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Antibody reactive with *Porphyromonas gingivalis* hemagglutinin in chronic and generalized aggressive periodontitis

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Background: Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobe, is found in periodontitis lesions and its presence in subgingival plaque significantly increases the risk for periodontitis. We have previously shown that patients with aggressive forms of periodontitis that are seropositive for *P. gingivalis* have less attachment loss than those that are seronegative. This suggests that antibody reactive with antigens of *P. gingivalis* may be protective and decrease disease severity and extent. Recent studies in the murine abscess model and in the host antibody response in chronic periodontitis patients suggest that antibody reactive with *P. gingivalis* hemagglutinin may be an important protective antibody response.

Objectives: In this study, we tested the hypothesis that there was a significant relationship between antibody reactive with *P. gingivalis* hemagglutinin and measures of periodontal attachment loss.

Methods: We determined the immunoglobulin G (IgG) antibody concentration reactive with recombinant *P. gingivalis* hemagglutinin in 117 chronic periodontitis and 90 generalized aggressive periodontitis patients. We also determined the IgG subclass distribution for antibody reactive with *P. gingivalis* hemagglutinin.

Results and Conclusions: We found IgG reactive with *P. gingivalis* hemagglutinin in both chronic periodontitis and generalized aggressive periodontitis patients. Most of this IgG antibody was of the IgG1 and IgG3 subclasses. Antibody reactive with *P. gingivalis* hemagglutinin, however, did not have a significant relationship with measures of periodontal attachment loss.

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Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobe, is frequently found in periodontitis lesions (1–4) and is a risk factor for periodontitis (5). We have previously shown that patients with aggressive forms of periodontitis that are seropositive for *P. gingivalis* have less attachment loss than those that are seronegative (6). This suggests that antibody reactive with antigens of *P. gingivalis* may be protective and decrease disease severity and extent. The antigen(s) important in this protective antibody response in humans are unknown. Recent work suggests that antibody reactive with *P. gingivalis* hemagglutinin is protective in rodent models (7–10). There is also a report that suggests that total immunoglobulin G (IgG) and IgG2 reactive with *P. gingivalis* hemagglutinin correlates with increased attachment loss, whereas IgG4 levels are inversely

Table 1. Patient demographics

Disease class	*n*	Age (mean ± SD)	Smoker (%)	Gender		Race		
				Male	Female	Caucasian	African-American	Hispanic
Chronic periodontitis	117	43.81 ± 11.29	34	46	71	39	78	0
Generalized aggressive periodontitis	90	28.32 ± 6.09	41	38	52	30	59	1

related to attachment loss (11). In this study, we tested the hypothesis that there is a significant relationship between antibody reactive with *P. gingivalis* hemagglutinin and measures of periodontal attachment loss. We examined total IgG and IgG subclasses reactive with *P. gingivalis* hemagglutinin in chronic periodontitis patients and extended the previous work to include patients with generalized aggressive periodontitis.

Materials and methods

Human subjects

Subjects for this study comprised 117 chronic periodontitis patients and 90 generalized aggressive periodontitis patients who were clinically characterized in the Virginia Commonwealth University Clinical Research Center for Periodontal Diseases (see Table 1). These patients were classified as follows.

(i) Chronic periodontitis – subjects 35 years or older with chronic periodontitis generalized to all four quadrants, although cuspid and incisor involvement may be absent. Subjects had three out of four of their first molars with pocket depths in interproximal sites of 5–7 mm with approximately the same loss of attachment. Subjects ranged in age from 35 to 55 years of age.

(ii) Generalized aggressive periodontitis – subjects of 35 years or less with a generalized pattern of severe destruction, with attachment loss of at least 5 mm on eight or more teeth, at least three of which were not first molars or incisors.

Patients having systemic diseases or receiving long-term treatment with antibiotics, nonsteroidal anti-inflammatory drugs, corticosteroids, or immunosuppressive therapy that might affect their immune responsiveness, were excluded from this study.

Preparation of *P. gingivalis* hemagglutinin

Hemagglutinin A protein was overexpressed in *Escherichia coli*, purified and used as the antigen in an enzyme-linked immunosorbent assay (ELISA). The QIAexpress® system (Qiagen, Valencia, CA, USA) was used. First, pVA2601 was constructed by cloning the 1.3 kb repeat region from *hagA* (12). The recombinant plasmid was then introduced into *E. coli* M15 cells containing the pREP4 repressor plasmid, thus creating the V2601 strain. Overnight culture of V2601 was used to inoculate 1 liter of Luria Bertani media. That culture was grown at 37°C until OD_{660} was equal to 0.6–0.7. Expression was induced by adding isopropyl-β-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated for an additional 2 h. Bacterial cells were pelleted, resuspended in Buffer A (50 mM $Na_2H_2PO_4$; 300 mM NaCl at pH 8.0) and disrupted by sonication (3 × 10 s, with cooling in between sonication). The mixture was centrifuged for 30 min at 40 *g* at 4°C. Supernatant was stored at 4°C and the pellet resuspended in Buffer B (100 mM $Na_2H_2PO_4$; 10 mM Tris-HCl; 3 M urea at pH 8.0), sonicated as described above and centrifuged at 20 *g* for 30 min at 4°C. This last supernatant underwent nickel affinity chromatography. The matrix was washed with Buffer C (same as B but pH 6.3) and His-tagged protein was eluted with Buffers D (pH 5.9) and E (pH 4.5).

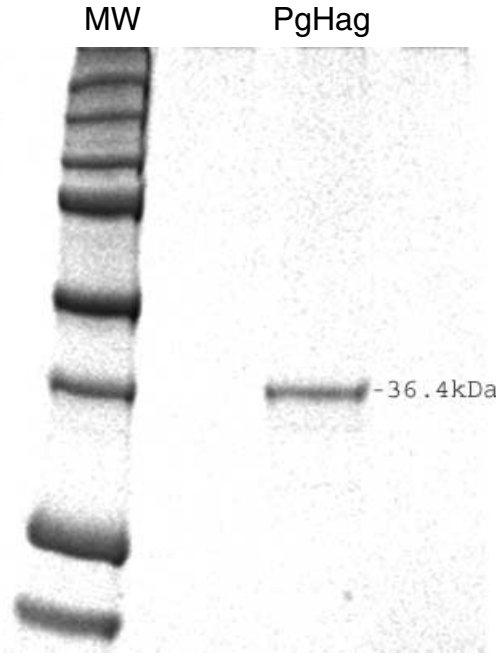

Fig. 1. Polyacrylamide gel of purified *Porphyromonas gingivalis* hemagglutinin. Lane 1, molecular weight standard (MW); lane 2, *P. gingivalis* hemagglutinin (PgHag).

Table 2. IgG antibody concentrations reactive with *Porphyromonas gingivalis* hemagglutinin and clinical variables in chronic periodontitis and generalized aggressive periodontitis patients (mean ± SD)

Anti-hemagglutinin (μg/ml)	*n*	Age (year)	Pocket depth (mm)	Mean attachment loss (mm)	% of teeth with attachment loss	
					≥ 2 mm	≥ 5 mm
0	111	36.11 ± 12.34	3.11 ± 0.97	2.19 ± 1.56	0.74 ± 0.27	0.33 ± 0.30
≤ 5	56	35.70 ± 11.97	3.12 ± 0.98	2.13 ± 1.66	0.73 ± 0.27	0.31 ± 0.30
> 5, ≤ 10	14	43.29 ± 11.42	3.22 ± 1.10	2.65 ± 1.88	0.78 ± 0.31	0.44 ± 0.38
> 10	26	41.12 ± 10.74	3.04 ± 0.75	2.28 ± 1.24	0.86 ± 0.18	0.34 ± 0.28

Most of our protein eluted in Buffer E. To verify the purity of the hemagglutinin protein, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with the denatured protein in order to determine the isolated protein to be 36.4 kDa (Fig. 1).

Enzyme-linked immunosorbent assay (ELISA)

Serum IgG antibodies to *P. gingivalis* hemagglutinin A were quantified by ELISA. Briefly, 100 ml of 0.015 M carbonate, pH 9.6, containing 250 ng protein/100 μl, were transferred to wells of a 96-well microtiter plate (Immulon 1, Dynatech Laboratories, Inc., Alexandria, VA, USA). The plates were incubated at 4°C for 16 h and then washed in tap water 20 times. Sera were serially diluted (from 1 : 100 to 1 : 3200) in diluent (equal volumes of distilled water containing 10% non-fat dry milk and phosphate-buffered saline, pH 7.2). Each of the serum dilutions were added to two antigen-coated wells and two uncoated wells. The final quantity of serum in the wells varied from 1/1000 ml in the first well to 1/32,000 ml in the final well. The uncoated wells served as a background control. After incubation and washing as described above, 100 μl of a 1 : 1000 dilution of alkaline phosphatase labeled goat anti-human IgG (heavy and light chain specific, Kirkegaard and Perry Laboratories, Inc., Gaithersberg, MD, USA) in diluent was added per well. After incubation for 16 h and washing, 100 μl of substrate (*p*-nitrophenyl phosphate, 1 mg/ml, in diethanolamine buffer) was added and incubated for 30 min at room temperature. The optical density (OD) at 405 nm for each well was then

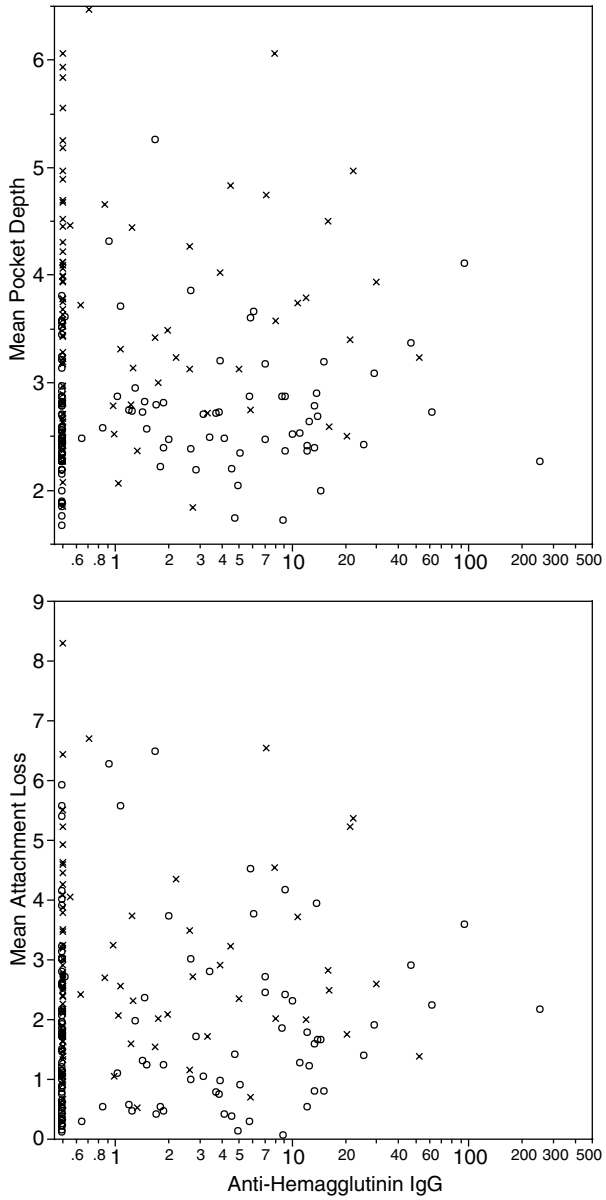

Fig. 2. Total IgG antibody concentration reactive with *Porphyromonas gingivalis* hemagglutinin (μg/ml) vs. mean pocket depth (mm) and mean attachment loss (mm); × = aggressive periodontitis patients, ○ = chronic periodontitis patients. The correlation coefficients between measures of attachment loss and seropositivity approached zero for pocket depth ($r = -0.03$, p-value = 0.6) and attachment loss ($r = 0.03$, p-value = 0.7).

measured using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The difference in OD (delta optical density between coated and uncoated wells) was the basic data unit.

Optical density readings were converted to an equivalent concentration of IgG antibodies by means of heterologous interpolation, as described elsewhere (13). Briefly, a standard curve was constructed using anti-NP-BSA human–mouse chimeric antibodies and NP-BSA as the antigen (Hybridoma Reagent Laboratory, Baldwin, MD, USA). A plot of OD vs. α-NP-BSA mouse–human IgG1 chimera was then utilized to estimate IgG concentration in patient sera using the corresponding OD values.

For determination of IgG subclass antibody reactive with *P. gingivalis* hemagglutinin, the method as described by O'Brien-Simpson was used (11). We initially used a single serum dilution of 1/500 but found some of our sera yielded optical densities that were above the range of our plate reader. The ELISA was therefore carried out as described above except 12 serum dilutions (1/100 to 1/204, 800) were used for each sample and the optical density at 405 nm was converted to ELISA units (EU) by comparing the optical density for each patient's serum to a standard curve generated using a reference serum that was arbitrarily set to a value of 100. The secondary antibodies for the four subclasses were alkaline phosphatase conjugated anti-human IgG1–4 monoclonal antibodies (IgG1 = 401,459; IgG2 = 401,462; IgG3 = 401,465; IgG4 = 401,468; Calbiochem, La Jolla, CA, USA).

Statistical analysis

Analysis of variance was performed in order to determine whether there was a relationship between log-transformed serum concentration of antibody reactive with *P. gingivalis* hemagglutinin and attachment loss [zero values were analyzed as log(0.5)]. The log-transformed values were used because the original scale values were strongly skewed. Transformed values satisfied the equal variability and normality assumptions of the analyses. The analysis controlled for pre-existing differences due to gender, age, smoking status, classification of periodontal disease and race. The distribution of antibody concentration was not different for the chronic periodontitis and generalized aggressive periodontitis subjects. The analysis was therefore completed separately for each group and then with the groups combined to increase the statistical power.

Results

Demographic information for the patients studied is found in Table 1. For these subjects the serum IgG reactive with the antigen ranged from undetectable to 255.5 μg/ml (mean ± SD = 7.1 ± 26.04 μg/ml for the chronic and 3.3 ± 7.53 μg/ml for the generalized aggressive periodontitis patients) (Table 3). Serum IgG reactive with *P. gingivalis* hemagglutinin was strongly skewed and not related to disease classification. Statistical analysis of the relationship between antibody reactive with *P. gingivalis* hemagglutinin and patterns of attachment loss indicated that seropositivity for this antigen was not significantly related to measures of attachment loss (Table 2 and Fig. 2). The correlation coefficients between measures of attachment loss and seropositivity approached zero for pocket depth ($r = -0.03$, p-value = 0.6) and attachment loss ($r = 0.03$, p-value = 0.7).

As shown in Table 3, the range of antibody responses for the four IgG subclasses was from undetectable to 129 EU for IgG1, 279 EU for IgG2, 182 EU for IgG3, 279 EU for IgG4. We found that in both chronic periodontitis and generalized aggressive periodontitis patients, IgG subclass antibody reactive with *P. gingivalis* hemagglutinin did not have a significant relationship with either probing depth or periodontal attachment loss (Fig. 3). The correlation coefficients between measures of attachment loss and seropositivity approached zero for pocket depth and attachment loss ($r = 0.07$ or less, p-value ≥ 0.33).

Discussion

In this study we examined antibody reactive with *P. gingivalis* hemagglutinin and looked for relationships between this antibody (both total IgG and IgG subclasses) and measures of attachment loss. We examined only subjects with either chronic or generalized aggressive periodontitis in the analysis. There were no significant relationships between anti-*P. gingivalis* hemagglutinin and measures of attachment loss. These results differ from a previous report that examined antibody reactive with *P. gingivalis* hemagglutinin in humans (11). In that study the investigators found that IgG reactive with this antigen was positively correlated with attachment loss. This finding may be the result of inclusion of healthy subjects in the analysis. We found that by including data from as few as 10 periodontally healthy subjects to the analysis we could create a significant positive

Table 3. IgG antibody reactive with *Porphyromonas gingivalis* hemagglutinin in chronic periodontitis and generalized aggressive periodontitis patients

Anti-hemagglutinin	Disease class	Mean	SD	Range
IgG total (μg/ml)	CP	7.09	26.04	0.0–255.5
	GAP	3.33	7.55	0.0–52.3
IgG1 (EU)	CP	29.39	61.49	5.9–128.6
	GAP	11.00	17.44	10.5–80.9
IgG2 (EU)	CP	2.70	12.86	0.0–278.9
	GAP	2.11	5.83	0.0–32.4
IgG3 (EU)	CP	12.33	24.55	2.5–139.1
	GAP	7.84	24.47	1.3–182.0
IgG4 (EU)	CP	4.73	28.26	0.0–278.9
	GAP	6.40	26.03	0.0–155.8

EU, ELISA units; CP, chronic periodontitis; GAP, generalized aggressive periodontitis.

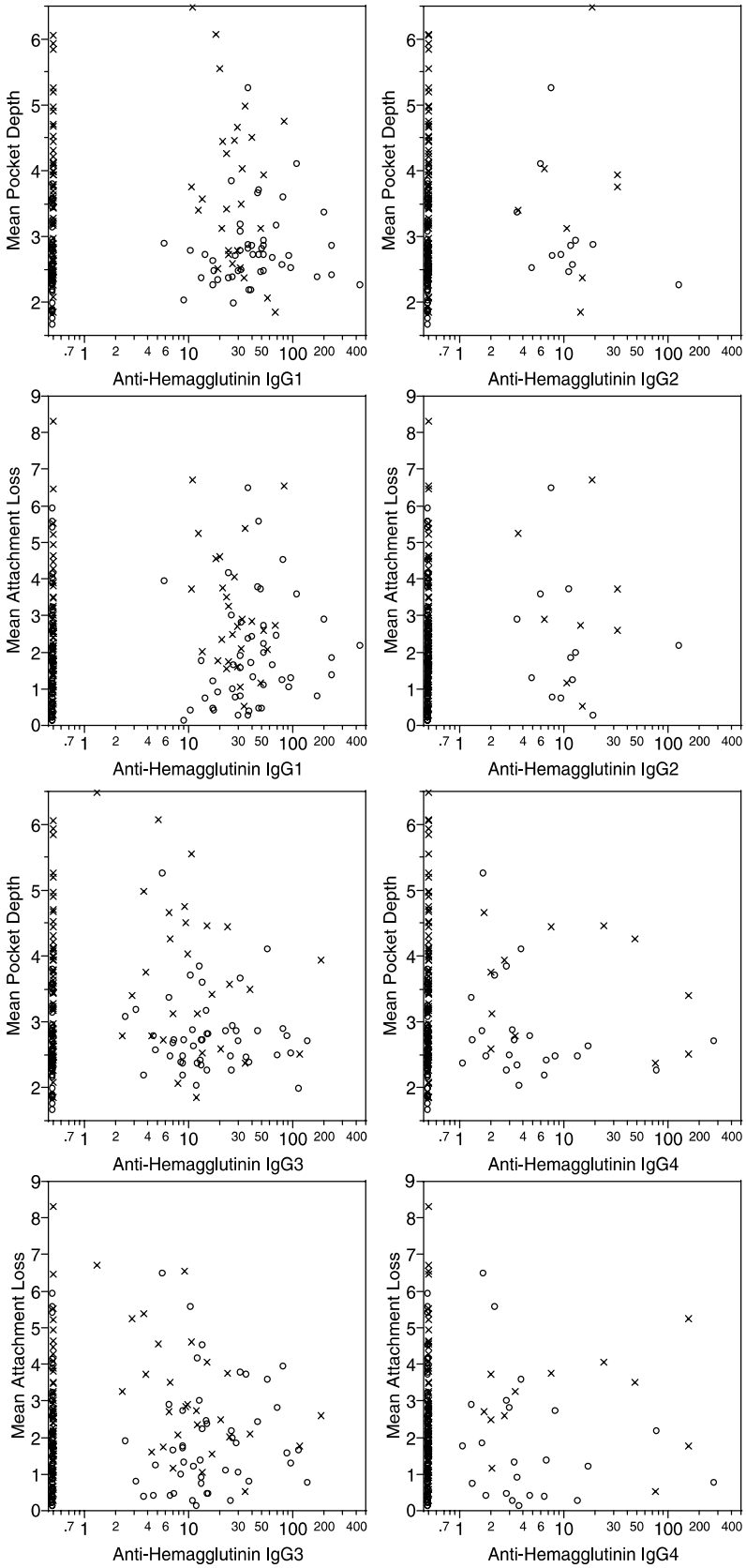

Fig. 3. Immunoglobulin G subclass antibody reactive with *Porphyromonas gingivalis* hemagglutinin (ELISA units) vs. mean pocket depth (mm) and mean attachment loss (mm); × = aggressive periodontitis patients, ○ = chronic periodontitis patients. ($r = 0.07$ or less, p-value ≥0.33 for IgG1-4, attachment loss and pocket depth).

correlation between anti-*P. gingivalis* hemagglutinin and attachment loss. We believe that since healthy subjects have both extremely low levels of antibody reactive with *P. gingivalis* hemagglutinin and no attachment loss, including these subjects produces spurious relationships between anti-*P. gingivalis* hemagglutinin and attachment loss. Since our goal was to determine relationships between anti-*P. gingivalis* hemagglutinin and measures of attachment loss, inclusion of healthy individuals was not informative and misleading.

We found significant amounts of IgG antibody reactive with *P. gingivalis* hemagglutinin in both chronic and generalized aggressive periodontitis. We found that IgG1 and IgG3 were the predominant subclasses reactive with *P. gingivalis* hemagglutinin as assessed by the prevalence of detectable antibody for each subclass. Detectable antibody for the subclasses IgG2 and IgG4 were found in only a small proportion of the subjects. A previous study found that the predominant IgG subclasses were IgG4 and IgG2 with only periodontitis subjects having IgG1 and IgG3 (11). They indicated that the levels of these IgG4 and IgG2 were quite variable. This study also found that individuals with high IgG4 typically had low IgG2 and those with high IgG2 had low IgG4. Further, they found that patients with high IgG4 had decreased disease severity. Perhaps the reason the results from this study are at odds with our own is due to the method of preparing the *P. gingivalis* hemagglutinin. We purified *P. gingivalis* hemagglutinin by expressing the protein as a His-tagged protein in *E. coli* and purification by affinity chromatography. In the work reported by O'Brien-Simpson, *P. gingivalis* hemagglutinin was purified directly from a *P. gingivalis* sonicate. Our *P. gingivalis* hemagglutinin preparation likely lacks the glycosylation present in protein synthesized by *P. gingivalis*. This may have resulted in our detecting minimal IgG4 and particularly IgG2 in our subjects' sera. The differing results and analyses may help identify important *P. gingivalis* hemagglutinin epitopes that elicit protective antibody in human patients. As IgG2 often dominates antibody responses to carbohydrate antigens perhaps the conflicting results between our results and that of O'Brien-Simpson indicate that the important epitope for *P. gingivalis* hemagglutinin includes sites on the *P. gingivalis* hemagglutinin protein that are glycosylated in *P. gingivalis* but not in *E. coli*.

Acknowledgements

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