Immunoglobulin G (IgG) Class, but Not IgA or IgM, Antibodies to Peptides of the *Porphyromonas gingivalis* Chaperone HtpG Predict Health in Subjects with Periodontitis by a Fluorescence Enzyme-Linked Immunosorbent Assay

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Chaperones are molecules found in all cells and are critical in stabilization of synthesized proteins, in repair/removal of defective proteins, and as immunodominant antigens in innate and adaptive immunity. Subjects with gingivitis colonized by the oral pathogen *Porphyromonas gingivalis* previously demonstrated levels of anti-human chaperone Hsp90 that were highest in individuals with the best oral health. We hypothesized that similar antibodies to pathogen chaperones might be protective in periodontitis. This study examined the relationship between antibodies to *P. gingivalis* HtpG and clinical statuses of healthy and periodontitis-susceptible subjects. We measured the humoral responses (immunoglobulin G [IgG], IgA, and IgM) to peptides of a unique insert (P18) found in *Bacteroidaceae* HtpG by using a high-throughput, quantitative fluorescence enzyme-linked immunosorbent assay. Indeed, higher levels of IgG class anti-*P. gingivalis* HtpG P18 peptide (P < 0.05) and P18c, consisting of the N-terminal 16 amino acids of P18 (P < 0.05), were associated with better oral health; these results were opposite of those found with anti-*P. gingivalis* whole-cell antibodies and levels of the bacterium in the subgingival biofilm. When we examined the same sera for IgA and IgM class antibodies, we found no significant relationship to subject clinical status. The relationship between anti-P18 levels and clinical populations and individual subjects was found to be improved when we normalized the anti-P18c values to those for anti-P18y (the central 16 amino acids of P18). That same ratio correlated with the improvement in tissue attachment gain after treatment (P < 0.05). We suggest that anti-*P. gingivalis* HtpG P18c antibodies are protective in periodontal disease and may have prognostic value for guidance of individual patient treatment.

Serum antibodies to periodontitis-associated pathogens are induced by the oral biofilm, an accumulation of microorganisms adherent to solid surfaces of the mouth (36, 50). Biofilms are clinically important, accounting for over 80% of microbial infections in the body, including those in oral soft and hard tissues. This “biofilm phenotype” is thought to contribute to the difficulty of treatment in periodontitis (33). The dynamics of the host response to bacterial biofilms plays a significant, albeit largely uncharacterized, role in preventing biofilm formation. Substantial work has been done to investigate the role that the biofilm mode of growth plays in resistance to antimicrobial agents (15); however, less has been published investigating the role of biofilm-induced antibody response by the human immune system (8).

*Porphyromonas gingivalis* is a gram-negative obligate anaerobe found with high frequency in the subgingival space of persons with periodontitis, where it participates in the initiation and maintenance of a chronic biofilm (15). This biofilm facilitates the long-term survival of *P. gingivalis* and induces an inflammatory response that is responsible for the destruction of the hard and soft tissue supporting structures of the teeth (52).

*P. gingivalis* produces a number of chaperones in response to environmental stresses and as essential tools in normal cellular processes. The role of those chaperones, like the *P. gingivalis* HSP90 homologue HtpG, in immune response dynamics has become an area of intense investigation (12). It has also been suggested that chaperones are probably important in the interaction between the host and the commensal microbial flora (17, 22, 46), functions important in the establishment and perpetuation of chronic inflammatory diseases. In addition, HtpG induces a strong humoral response that may have consequences in the pathogenesis of periodontitis (27).

We have described experiments that suggest that antibodies to HtpG may mitigate some of the induction of inflammatory chemokines through Toll-like receptor 4 (TLR4) and CD91 (41), receptors expressed on human mononuclear cells. Results from this laboratory have also suggested that high levels of anti-(*P. gingivalis*) HtpG antibodies could have protective qualities (44). In particular, we showed that a unique peptide segment of the HtpG molecule, which we term P18, seems to be of particular importance in this regard. P18 is 36 amino acids...
long (amino acid numbers 613 to 648) and is part of an unusual insert in HtpG molecules found in the *Cytophaga-Flavobacterium-Bacteroides* group. Little is known about the function of HtpG in these (or most other) bacteria (reviewed in reference 53). These molecules seem to provide protection from only a very high level of heat shock (~45°C) and are involved in tetrapyrrole biosynthesis (51). HtpG of *P. gingivalis* is minimally expressed on the bacterial surface, and an HtpG disruption mutation in *P. gingivalis* did not affect growth or adherence to mammalian cells (26, 47). The N-terminal 600 amino acids of HtpG contain some regions common to all molecules of the HSP90 group; however, P18 was found to be exclusive to *Bacteroides* spp. when examined by BLAST analysis (44). In fact, P18 contains segments of low homology even to other *Bacte- roidaceae* that may be unique to *P. gingivalis* (44). Our earlier study measured only immunoglobulin G (IgG) class antibodies to the whole P18 peptide in serum samples from 100 subjects. Here, we describe the results of an extended study of those subjects, using a quantitative enzyme-linked immunosorbent assay (ELISA) to measure IgG, IgA, and IgM to three internal segments of P18. Our results support the notions that the potentially protective qualities are apparently limited to IgG class antibodies and that IgG class antibodies to the N-terminal 16 amino acids of P18 appear to correlate best with the disease statuses of these subjects.

### MATERIALS AND METHODS

#### Subjects

Subjects. All work with human subjects was approved by the University of Michigan Institutional Review Board. Each subject gave individual written informed consent and was advised that withdrawal from the study was available at their discretion at any time. The condition of each subject was determined by clinical measurements. Probing pocket depth (PD), the vertical depth of the space around the tooth, was determined to the nearest mm at six sites around each tooth, and then the results were averaged for all sites in each subject (23). Clinical attachment loss (CAL) at the same sites was determined by measuring the distance between the cemento-enamel junction and the bottom of each tooth, and then the results were averaged for all sites in each subject (23). The condition of each subject was determined by formed consent and was advised that withdrawal from the study was available at any time. The condition of each subject was determined by clinical measurements. Probing pocket depth (PD), the vertical depth of the space around the tooth, was determined to the nearest mm at six sites around each tooth, and then the results were averaged for all sites in each subject (23).

#### Bacterial strains and culture conditions.

*Porphyromonas gingivalis* (ATCC 33277) was obtained from the American Type Culture Collection and was maintained by weekly transfer in an anaerobic chamber (Gay Manufacturing, Grass Lake, MI) at 37°C on preduced anaerobically sterilized brucella agar plates (Anaerobe Systems, Morgan Hill, CA) in a 5% hydrogen-10% carbon dioxide-85% nitrogen atmosphere. Broth cultures were grown in a mixture of 50% brain heart infusion broth and 50% tryptic soyic broth (both made according to the manufacturer’s instructions), supplemented with 5 g/liter yeast extract (BD Diagnosic Systems), 0.01 g/liter sodium bisulfite, 5 mg/liter hemin, and 5 μg/liter vitamin K.

#### P18 peptide and subpeptides.

A 36-amino-acid segment (amino acids 613 to 648) [KPPPPFPVEAENKVEAQKTEGSNDIOLKTY] of the *P. gingivalis* HtpG molecule (P18) encompassing the N-terminal half of the *Bacteroides*’s “insert” (25) was synthesized at the University of Michigan Department of Chemistry. The peptide was purified by high-performance liquid chromatography and purity assessed by matrix-assisted laser desorption ionization–time of flight mass spectroscopy. The peptide was found to be more than 95% pure and of the correct molecular weight. While there is considerable variability in the minimum recognizable peptide epitope, most would agree that something in the range of 10 to 20 amino acids is reasonable. To that end, we chose three peptides that are part of the P18 segment to test in the anti-P18 assay: those consisting of the N-terminal (P18o [KPPPPFPVEAENKVEAQKTEGSNDIOLKTY]), the C-terminal (P18i [VEQ AKTEGSNDIOLK]) and the central (P18r [KAEKNNVEAQKTEG]) 16 amino acids in the segment. These peptides were synthesized by EOBiologics (Westfield, IN) and their sequences and purity confirmed by mass spectroscopy done by the manufacturer.

#### Serum ELISA for anti-HtpG peptide antibodies.

*P. gingivalis* HtpG peptides were dissolved in 0.2 M sodium carbonate-bicarbonate buffer (pH 9.5) at 10.0 μg/ml, loaded into the wells of 384-well black microtiter plates (25 μl/well), and incubated overnight at 4°C. The wells were then washed three times with phosphate-buffered saline (PBS; pH 7.5), filled with 1% bovine albumin (PBS-BSA), and incubated overnight at 4°C. After an additional three washes with PBS plus 0.125% Nonidet P-40 (NP-40), 25 μl of each serum sample (diluted 1:100 or 1:32,000 in PBS-BSA) was added to the plate in triplicate and incubated at 4°C overnight. The plates were then washed three times with PBS plus 0.125% NP-40, followed by addition of 25 μl of goat anti-human IgG (λ-chain specific), IgA (κ-chain specific), or IgM (λ-chain specific) labeled with alkaline phosphatase (1 μg/ml in PBS-BSA). After incubation at 4°C overnight, the plates were washed again and 50 μl of alkaline phosphatase substrate buffer (4-methylumbelliferyl phosphate [10 μg/ml] in 0.1 M Tris, pH 9.5, plus 1 mM MgCl2) was added. The number of relative fluorescence units (RFU) for each well was determined using a Genios (Tecan, Switzerland) filter-based microtiter plate reader (excitation wavelength, 360 nm; emission wavelength, 540 nm). The antibody bound to HtpG-coated wells was calculated using a standard curve run on each of the assay plates. Briefly, 48 wells on each plate were coated with goat anti-human IgG, IgA, and IgM (KPL, Gaithersburg, MD) and blocked with PBS-BSA and 25 μl of human IgG, IgM, or IgA was added (1 μg/ml to 0.06 ng/ml in PBS-BSA) in triplicate. After an overnight incubation at 4°C, alkaline phosphatase (25 μl)-labeled goat anti-IgG, IgA, or -IgM was added, incubated, and washed, and the number of RFU was obtained exactly as described for the serum samples.

#### Subgingival-plaque collection and analysis.

Subgingival plaque was collected and immediately placed in a labeled vial containing 500 μl of stabilizing buffer to prevent degradation (RNA Protect; Ambion, Austin, TX). After vortexing for 30 s, the samples were stored at 4°C until they were sent to the laboratory for analysis as described previously (42). Colonization of plaque samples was evaluated by real-time PCR as described previously, using primers specific for the species-specific segments of the 16S rRNA genes of *P. gingivalis* (43). The percentage of the total flora for each species was calculated by dividing the number of target organisms by the total number of bacteria as determined by real-time PCR using 16S rRNA primers that reacted with all bacterial species (42).

#### Competitive inhibition ELISA.

To explore the specificity of the ELISA, competitive inhibition experiments, based on the standard ELISA procedure, were undertaken. The anti-P. gingivalis HtpG P18 subpeptide antibody concentrations in the subjects’ sera were first determined as described above. Sera (200-μl diluted as described above) were combined with 1 μg of lophylolamin recombinant HtpG (HhtgP) (44), incubated for 1 h at room temperature with shaking, and stored overnight at 4°C. The samples were cen-

### TABLE 1. Clinical characteristics of subjects

<table>
<thead>
<tr>
<th>Clinical status (no. of subjects)</th>
<th>PD (mm)</th>
<th>CAL (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (49)</td>
<td>1.6 (1.14–2.21)</td>
<td>0.21</td>
</tr>
<tr>
<td>Periodontitis (50)</td>
<td>2.6 (1.58–5.47)</td>
<td>0.71</td>
</tr>
<tr>
<td>Periodontitis susceptible (50)</td>
<td>3.5 (1.58–5.47)</td>
<td>1.07</td>
</tr>
<tr>
<td>Healthy (49)</td>
<td>1.6 (1.14–2.21)</td>
<td>0.21</td>
</tr>
<tr>
<td>Periodontitis (50)</td>
<td>2.6 (1.58–5.47)</td>
<td>0.71</td>
</tr>
<tr>
<td>Periodontitis susceptible (50)</td>
<td>3.5 (1.58–5.47)</td>
<td>1.07</td>
</tr>
</tbody>
</table>

* The P values for both PD and CAL were <0.001 (t test).
trifuged at 6,000 × g for 45 min to remove the antigen-antibody complexes. A 25-μl volume of each absorbed serum sample was assayed in triplicate as described above.

Data analysis and standard curve fitting. A four-parameter logistic-log curve fitting model was used to develop a standard curve equation (ELISA for Windows; CDC [38]). Statistical analyses of the results were done with Statistica 8 (StatSoft, Tulsa, OK). For all analyses, values for clinical parameters observed within a subject and then across subjects in the clinical groups were averaged. Differences among clinical groups were evaluated using analysis of variance (ANOVA) or the t test methodology. Correlation coefficients were computed to examine the relationships between antibody levels and clinical parameters. For these analyses, the values for the outcome variables were Ig assay levels determined for each subject. The values for the clinical variables were average full-mouth PD and CAL measurements.

RESULTS

Colonization of plaque by *P. gingivalis*. We determined the level of *P. gingivalis* in pooled plaque samples from each subject. Subjects were grouped as either healthy or periodontitis susceptible by clinical measurements taken at the time the plaque samples were collected. Samples were considered positive if they contained more than 100 copies of the *P. gingivalis* 16s rRNA gene and contained more than 1,000 total organisms as determined by quantitative reverse transcription-PCR. Proportions were calculated by dividing the number of copies of the *P. gingivalis* 16s rRNA gene by the total number of copies of 16S rRNA. Of the 49 samples from healthy individuals, 71% (35 of 49) were positive, and the proportion of *P. gingivalis* in positive samples was 0.11. Of the 11 samples from the periodontitis-susceptible population, 86% (42 of 49) were positive for *P. gingivalis*; the proportion of *P. gingivalis* (0.98) was significantly higher (P < 0.05) than that observed for the healthy subject group (Table 2).

Levels of IgG antibodies to *P. gingivalis* HtpG peptides are higher in healthy subjects. Serum antibodies (IgG, IgA, and IgM) binding to the P18 peptides were measured using the ELISA. Preliminary checkerboard experiments demonstrated that different serum dilutions and enzyme-conjugated anti-Ig subclass antibody dilutions were required for optimal sensitivity and specificity for each serum antibody class. Serum dilutions of 1:100, 1:1,000, and 1:3,200 were found to be best for IgA, IgM, and IgG, respectively. Numbers of RFU were converted to μg/ml by using a standard curve run on each plate. The mean values for the healthy and periodontitis-susceptible subject groups were compared for each antibody class and peptide combination; the IgG values for the P18 whole peptide (36 amino acids) and the P18α subpeptide (16 amino acids) were significantly elevated in the healthy subjects in comparison with the levels in the periodontitis-susceptible subjects (ANOVA; P < 0.05). In a similar comparison, IgM class antibody and IgA class antibody levels were not significantly different for the whole P18 peptide, P18α, P18β, or P18γ (data not shown). In the healthy subjects, there was a significantly higher level of anti-P18α IgG than of P18β or P18γ (ANOVA; P < 0.05). There was no significant difference in levels of antibodies to the subpeptides in the periodontitis-susceptible group (Table 3).

Levels of IgG antibodies to *P. gingivalis* HtpG peptides do not correlate with total serum IgG values. To confirm that the levels of anti-*P. gingivalis* HtpG peptides were not simply a reflection of higher or lower total IgG levels, we compared those values. There was no significant difference between mean total IgG values for the healthy and periodontitis-susceptible groups (10.6 mg/ml and 11.1 mg/ml, respectively [t test; P = 0.751]). In addition, we sought correlations between total IgG and anti-*P. gingivalis* HtpG peptide values for each individual serum sample. The Pearson r values were all small, and none were statistically significant (Table 3).

Levels of anti-HtpG peptides are inversely correlated with those of *P. gingivalis* colonization and antibody to whole *P. gingivalis* cells. We compared the mean levels of antibodies to *P. gingivalis* whole cells, the P18 peptide, and the P18 subpeptides and the levels of colonization by *P. gingivalis* in the healthy and periodontitis-susceptible subjects. As expected, we found significantly higher levels of anti-*P. gingivalis* cells (P < 0.05) and higher proportions of bacteria (P < 0.05) in the periodontitis-susceptible group than in the healthy group. However, there were higher levels of antibodies to the whole P18 peptide (P < 0.05) and the P18α subpeptide (P < 0.05) in the healthy group than in the periodontitis-susceptible group. There was no difference found between the subject groups when anti-P18β or -P18γ antibodies were examined (Table 3).

Adsorption of serum with rHtpG reduces binding to the P18 peptides. Serum samples were adsorbed with rHtpG to determine if binding to the P18 peptide and subpeptides was altered. The rHtpG was harvested and purified under native conditions to preserve the secondary structure of the molecule.

### Table 2. *P. gingivalis* colonization of subject plaque

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of healthy subjects positive</td>
<td>35</td>
</tr>
<tr>
<td>No. of periodontitis-susceptible subjects positive</td>
<td>42</td>
</tr>
<tr>
<td>Total no. of subjects</td>
<td>98</td>
</tr>
<tr>
<td>Total no. of healthy subjects</td>
<td>49</td>
</tr>
<tr>
<td>Total no. of periodontitis-susceptible subjects</td>
<td>50</td>
</tr>
<tr>
<td>% of healthy subjects positive</td>
<td>71.4</td>
</tr>
<tr>
<td>% of periodontitis-susceptible subjects positive</td>
<td>84</td>
</tr>
<tr>
<td>Mean % <em>P. gingivalis</em> in healthy group</td>
<td>0.11</td>
</tr>
<tr>
<td>Mean % <em>P. gingivalis</em> in periodontitis-susceptible group</td>
<td>0.98</td>
</tr>
<tr>
<td>P (t test) for mean % <em>P. gingivalis</em></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 3. Serum IgG antibody levels in subject groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for indicated group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean log10 anti-<em>P. gingivalis</em> whole-cell concn (μg/ml)</td>
<td>3.662</td>
<td>3.938</td>
</tr>
<tr>
<td>Mean <em>P. gingivalis</em> proportion (%)</td>
<td>0.106</td>
<td>0.977</td>
</tr>
<tr>
<td>Mean log10 anti-P18 concn (ng/ml)</td>
<td>4.548</td>
<td>4.415</td>
</tr>
<tr>
<td>Mean log10 anti-P18α concn (ng/ml)</td>
<td>4.152</td>
<td>3.954</td>
</tr>
<tr>
<td>Mean log10 anti-P18β concn (ng/ml)</td>
<td>3.853</td>
<td>3.865</td>
</tr>
<tr>
<td>Mean log10 anti-P18γ concn (ng/ml)</td>
<td>3.677</td>
<td>3.817</td>
</tr>
<tr>
<td>Mean total IgG concn (mg/ml)</td>
<td>10.6 ± 11.1</td>
<td>0.751</td>
</tr>
<tr>
<td>r value for total IgG and anti-P18α</td>
<td>0.023</td>
<td>0.584</td>
</tr>
<tr>
<td>r value for total IgG and P18β</td>
<td>0.093</td>
<td>0.749</td>
</tr>
<tr>
<td>r value for total IgG and P18γ</td>
<td>-0.10</td>
<td>-0.064</td>
</tr>
</tbody>
</table>

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and 200 µl of each diluted serum sample combined with 1 µg of rHtpG. Given the dilution of the serum samples, there was about a 5:1 molar excess of rHtpG to IgG. When the serum samples were tested against the P18 peptide and subpeptides and compared with unabsorbed serum, there was found to be an average of >90% reduction in bound IgG to all three molecules (ANOVA; \( P < 0.05 \)). Anti-P18 peptide antibody levels were significantly reduced in both the healthy and the periodontitis-susceptible subject groups (Table 4).

**Normalization of anti-*P. gingivalis* HtpG levels to those for internal peptides.** The results of many diagnostic assays are expressed as normalized values. Therefore, we examined the relationship of each of the anti-P18 subpeptide values in both subject groups after normalization to the values for the other subpeptides. Three possible ratios for each of the anti-P18 subpeptide values were calculated and the means for subject groups compared. The mean values for the P18α/P18γ subpeptide ratio were significantly higher for the healthy subjects than for the periodontitis-susceptible subjects (\( P = 0.0005; t \) test and ANOVA). There was no significant difference between the ratios for the other subpeptides (Fig. 1).

### Table 4. IgG levels in subject group sera absorbed with rHtpG

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clinical status</th>
<th>Mean ± SD log10 serum IgG antibody level (ng/ml)</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonabsorbed</td>
<td>Absorbed</td>
<td></td>
</tr>
<tr>
<td>Anti-P18</td>
<td>Healthy</td>
<td>4.9 ± 0.5</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Periodontitis-susceptible</td>
<td>4.8 ± 0.5</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>Anti-P18α</td>
<td>Healthy</td>
<td>4.2 ± 0.5</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Periodontitis-susceptible</td>
<td>3.9 ± 0.3</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Anti-P18β</td>
<td>Healthy</td>
<td>3.8 ± 0.3</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Periodontitis-susceptible</td>
<td>3.8 ± 0.6</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Anti-P18γ</td>
<td>Healthy</td>
<td>3.7 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Periodontitis-susceptible</td>
<td>3.8 ± 0.2</td>
<td>3.1 ± 0.3</td>
</tr>
</tbody>
</table>

\( ^a P \) values are for comparison of nonabsorbed sera to sera absorbed with rHtpG (t test).

Improved clinical measurements were found in subjects with higher anti-P18α/anti-P18γ ratios after treatment. Subjects in each group were given appropriate treatment (scaling/
root planning for periodontitis-susceptible individuals and prophylactic cleaning for healthy subjects) and the results recorded 6 months later. We calculated the percents improvement for PD and CAL. Proportions were used to take account of the well-known phenomenon that the greatest numerical improvement is always found in the most-damaged sites. The healthy group (with the highest anti-P18α/anti-P18γ ratios) had significantly higher percents gain of attachment than the periodontitis-susceptible group at 6 \( (P = 0.0004) \) and 12 \( (P = 0.006) \) months (t test). No significant difference in percent PD reduction was observed at either point. Pearson R correlations were calculated, and a significant correlation between percent CAL recovery (but not percent PD reduction) and anti-P18α/anti-P18γ ratio was found (Fig. 3).

FIG. 2. P18α/P18γ antisubpeptide ratio correlates with clinical measurements at the baseline. Pearson R correlations were calculated for PD and CAL by using a linear fit for either PD (filled circles) or CAL (open squares) to scatter plots. The associated correlation coefficients \( (r \text{ values}) \) and probabilities \( (P \text{ values}) \) are displayed in the boxed insert.

FIG. 3. Correlation of anti-P18α/anti-P18γ ratio with tissue improvement after periodontitis treatment. Pearson R correlations between percents reduction of PD (filled circle) and recovery of CAL (filled squares) (6 months after treatment) and anti-P18α/anti-P18γ ratio are shown. The associated correlation coefficients \( (r \text{ values}) \) and probabilities \( (P \text{ values}) \) are displayed in the boxed insert.
Antibodies to HtpG and P18 peptides appear to be involved in an indirect anti-inflammatory function not associated with direct antibacterial activity, such as opsonization. More likely, they are involved in some sort of effect on innate and/or adaptive immunity, possibly at the level of the antigen-presenting cells. Antibodies to P. gingivalis whole-cell antigen are elevated in periodontitis subjects with more-extensive disease, whereas anti-P18 antibodies are elevated in subjects with less disease. Second, P. gingivalis HtpG induces chemokines in human macrophage and endothelial cells in vitro; however, serum antibodies from subjects with anti-HtpG activity can significantly reduce production of CXCL8. We hypothesize that serum antibodies to HtpG prevent the induction of CXCL8 and thereby prevent or reduce inflammatory infiltrate-mediated tissue destruction, linking these antibodies to the pathogenic mechanisms of the disease.

Periodontal disease is often diagnosed using the clinical measurements of PD, CAL, bleeding on probing, gingival inflammation, and the radiographic pattern and extent of alveolar bone loss. Other local and environmental factors may be considered, such as age, microbial burden, and tooth mobility. Case definitions of periodontal disease are generally based on measurements of PD and CAL. CAL is considered the gold standard for periodontal disease severity and progression over time; however, CAL is not reflective of current disease severity. Therefore, both PD and CAL should be used as clinical measures of disease severity and progression, hence our use of those measures to compare our antibody results between groups in this report. During that analysis, we have used a somewhat unconventional approach to compare the changes in clinical measures between our subject groups. The variables of CAL and PD change are usually reported in millimeters. This presents a problem in studies like this one, where there are striking differences in the potentials of the subject groups to recover or increase CAL or reduce or increase PD due to disease progression or therapeutic intervention. For example, subjects with 1 mm of CAL cannot recover more than that in response to periodontal treatment; however, subjects with 5 mm of CAL can recover 2 to 3 mm in response to treatment. In the former case, the 1-mm CAL recovery would represent essentially 100% wound healing, but 3 mm of CAL recovery would represent only 60% healing in the latter case. To facilitate comparison, we have converted the CAL and PD changes to percentages of recovery or increase of CAL and reduction or increase of PD relative to the baseline level to attempt to reflect the changes in terms of wound healing rather than traditional dental experiential/treatment-based criteria. We have also assumed that periodontal disease with CAL is an extension of gingivitis. And while the professional consensus is that all periodontitis-susceptible subjects previously had non-CAL periodontal disease (gingivitis), it is also true that only a proportion of those individuals progresses to disease with CAL.

Although there was a trend for anti-P18 antibodies of all Ig classes to be lower in the periodontitis-susceptible group, only IgG class antibodies were significantly different. We believe that this indicates that the assay is identifying as significant antibody levels of relatively long duration, not recently stimulated antibodies as would be expected if IgM antibodies were closely related to the disease process. This result may also indicate that the IgA antibody response, which is vigorous in oral fluids such as saliva, is not substantially translated into circulating anti-P18 and agrees with previous reports that serum IgA class antibodies to P. gingivalis have little or no diagnostic or prognostic value. However, IgA antibodies are very effective as antimicrobial antibodies. This may also explain why the IgG class antibodies are better correlated with CAL than with PD; substantially more tissue healing time is required for the reattachment of gingival tissue than for the reduction of PD.

Total IgG levels were similar in both subject groups (Fig. 3), as has been reported by others (1, 14, 28). We were, however, concerned that variation in the total IgG might influence the results of our antipeptide assays, so we examined the data for any evidence of correlations between those values for each subject. There was no evidence of correlation, which increases our confidence in the specificity of the assay. The antibody binding to the peptides is also not artificial, since whole HtpG adsorbs antiserum antibodies. In addition, the differential binding of anti-P18 antibodies to the P18 subpeptide and to the P18 subpeptide suggests that the N-terminal portion constituting P18 may be the most important of the three subpeptides tested. We believe that Ig subclass analysis of anti-P18 and further peptide mapping will allow us to better characterize this interaction.

Normalization by comparison of ratios of antibodies to P18 epitopes is an approach frequently used to reduce subject-to-subject variation in Ig response levels. It is used in diagnostic settings for diseases as varied as glomerulosclerosis (10), diseases caused by Helicobacter pylori infections (29), and bronchiectasis (18) and for evaluation of Ig receptor effectiveness (32). In this case, we have chosen to normalize the anti-P18 levels, which trend to be higher in healthy subjects, to the anti-P18 values, which show no such trend. The resultant ratio is significantly higher in the healthy subjects and highly correlated with disease state in individual subjects. In addition, the normalized values correlate with improvements in tissue attachment (CAL recovery) after treatment, which suggests that the ratio may have prognostic value.

Analysis of serum antibody is an area of testing that has traditionally been underutilized by the dental profession. Levels of antimicrobial antibodies found in serum tend to be higher in individuals with periodontitis than in those without periodontitis, but the elevations seem to have little prognostic value (49), although long-term monitoring may be more useful (39). Tests for antibodies can be simple to use and inexpensive; however, there is general agreement that a significant and specific antigenic target for such an assay remains to be discovered. Such a target must be specific for the disease process for diagnostic effectiveness and simultaneously involved in the pathogenic mechanism to have prognostic value. Assays for a qualified target, such as the P18 subpeptides, would indicate/justify aggressive adjunct periodontitis treatment and provide a simple method for monitoring that treatment. In the long term, these subpeptides might be found to be effective vaccine candidates (21). Since the P18 subpeptides appear to be unique antigens, they could be used as vaccines without fear of inducing inappropriate responses to human Hsp90.
Measurement of anti-P18 antibodies may have diagnostic potential; the sensitivity of the assay is high for identifying healthy and periodontitis-susceptible individuals. However, such determinations are easily made using conventional periodontal examination techniques. More important is the finding that individuals with good results from periodontal treatment had higher levels of anti-P18 antibodies before that treatment. These data support the notion of prognostic value for the test but do not prove it; analysis of serum samples from other subject groups and longitudinal/treatment studies are currently being completed as we attempt to substantiate that goal. This potential may be due to the “time-averaged” nature of the adaptive immune response that probably incorporates elements of immune memory. Alternatively, the response may have significance because of the target antigen, HtpG. Chaperones are unique antigens; they are highly conserved but elicit strong immune responses. However, they are mainly intercellular molecules exposed when cells are destroyed; they are antigens associated with bacterial destruction in this case. This is similar to the case of human chaperones that are “danger signals” because of their release by necrotic as opposed to apoptotic human cell destruction (7). HtpG (30), like other chaperones, binds to the “chaperone receptor” CD91 (3) and TLR4. The CD91 endocytic receptor is involved in antigen processing and major histocompatibility complex antigen presentation (4, 5); TLR4 is involved in expression of B-cell co-stimulatory molecules CD40, CD80, and CD86, which are up-regulated in monocytes treated with HtpG (C. E. Shelburne, unpublished observation). Together, they may provide the pathway that results in the anti-P18 antibodies described here.

The data reported here demonstrate that antibody responses to the HtpG subpeptides do not follow the same pattern as do antibodies to whole P. gingivalis cells or other P. gingivalis antigens (11, 24, 45, 48). Also, the P18a epitope, especially with normalization to the values for the P18y peptide, yields statistically superior relationships to the clinical statuses of periodontitis subjects in comparison to antibodies to the whole P18 peptide. The biological processes that are reflected in these results remain to be elucidated but are almost certain to yield important information about the pathology of periodontitis and, because of the ubiquitous nature of the HtpG chaperone family, of other chronic bacterial infections.

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