with periodontal health, possibly through their ability to neutralize *P. gingivalis* hemoglobin capture. The data also suggest that inadvertent or therapeutic inoculation of *P. gingivalis* in the plaque may contribute to generation of neutralizing anti-HA2 IgG and improvement of periodontal prognosis.

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Key words: antibodies; gingipains; hemagglutinin; real-time polymerase chain reaction; serum; *Porphyromonas gingivalis*

Accepted for publication January 5, 2004

Periodontal disease affects the majority of adults to some degree and may be associated with significant systemic morbidity (1, 2), including dental infection and loss of teeth (3). *Porphyromonas gingivalis* is implicated as an important periodontal pathogen by its high incidence and relative levels in human disease (4–7), and by its virulence in mono-infected animals (8, 9).

P. gingivalis has a requirement for an exogenous source of porphyrin-associated iron and has been shown to utilize heme or hemoglobin most efficiently (10–14). A 15 kDa protein domain (HA2), expressed primarily within the multi-domain gingipain (Kgp and RgpA) and hemagglutinin molecules of *P. gingivalis*, has recently been implicated as the microorganism's predominant high-affinity hemoglobin-binding receptor (15–18). With high-affinity binding to the heme moiety (19), this HA2 may also have a significant role in heme binding and uptake (18, 20, 21), a role that may be attributed to the HA2 domain of Kgp (22, 23).

Immunization with recombinant HA2 (rHA2) has been shown to confer some protection in a rat model of periodontitis (24), and this protection was

associated with high and sustained generation of anti-HA2 immunoglobulin G (IgG) antibodies. Assessment of the anti-HA2 antibody levels in patients might therefore be of considerable value in the understanding of protective mechanisms and the development of protective strategies for our patients.

In this report, we present pre- and post-therapy data demonstrating a host humoral immune response directed towards the potentially fundamental virulence factor, HA2, in patients with varying degrees of periodontitis severity.

Material and methods

Subjects and clinical procedures

Participants were conveniently recruited from patients presenting for dental care to the United Dental Hospital in Sydney, Australia. The criteria for acceptance into this study were: (i) no professional periodontal treatment within the prior 3 years, and (ii) no use of antibiotics within the prior 6 months. With informed consent, 5–15 μl marginal and submarginal dental plaque were collected in 300 μl of phosphate-buffered saline containing 10 mM sodium azide from the site with most advanced periodontitis as determined by radiograph analysis. Sample site plaque collection was done prior to any probing or treatment. Periodontal diagnosis scores were assigned to participants according to The American Dental Association case-type classification (0–3). All participants showed signs of gingival inflammation and periodontitis (diagnosis scores 1–3, loss of attachment 5–11 mm, formation of periodontal pockets with probing depths of 4–11 mm). Blood samples were obtained from 22 participants immediately following the initial examination and prior to commencement of therapy. Non-surgical periodontal therapy was administered after the initial examination and consisted of root planing one quadrant under local anesthesia. Post-therapy serum was collected from 15 participants between 1 and 22 weeks following root planing.

Serum IgG isolation

All of the participant sera were separated from clotted blood by centrifugation at 1000 *g* for 20 min and stored at −70°C until used. Once thawed for use, sodium azide was added to a final concentration of 10 mM and the serum samples were kept at 4°C. The IgG fraction was isolated from each patient sera by protein-G affinity chromatography. Protein-G columns (Pharmacia, Amersham Biosciences, Upsala, Sweden) were equilibrated with 50 mM Tris, 25 mM NaCl, 1 mM $CaCl_2$, 10 mM NaN_3, pH 7.4 then loaded with a 1/10 dilution of patient sera in the same buffer. Columns were washed with eight column volumes of equilibration buffer then bound IgG was eluted with 0.1 M glycine, pH 2.7. IgG fractions were adjusted to pH 8.4 with a 1/20 volume of 2 M Tris buffer, pH 8.4. IgG concentrations were determined by absorbance at 280 nm (extinction coefficient = 1.4) and eluants were each diluted to a final IgG concentration of 460 μg/ml with equilibration buffer, pH 8.4 for use in neutralization assays.

Serum titer determination

To detect serum immunoglobulin, alkaline-phosphatase conjugated goat anti-human IgG, IgM, or IgA antibody preparations were used in ELISAs performed as previously described (19). Because of the low serum antibody reactivity with the rHA2, serum dilutions were incubated overnight at 4°C. Serum titers to rHA2 or the gingipains were determined from an estimation of the equilibrium point of binding curves established with 10-fold dilutions using a four-parameter logistic algorithm. After estimating maximal and minimal signal at 414 nm for each dilution curve, the point on the curve representing 50% maximal signal (equilibrium point) was extrapolated to the sample dilution on the *x*-axis corresponding to that signal, the inverse of which was used as titer for these data. The concentrations (ng/ml) of specific anti-HA2 antibodies were determined by assessing the samples simultaneously with a calibrated pool of human sera and interpolating from a standard curve, also established using a four-parameter logistic algorithm.

Hemoglobin-binding inhibition assays

The hemoglobin-binding assay, described in an earlier report (19), was a variant of the ELISA in which hemoglobin was coated onto the wells then subsequently allowed to bind the gingipains in phosphate-buffered saline containing 0.1% Tween 20 (phosphate-buffered saline/Tween). IgG fractions of 460 μg/ml (described above) were pre-incubated with 1/3 volume 1 nM RgpA, or Kgp, in phosphate-buffered saline/Tween at room temperature in microtiter plates that had been blocked in phosphate-buffered saline/Tween. Separate microtiter plates were coated with 5 μg/ml human hemoglobin then blocked. The pre-incubated mixtures were added to the hemoglobin-coated plates and incubated for 1 h at room temperature. Levels of gingipains binding to hemoglobin were detected with monoclonal antibody (mAb) 2B2 followed by a rabbit anti-mouse AP conjugate and developed as described for ELISA.

mAb 2B2 was prepared in mice against gingipains as described (25), and recognizes the HA3 and HA1 domains of the gingipains in non-denatured samples (26).

Percentage inhibition of gingipain hemoglobin-binding was calculated relative to the maximal gingipain binding within the cohort, essentially allowing normalization of the data to the IgG sample with least neutralizing activity. Experiments were performed twice and data averaged for analysis.

P. gingivalis culture, rHA2 expression and protein purification

P. gingivalis ATCC 33277 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was grown as previously described (27). The rHA2 domain was cloned, expressed, purified and characterized as previously described (19). The gingipains RgpA and Kgp were purified

Table 1. Primers–probe set used in this study

Primers or probe	Sequence (5′–3′)	T_m (°C)	Reference
Porphyromonas gingivalis forward primer	TCGGTAAGTCAGCGGTGAAAC	58.8	(27)
P. gingivalis reverse primer	GCAAGCTGCCTTCGCAAT	58.7	(27)
P. gingivalis probe	[6-FAM] CTCAACGTTCAGCCTGCCGTTGAAA [TAMRA][a]	68.8	(27)
Universal forward primer	TCCTACGGGAGGCAGCAGT	59.4	(29)
Universal reverse primer	GGACTACCAGGGTATCTAATCCTGTT	58.1	(29)
Universal probe	[6-FAM] CGTATTACCGCGGCTGCTGGCAC [TAMRA][a]	69.9	(29)

[a]6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

from log-phase culture as previously described (28).

Design of primers–probe sets

The design of a universal primers–probe set and a *P. gingivalis* species-specific primers–probe set has previously been reported by us (27, 29). The universal primers–probe set (Table 1) generated a 466 bp amplicon spanning residues 331–797 on the *Escherichia coli* 16S ribosomal RNA (rRNA) gene (GenBank Accession no. ECAT1177T) with an internal site for the dual-labeled fluorogenic probe. The *P. gingivalis* species specific primers–probe set (Table 1) generated a 150 bp amplicon spanning nucleotides 589–739 in the *P. gingivalis* 16S rDNA sequence (portion of the genome encoding the rRNA; GenBank Accession no. L16492) with an internal site for the dual-labeled fluorogenic probe. The primers–probe sets fulfilled the recommended guidelines set by Applied Biosystems (Foster City, CA, USA). All the probes and primers were synthesized by Applied Biosystems.

Real-time polymerase chain reaction (PCR)

Bacterial DNA was isolated from sample site plaque (described above) using QIA Amp DNA Mini kit (QIAGEN, Clifton Hill, Victoria, Australia) according to the manufacturer's instructions. This technique efficiently extracts DNA from Gram-negative and anaerobic bacteria (27). Amplification and detection of DNA by real-time PCR made use of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using a 96-well plate format. The PCR was carried out in triplicate, in a 25 μl reaction volume containing a final concentration of 100 nM of each of the *P. gingivalis* primers–probe set or 300 nM of the universal primers and 100 nM universal probe (Table 1) using the TaqMan® PCR Core Reagents Kit (Applied Biosystems). The reaction conditions for amplification of DNA were 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Data was analyzed using the Sequence Detection System 6.1 supplied by Applied Biosystems. Purified DNA from plaque samples was diluted 10-fold before relative estimation of DNA on a *P. gingivalis* DNA standard graph. Known quantities of *P. gingivalis* DNA (3600 pg to 0.36 pg) were used to establish a standard graph for relative estimation of DNA in the plaque samples. The amount of *P. gingivalis* present in the samples were expressed as a percentage of the measured Gram-negative and anaerobic Gram-positive organisms in the samples (% *P. gingivalis*), as detected by the universal primers (27).

Data analysis

All data was entered into and analyzed with the JMP™ statistical software (version 5.0.1a). Values of serum titers were adjusted for normalcy of distribution by log conversion of values. Values of HgA1c (a measurable form of non-enzymatically glycated hemoglobin which reflects long-term blood glucose levels) were adjusted for normalcy of distribution by conversion with the following formula:

$$\log[\mathrm{HgA1c}/(50 - \mathrm{HgA1c})] \quad (1)$$

When assessing statistical interactions with periodontitis severity, variability attributable to HgA1c, smoking (pack-years), and age, were always included in the analyses due to their known interactive effect on periodontal disease severity as follows:

$$S_j = \alpha + \beta_1 S_j + \beta_2 H_j + \beta_3 M_j + \beta_4 A_j + u_j \quad (2)$$

where S_j was the periodontitis severity score measured for the jth person; H_j was the HgA1c level measured for the jth person; M_j was a dummy variable for smoking which receives a value of 1 if the jth person smokes, a value of 0 otherwise; A_j was the age of the jth person; u_j was a normally and independently distributed stochastic disturbance for the jth person; $\beta_1 \ldots \beta_4$ were the least squares coefficients estimated in the equation for the jth person; and where $j = 1,\ldots, m_i$; and m_i were the number of subjects in the data set for the equation. In accordance with the overall hypothesis being tested, a cross-sectional interpretation of post-therapy anti-HA2 antibody titers resulting from therapeutic inoculation (root planing) would require a consideration of *P. gingivalis* in the plaque, as well as the amount of time lapsed between inoculation and measurement. Therefore, these covariates were similarly added to the least-squares analysis of the relationship between pre-therapy IgG anti-HA2 titers and post-therapy IgG anti-HA2 titers.

Materials

All chemicals and compounds were purchased from Sigma Corp., New South Wales, Australia unless otherwise specified in the text.

Results

Of the 22 participants included in this analysis, the mean age was 49 ± 9 years, the mean diagnosis score was 2.6 ± 0.6, the mean sample site pocket depth was 6.6 ± 2.0 mm, and the mean sample site attachment loss was 8.1 ± 1.9 mm.

Total serum anti-HA2 antibody levels were low, estimated to be < 50 ng/ml in all of the serum samples. Serum IgM anti-HA2 titers predominated within the pre-therapy serum of untreated patients, followed by titers of the IgG isotype then the IgA isotype (Fig. 1).

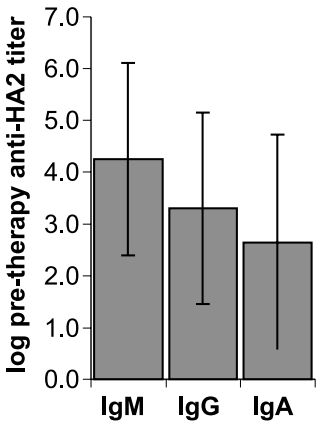

Fig. 1. Immunoglobulin M (IgM), IgG, and IgA pre-therapy serum anti-hemoglobin-binding domain (anti-HA2) titers. Log values of pre-therapy anti-HA2 serum titers are represented for IgM, IgG, and IgA. Means are displayed, and error bars depict standard deviations.

Pre-therapy serum antibody titers (total IgM, IgG, IgA) specific for the HA2 domain varied inversely with periodontal disease severity in untreated patients. An inverse relationship with periodontal diagnosis was present ($p = 0.039$, ordinal logistic fit controlling for age, smoking, and HgA1c) (Fig. 2a). An index of attachment loss was also inversely related to pre-therapy anti-HA2 antibody titers ($p = 0.028$, least squares analysis) (Fig. 2b).

IgG antibody titers specific for the HA2 domain partially accounted for this inverse relationship with periodontal disease severity in untreated patients (Fig. 3).

Serum IgG antibody titers specific for the HA2 domain varied directly with an estimate of percentage *P. gingivalis* in plaque in untreated patients ($p = 0.025$, linear regression analysis) (Fig. 4).

After non-surgical periodontal therapy, mean post-therapy anti-HA2 serum titers for either the IgM, IgG, or IgA isotypes did not significantly increase with respect to pre-therapy titers. Unexpectedly, pre- and post-therapy anti-HA2 IgG titers were not correlated well within the subjects ($R^2 = 0.06$, $p = 0.374$, least squares analysis). Recognizing that the relative level of *P. gingivalis* in plaque, and the number of days between therapy and serum collection, might be relevant in assessment of post-therapy titers, and including these as covariates in analysis, pre-therapy anti-HA2 IgG titers were able to significantly predict post-therapy anti-HA2 IgG titers ($p = 0.012$, least squares analysis) (data not shown). The relevance of these covariates suggested that a typical dose- and time-dependent, humoral immune response specific for the HA2 domain could occur following non-surgical periodontal therapy.

Higher post-therapy anti-HA2 IgG titers corresponded functionally to higher neutralization of hemoglobin binding by Kgp (but not RgpA) ($p = 0.008$, least squares analysis), but, unexpectedly, only when including serum HgA1c levels in the model (Fig. 5). In the same analysis, pre-therapy anti-HA2 IgG antibodies only showed a weak trend towards neutralizing Kgp hemoglobin-binding (data not shown).

Poor neutralization of Kgp hemoglobin-binding activity by post-therapy serum IgG was found in those with severe, generalized periodontal disease ($p = 0.01$, ordinal logistic fit, controlling for age, smoking, and HgA1c) (Fig. 6).

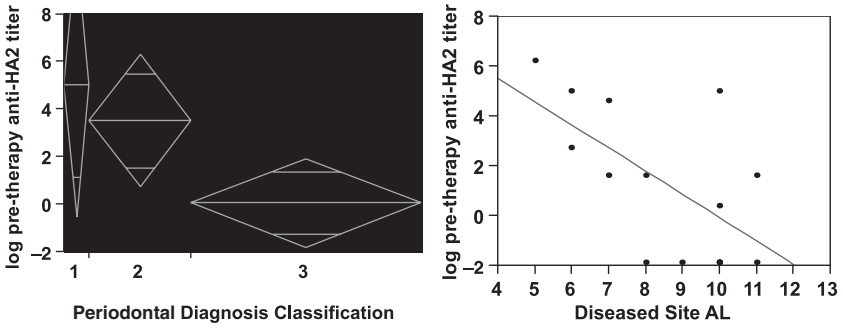

Fig. 2. Pre-therapy serum antibody titers specific for hemoglobin-binding domain (HA2) varied inversely with periodontal disease severity. (a) Total pre-therapy anti-HA2 antibody titers (log values) of the first 14 participants with periodontal diagnosis classification of either mild (1), moderate (2), or severe (3) disease are depicted. Group means and 95% confidence intervals (CI) are represented within the diamonds, where width represents relative sample size. $p = 0.039$, ordinal logistic fit controlling for age, smoking, and HgA1c. (b) Total pre-therapy anti-HA2 antibody titers (log values) of the first 14 participants with attachment loss (AL) indices ranging from 5 to 11 mm. Line represents least squares best fit. $p = 0.028$, least squares analysis, controlling for age, smoking, HgA1c levels.

Discussion

In moving towards a clearer understanding of periodontal disease pathogenesis, the immunogenicity of known microbial virulence factors should be examined. In a limited cohort, we have begun to measure natural serum titers to the HA2 domain of *P. gingivalis*, which is highly conserved in all *P. gingivalis* strains studied, is structurally unique, and functions critically to scavenge essential porphyrin from the environment (18). It is speculated that a major function of the hemagglutinin region of gingipains, which includes the HA2 domain, is to agglutinate erythrocytes for lysis (possibly by the proteolytic domain), to bind hemoglobin for proteolysis, then to capture released heme as an essential metabolite (11, 17). We have previously shown that the HA2 domain is essential to the gingipains

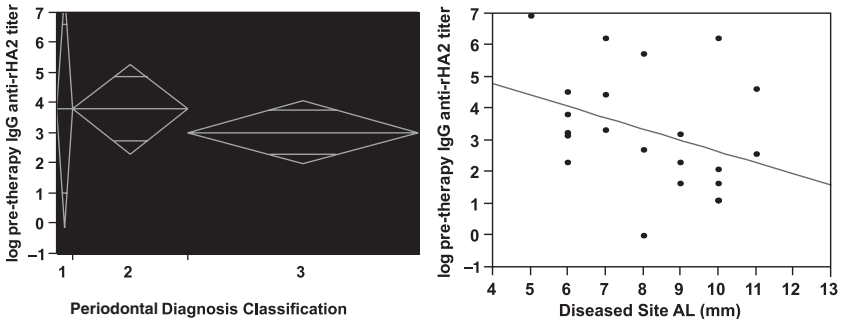

Fig. 3. Pre-therapy serum immunoglobulin G (IgG) antibody titers specific for hemoglobin-binding domain (HA2) varied inversely with periodontal disease severity. (a) Pre-therapy anti-HA2 IgG antibody titers (log values) of all participants with periodontal diagnosis classification of either mild (1), moderate (2), or severe (3) disease are depicted. Group means and 95% confidence intervals (CI) are represented within the diamonds, where width represents relative sample size. (b) Pre-therapy anti-HA2 IgG antibody titers (log values) of all participants with attachment loss (AL) indices ranging from 5 to 11 mm. Line represents least squares best fit demonstrating the inverse trend of anti-HA2 antibody titers with increasing AL severity.

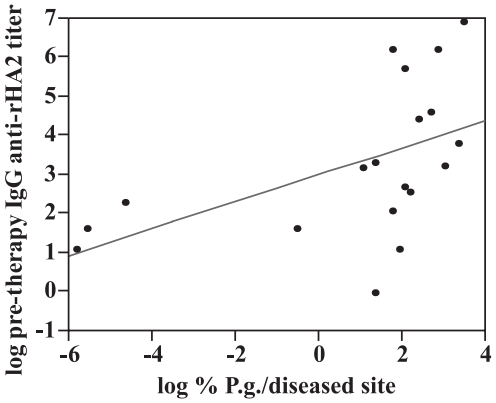

Fig. 4. Serum antibody titers specific for hemoglobin-binding domain (HA2) varied directly with *Porphyomonas gingivalis* in plaque. Log values of pre-therapy anti-HA2 immunoglobulin G (IgG) titers (*y*-axis) are shown in direct association with percentage *P. gingivalis* (*x*-axis) (determined by real-time PCR of plaque from a site with most advanced periodontitis as described in Materials and Methods). $p = 0.025$, linear regression analysis. Note that the direct association remained significant ($p = 0.031$) when the three outlier subjects were excluded from the analyses (not shown).

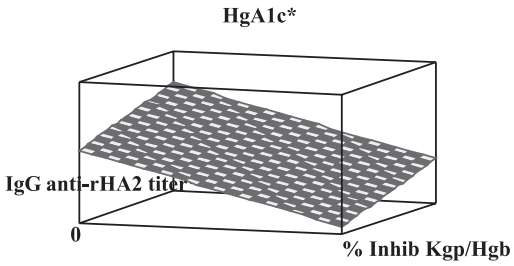

Fig. 5. Neutralization activity by post-therapy immunoglobulin G (IgG) of Kgp hemoglobin-binding is related directly to post-therapy anti-HA2 IgG and indirectly to HgA1c levels. Planar relationship between post-therapy anti-HA2 IgG titers (log values) (*z*-axis), percentage inhibition of hemoglobin binding by Kgp with post-therapy IgG (*x*-axis) and HgA1c levels (log conversion as described in Materials and Methods) (*y*-axis) of patients is depicted. $p = 0.008$ for the model, least squares analysis. *Designates log values of data.

for tight binding of hemoglobin and hemin (19), and that the entire organism could rely heavily on this protein domain for normal growth, and by extension, competitive growth (18).

It was, therefore, not entirely surprising that the natural humoral anti-HA2 levels were low. Success of an opportunistic pathogen would be compromised if any of the critical bacterial extracellular proteins, such as the hemoglobin/heme-binding HA2 domain of *P. gingivalis*, were not protected from effective immune targeting by the host. We have previously presented data indicating that the HA2 domain within the gingipains is at least partially protected from antibody detection, and, by extension, immunological detection (19). Accordingly, the HA2 domain appears to generate a weaker humoral antibody response than the other gingipain hemagglutinin domains HA1, HA3, and HA4 in chronic adult periodontitis (26). Alternatively, those individuals inherently able to generate effective anti-HA2 humoral antibodies might be most protected from *P. gingivalis* and, theoretically, have less aggressive adult periodontitis, as these data have suggested.

It was intriguing that in our patient group, which had a limited history of prior periodontal therapy, those presenting with less periodontal disease had higher antibody titers against HA2 than did those members of the group with more aggressive adult periodontitis, who had significantly lower anti-HA2 titers. In another report, there was no correlation of the other gingipain hemagglutinin domains HA1, HA3, or HA4 with parameters of disease severity in periodontitis patients (26). This suggested to us that the ability to generate relatively higher anti-HA2 antibody titers during the course of chronic periodontal infection would confer relative protection.

We extended this finding to test the hypothesis that those who generated relatively higher anti-HA2 IgG antibody titers following periodontal therapy might be those with the lesser degree of periodontitis. However, periodontal health was not directly associated with higher post-therapy

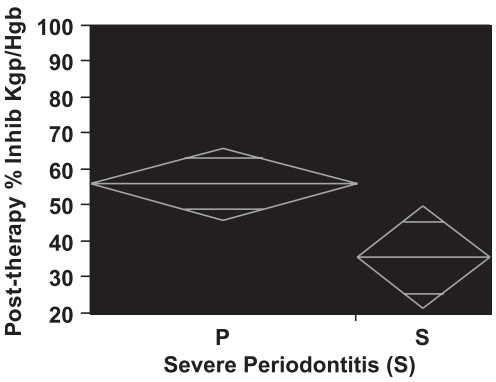

Fig. 6. Percentage inhibition of hemoglobin binding by Kgp with post-therapy IgG (*y*-axis) of participants with a generalized form of severe adult periodontitis (S) vs. those diagnosed with less severe periodontitis (P). Group means and 95% confidence intervals (CI) are represented within the diamonds, where width represents relative sample size. $p = 0.01$, ordinal logistic fit, controlling for age, smoking, and HgA1c.

anti-HA2 IgG titers. Rather, the improved functionality of the post-therapy anti-HA2 IgG titers relative to their ability to neutralize the function of the HA2 domain, namely hemoglobin binding of Kgp, was directly related to the periodontal health of the subjects. Because the binding affinity and neutralization activity are functionally related (30) and are generally higher in the memory responses [for review see (31)], these data could be interpreted to fit the classic model of humoral antibody responses to antigen.

In further support of the therapeutic inoculation model was the relevance of percentage *P. gingivalis* in the plaque, and the time-dependence of sampling. Enhancement of anti-*P. gingivalis* antibodies post-therapy would not be expected to be as robust if there were limiting proportions of *P. gingivalis* in the periodontal pockets. Likewise, the time-dependence of the IgG response subsequent to immunization, rising within weeks then falling over a period of months, is well known, so the importance of these covariates was expected.

A low and chronic level of antigen presentation, as could occur in the untreated patient, might be expected to maintain a high level of specific IgM, which we found in our pre-therapy titers, and also to evoke some potential for immunologic memory. As an example, in mice, pre-immune serum IgM levels specific for the *Streptococcus mutans* Antigen I/II were significantly higher than corresponding IgG or IgA levels (32), and three oral immunizations with this antigen produced a significant dose-dependent rise in specific levels of IgG and IgA, but not IgM (33). Also, in rats, a local immunization with whole *P. gingivalis* into the gingiva was shown to produce an IgM response that was two-fold greater than the corresponding IgM response after 1 week, with IgG levels surpassing the IgM levels over the subsequent weeks and months (34). Therefore, single therapeutic inoculation of the relatively naive patient, as we can consider our mechanical periodontal therapy, might be expected to evoke high IgM levels characteristic of an initial immunization, but also IgG antibodies with potentially greater specificity, each of which we tended to see in the secondary titers.

The statistical interaction of HgA1c levels with the functional secondary antibody response is difficult to interpret at this time. Diabetes control, reflected in HgA1c levels, is known to affect periodontal disease progression (35), and a significant body of literature implicates the non-enzymatic glycation of various cellular and extracellular biomolecules [for example (36, 37)]. Therefore, HgA1c, which is a well-known quantitative marker for chronic hyperglycemia and non-enzymatic glycation, was tested in the model. Its significance in this model suggests that variability in either post-therapy anti-HA2 titers or neutralization activity were closely linked to variability in HgA1c levels. There is evidence that hyperglyemic conditions and non-enzymatic glycation may also affect lymphocyte activities (38, 39), but alteration of the binding or neutralization capacity of IgG has not been established.

Recently, an analysis of anti-*P. gingivalis* PrtC (collagenase) serum antibodies measured 2 years after clinical therapy in 34 periodontitis patients demonstrated less progression of attachment loss in patients *without* detectable anti-PrtC antibodies (40). This suggested that although anti-HA2 or other antimicrobial serum antibodies could be protective against periodontitis, clinical therapy and a maintained eradication of the microbes would ultimately result in undetectable levels of serum antibodies.

Data such as these presented in this report can provide information on the immunogenicity of a particular periodontal antigen in humans, and on the role of the humoral response relative to that antigen and periodontal disease. The host response of other virulence factors has been examined, and indeed, several, including the HA2 domain (24), are being tested in animal trials as vaccine candidates (41–46). The longitudinal data from this limited patient cohort suggested that generating and maintaining higher anti-HA2 serum antibody titers might be protective against periodontal bone loss.

Acknowledgements

This research was funded by a grant from the National Health and Medical Research Council of Australia. The authors thank Patrick Hardigan, Ph.D. at Nova Southeastern University, for guidance in statistical analysis of these data.

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