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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Received 1 July 2009/Returned for modification 30 July 2009/Accepted 23 September 2009

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 pathogenic 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 P18a, 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-P18a values to those for anti-P18γ (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 P18a 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 monocytes 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

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† Published ahead of print on 30 September 2009.
TABLE 1. Clinical characteristics of subjects*  

| Clinical status (no. of subjects) | PD (mm)     | CAL (mm)    | SD   |
|----------------------------------|-------------|-------------|------|
| Healthy (49)                     | 1.6 (1.14–2.21) | 0.21        | 0.68 (0.01–1.68) | 0.52 |
| Periodontitis susceptible (50)   | 2.6 (1.58–5.47) | 0.71        | 2.24 (0.76–5.58) | 1.07 |

* The P values for both PD and CAL were <0.001 (t test).

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 *Bacteroidaceae* 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.** 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 pocket to the nearest mm and averaging as for PD (52).

Subjects were recruited at the Michigan Center for Oral Health Research, and a complete description of the subject groups (age, gender, ethnicity, and smoking status, etc.) is given in the work of Ramsayer et al. (40). Subject inclusion was based on the following criteria: possession of at least 20 teeth, no periodontal treatment or antibiotic-related therapy for medical or dental reasons for 3 months before inclusion in the study, no history of long-term treatment with medications known to affect periodontal status, and no history of metabolic bone disease. Healthy control subjects (n = 49) were recruited on the basis of the following criteria: average of less than 3 mm of CAL, no PD greater than 4 mm, no radiographic bone loss, and fewer than 20 sites with bleeding on probing. Periodontitis-susceptible subjects (n = 50) exhibited at least four sites with evidence of radiographic bone loss, a mean CAL of >3 mm, a mean PD of >4 mm, and bleeding on probing (Table 1). Colonization of plaque samples collected simultaneously with the serum samples was evaluated for *P. gingivalis* by a real-time PCR assay as described previously (43), using primers specific for the *P. gingivalis* 16S rRNA gene. Clinical examinations were conducted at the baseline and at 6-month and 12-month intervals (40). The serum samples analyzed in this report were collected at the baseline.

**Reagents.** All chemicals and antibodies were purchased from Sigma Chemical unless otherwise noted in the text.

**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 (Coy Manufacturing, Grass Lake, MI) at 37°C on prerduced anaerobically sterilized bruccella 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% trypticase soy broth (both made according to the manufacturer’s instructions), supplemented with 5 g/liter yeast extract (BD Diagnostic Systems), 0.01 g/liter sodium bisulfite, 5 mg/liter hemin, and 5 mg/liter vitamin K.

**P18 peptide and subpeptides.** A 36-amino-acid segment (amino acids 613 to 648) of *P. gingivalis* HtpG molecule (P18) encompassing the N-terminal half of the *Bacteroidaceae* “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 spectrometry. 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 (P180 [PPEEIPVATKEAKENNAKKEGN9253]), the C-terminal (P181 [VEQ AKTEG9262]) and the central (P182 [KEAKEN9262]AKEG)) 16 amino acids in the segment. These peptides were synthesized by EZ Biolabs (Westfield, IN) and their sequences and purity confirmed by mass spectrometry 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 PBS containing 1% bovine serum 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 (γ-chain specific), IgA (α-chain specific), or IgM (μ-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 [1 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 protocol with the species-specific modifications, 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 lipophilic recombinant HtpG (HtGP) (44), incubated for 1 h at room temperature with shaking, and stored overnight at 4°C. The samples were cen-
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 r 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 per milliliter in periodontitis-susceptible group. The rHtpG was harvested and purified under native conditions to preserve the secondary structure of the molecule.

Levels of IgG antibodies to P. gingivalis 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 IgG antibodies to the whole P18 peptide (P < 0.05) and the P18a subpeptide (P < 0.05) in the healthy group than in the periodontitis-susceptible group.

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 IgG antibodies to the whole P18 peptide (P < 0.05) and the P18a subpeptide (P < 0.05) in the healthy group than in the periodontitis-susceptible group.

Adsorption of serum with rHtpG reduces binding to the P18 peptide. 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.
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-\textit{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).

\textit{Anti-\textit{P. gingivalis} HtpG antibody ratios correlate with disease groups.} We calculated the correlation of antisubpeptide ratios and disease status. Antibody ratios were higher in the healthy subjects than in the periodontitis-susceptible subjects, and there was a significant correlation (Pearson’s \( R = 0.4251 \); \( P = 0.0002 \)) between the P18α/P18γ ratio and the health statuses of the subjects. There was a trend for the P18α/P18β ratio to demonstrate a similar correlation, but this correlation was lower (Pearson’s \( R = 0.1586 \)) and not significant (\( P = 0.1896 \)). No trend was observed with the P18β/P18γ ratio (Pearson’s \( R = 0.0387 \); \( P = 0.7502 \)).

P18α/P18c antisubpeptide ratio correlates with clinical measurements at the baseline. The correlation of anti-P18α/anti-P18γ ratios for individual subjects was calculated for clinical measurements taken at the time the serum samples were collected (baseline). For PD, there was a significant correlation with the anti-P18α/anti-P18γ ratio, and there was a similar \( r \) value for CAL, which almost reached statistical significance. The mean values for the clinical measures were all lower for the periodontitis-susceptible individuals (Fig. 2).

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/}

![FIG. 1. Normalization of anti-\textit{P. gingivalis} levels to those for internal peptides. Values for anti-P18 peptide antibodies to the three subpeptides of P18 were normalized by dividing the concentration of each antisubpeptide antibody by the same values for the other individual subpeptides: anti-P18α/anti-P18γ (clear bars), anti-P18α/anti-P18β (hatched bars), and anti-P18β/anti-P18γ (filled bars). Means for all subjects were calculated and those for healthy and periodontitis-susceptible subjects compared by ANOVA (boxed insert). Point, mean; box, mean ± standard error; diamond, mean ± 1.96 × standard error.](image-url)
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 values) and probabilities (P 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 values) and probabilities (P 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 \textit{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, \textit{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 (44). 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 (35). CAL is considered the gold standard for periodontal disease severity and progression over time; however, CAL is not reflective of current disease severity (35). Therefore, both PD and CAL should be used as clinical measures of disease severity and progression (2, 13), 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 (30, 37). 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 (6, 9) 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 progress to disease with CAL (19, 31, 34).

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 \textit{P. gingivalis} have little or no diagnostic or prognostic value (16). However, IgA antibodies are very effective as antimicrobial antibodies (20). 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 artifactual, since whole HtpG adsorbs antipeptide antibodies. In addition, the differential binding of anti-P18 antibodies to the P18s and to the P18\textgamma subpeptide suggests that the N-terminal portion constituting P18\textalpha may be the most important of the three subpeptides tested. We believe that Ig subclass analysis of anti-P18s 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 \textit{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-P18s levels, which trend to be higher in healthy subjects, to the anti-P18\textgamma 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 costimulatory 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 individuals with normalization to the values for the P18/H9253 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.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Florence Y. -P. An. This work was supported by funds from the Department of Cariology, Restorative Sciences and Endodontics, University of Michigan School of Dentistry (D.G.S.), and NIH/NIDCR DE11117 (D.E.L.). Subject sample collections were supported by NIH grants NIH/NIDCR U01-DE-14961 and NIH/NCRRC M01-RR000042 (W.G.R.).

REFERENCES

1. Al-Ghamdi, H. S., and S. Anil. 2007. Serum antibody levels in smoker and non-smoker Saudi subjects with chronic periodontitis. J. Periodontol. 78: 1043–1050.
2. Armitage, G. C. 2003. Diagnosis of periodontal diseases. J. Periodontol. 74:1237–1247.
3. Banu, S. R., R. J. Binder, T. Ramalingam, and P. K. Srivastava. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calretici-
4. Binder, R. J., and P. K. Srivastava. 2004. Essential role of CD91 in re-
5. Binder, R. J., R. VATNER, and P. Srivastava. 2004. The heat-shock protein recep-
6. Bosch, J. A., C. G. Engeland, J. T. Cacioppo, and P. T. Marucha. 2007. Depressive symptoms predict mucosal wound healing. Psychosom. Med. 69:597–605.

7. Calderwood, S. K., S. S. Mambula, P. J. Gray, Jr., and J. R. Theriault. 2007. Extracellular heat shock proteins in cell signaling. FEBS Lett. 581:3689–
8. Ebersole, J. L., D. Cappelli, and S. C. Holt. 2001. Periodontal diseases: to protect or not to protect is the question? Acta Odontol. Scand. 59:161–166.
9. Englund, C. G., B. Sabzehei, and P. T. Marucha. 2009. Sex hormones and mucosal wound healing. Brain Behav. Immun. 23:629–635.
10. Fu, L. W., L. Y. Yang, W. P. Chen, S. J. Tsai, and C. Y. Lin. 1998. IgG subclass/ IgM ratio and response to therapy in focal segmental glomerulo-
11. Gemmell, E., B. Polak, R. A. Reinhardt, J. Eccleston, and G. J. Seymour. 1995. Antibody responses of Porphyromonas gingivalis infected gingivitis and periodontitis subjects. Oral Dis. 1:63–69.
12. Goulben, F., D. Grenier, and D. Dufand. 2003. Oral microbial heat-shock proteins and their potential contributions to infections. Crit. Rev. Oral Biol. Med. 14:399–412.
13. Greenstein, G. 1997. Contemporary interpretation of probing depth assessments: diagnostic and therapeutic implications. A literature review. J. Peri-
14. Gunsolley, J. C., J. P. Pandey, S. M. Quinn, J. Tew, and H. A. Schenkein. 1997. The effect of race, smoking and immunoglobulin allotypes on IgG subclass concentrations. J. Periodontal Res. 32:381–390.
15. Halilaj, A. D., and S. O. Soccolich. 2006. Introduction to microbial aspects of periodontal biofilm communities, development and treatment. Periodon-
16. Haywood, S. J., D. L. Fishel, C. E. Christan, J. P. Benimoulin, and A. Kage. 2003. Salivary IgA in response to periodontal treatment. Eur. J. Oral Sci. 111:203–208.
17. Henderson, B., and M. Wilson. 1998. Commensal communism and the oral cavity. J. Dent. Res. 77:1674–1683.
18. Hill, S. L., J. L. Mitchell, D. Burzett, and R. A. Stockley. 1998. IgG sub-
19. Lamster, I. B., D. S. Harper, L. A. Fiorello, R. L. Oshrnan, R. S. Celenti, and J. M. Gordan. 1987. Immunological and cytoplasmic enzyme activity, crevicular fluid volume, and clinical parameters characterizing gingival sites with shal-
20. Lappin, D. F., A. M. McGregor, and D. F. Kinane. 2003. The systemic immune response is more prominent than the mucosal immune response in the pathogenesis of periodontal disease. J. Clin. Periodontol. 30:778–786.
21. Lee, J. Y., N. N. Yi, U. S. Kim, J. S. Choi, S. J. Kim, and J. I. Choi. 2006. Porphyromonas gingivalis heat shock protein vaccine reduces the alveolar bone loss induced by multiple periodontalperiodontalpathogenic bacteria. J. Periodontal Res. 41:10–14.
22. Liu, B., Y. Yang, J. Dai, R. Medzhitov, M. A. Freundgen, P. L. Zhang, and Z. Li. 2006. TLR4 up-regulation at protein or gene level is pathogenic for lupus-like autoimmune disease. J. Immunol. 177:6880–6888.
23. Loe, H., E. Theilade, and S. B. Jensen. 1965. Experimental gingivitis in man. J. Periodontol. 36:177–187.
24. Lopatin, D. E., and E. Blackburn. 2003. Essential role of CD91 in re-
25. Lopatin, D. E., A. Combs, D. G. Sweier, J. C. Fenno, and S. Dhamija. 2003. Characterization of heat-inducible expression and cloning of HtpG (Hsp90 homologue) of Porphyromonas gingivalis. Infect. Immun. 61:1980–1987.
26. Logatian, D. E., J. Jaramillo, C. A. Edwards, N. Van Poperin, A. Combs, and C. E. Shelburne. 1999. Cellular localization of a Hsp90 homologue in Por-
27. Lu, H., M. Wang, J. C. Gunsolley, H. A. Schenkein, and J. G. Tew, 1994. Serum immunoglobulin G subclass concentrations in periodontally healthy and diseased individuals. Infect. Immun. 62:1677–1687.
28. Mitchell, H. M., R. Ally, A. Wadee, M. Wiseman, and I. Segal. 2002. Major differences in the IgG subclass response to Helicobacter pylori in the first and third worlds. Scand. J. Gastroenterol. 37:517–522.
29. Moles, D. R., M. C. Downer, and P. M. Spaight. 2002. Meta-analysis of measurements of performance reported in oral cancer and precursor screening studies. Br. Dent. J. 192:332, 340–344.
30. Muller, H. P., S. Stadtermann, and A. Heinecke. 2002. Longitudinal associa-
31. Nimmerjahn, F., and J. V. Ravetch. 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 310:1510–
32. Offenbacher, S., S. P. Barros, R. E. Singer, K. Moss, R. C. Williams, and J. D. Beck. 2007. Periodontal disease at the biofilm-gingival interface. J. Periodontol. 78:1911–1925.

1772 SWIEGER ET AL. CLIN. VACCINE IMMUNOL.
34. Page, R. C. 1986. Current understanding of the aetiology and progression of periodontal disease. Int. Dent. J. 36:153–161.
35. Page, R. C., and P. I. Eke. 2007. Case definitions for use in population-based surveillance of periodontitis. J. Periodontol. 78:1387–1399.
36. Paster, B. J., I. Olsen, J. A. Aas, and F. E. Dewhirst. 2006. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol. 2000 40:80–87.
37. Pihlstrom, B. L., R. B. McHugh, T. H. Oliphant, and C. Ortiz-Campos. 1983. Comparison of surgical and nonsurgical treatment of periodontal disease. A review of current studies and additional results after 6 1/2 years. J. Clin. Periodontol. 10:524–541.
38. Quinn, C. P., V. A. Semenova, C. M. Elie, S. Romero-Steiner, C. Greene, H. Li, K. Stamey, E. Steward-Clark, D. S. Schmidt, E. Mothershed, J. Pruckler, S. Schwartz, R. F. Benson, L. O. Helsel, P. F. Holder, S. E. Johnson, M. Kellum, T. Messmer, W. L. Thacker, L. Besser, B. D. Plikaytis, T. H. Taylor, Jr., A. E. Freeman, K. J. Wallace, J. Walls, M. Bronsdon, G. M. Carlone, M. Bajani-Ari, D. A. Ashford, D. S. Stephens, and B. A. Perkins. 2002. Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin protective antigen. Emerg. Infect. Dis. 8:103–1110.
39. Rams, T. E., M. A. Listgarten, and J. Slota. 2006. Actinobacillus actinomy- cetemcomitans and Porphyromonas gingivalis subgingival presence, species-specific serum immunoglobulin G antibody levels, and periodontitis disease recurrence. J. Periodontal Res. 41:228–234.
40. Ramsreiter, C. A., J. S. Kinney, A. E. Herr, T. Braun, J. V. Sugai, C. A. Shelburne, L. A. Rayburn, H. M. Tran, A. K. Singh, and W. V. Giannobile. 2009. Identification of pathogen and host-response markers correlated with periodontitis disease recurrence. J. Periodontal Res. 40:436–446.
41. Shelburne, C. E., M. D. Coopamah, D. G. Sweier, F. Y.-P. An, and D. E. Lopatin. 2007. HtpG, the Porphyromonas gingivalis HSP-90 homologue, induces the chemokine CXCL5 in human monocyte and microvascular vein endothelial cells. Cell. Microbiol. [Epub ahead of print.] doi:10.1111/j.1462-5822.2007.00897.x.
42. Shelburne, C. E., R. M. Gleason, G. Germain, L. F. Wolff, B. H. Mullally, W. A. Coulter, and D. Lopatin. 2002. Quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis of Porphyromonas gingivalis gene expression in vivo. J. Microbiol. Methods 49:147–156.
43. Shelburne, C. E., A. Prabhu, R. M. Gleason, B. H. Mullally, and W. A. Coulter. 2000. Quantitation of Bacteroides forsythus in subgingival plaque comparison of immunoassay and quantitative polymerase chain reaction. J. Microbiol. Methods 39:97–107.
44. Shelburne, C. E., P. S. Shelburne, V. M. Dhople, D. G. Sweier, W. V. Giannobile, J. S. Kinney, W. A. Coulter, B. H. Mullally, and D. E. Lopatin. 2008. Serum antibodies to Porphyromonas gingivalis chaperone HtpG predict health in periodontitis susceptible patients. PLoS ONE 3:e1984.
45. Sims, T. J., R. E. Schifferle, R. W. Ali, N. Shaug, and R. C. Page. 2001. Immunoglobulin G response of periodontitis patients to Porphyromonas gingivalis capsular carbohydrate and lipopolysaccharide antigens. Oral Microbiol. Immunol. 16:193–201.
46. Sugimoto, S., M. Abdallah Al, and K. Sonomoto. 2008. Molecular chaperones in lactic acid bacteria: physiological consequences and biochemical properties. J. Biosci. Bioeng. 106:324–336.
47. Sweier, D. G., A. Combs, C. E. Shelburne, J. C. Fenno, and D. E. Lopatin. 2003. Construction and characterization of a Porphyromonas gingivalis htpG disruption mutant. FEMS Microbiol. Lett. 225:101–106.
48. Tabet, K., K. Yamazaki, H. Hotokezaka, H. Yoshie, and K. Hara. 2000. Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. Clin. Exp. Immunol. 120:285–293.
49. Takeuchi, Y., M. Maramaki, T. Nagasawa, M. Umeda, S. Oda, and I. Ishikawa. 2008. Molecular chaperone G subclass antibody profiles in Porphyromonas gingivalis-associated aggressive and chronic periodontitis patients. Oral Microbiol. Immunol. 21:314–318.
50. Teles, R. P., A. D. Hassajee, and S. S. Socransky. 2006. Microbiological goals of periodontal therapy. Periodontol. 2000 42:180–218.
51. Watanabe, S., T. Kobayashi, M. Saito, M. Sato, K. Nimura-Matsune, T. Chiba, and M. Yoshikawa. 2007. Studies on the role of HtpG in the tetrapyrrole biosynthesis pathway of the cyanobacterium Synechococcus elongatus PCC 7942. Biochem. Biophys. Res. Commun. 352:36–41.
52. Wolff, L. F., W. F. Liljemark, B. L. Pihlstrom, E. M. Schaffer, D. M. Aeppli, and C. L. Bandt. 1988. Dark-pigmented Bacteroides species in subgingival plaque of adult patients on a rigorous recall program. J. Periodontal Res. 23:170–174.
53. Zuehlke, A., and J. L. Johnson. 2009. Hsp90 and co-chaperones twist the functions of diverse client proteins. Biopolymers. [Epub ahead of print.] doi:10.1002/bip.21292.