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Induction of interleukin (IL)-1β, IL-10, IL-8 and immunoglobulin G by *Porphyromonas gingivalis* HmuY in humans

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Background and Objective: Porphyromonas gingivalis, an anaerobic gram-negative bacterium, is associated with chronic periodontitis. This study was undertaken to evaluate the production of interleukin (IL)-1β, IL-8 and IL-10 by human peripheral blood mononuclear cells (PBMC) stimulated with *P. gingivalis* antigens and to assess the levels of serum immunoglobulin (Ig)G, IgA and IgG subclasses raised against *P. gingivalis* HmuY protein.

Material and Methods: PBMC from patients with chronic periodontitis (CP) and from nonperiodontitis (NP) control subjects were stimulated with *P. gingivalis* antigens, and the cytokine levels in the culture supernatants were determined by ELISA. The specificity of serum antibodies raised against HmuY was analyzed by Western blotting and by ELISA.

Results: Compared with the NP controls, the CP patients produced higher levels of total serum IgG and IgG1 specific for *P. gingivalis* HmuY. No differences were found between CP and NP groups in the production of IL-1β and IL-8 by PBMC stimulated with total *P. gingivalis* antigens. Only *P. gingivalis* lipopolysaccharide (LPS) induced higher levels of IL-10 in the CP group. Higher levels of IL-1β and IL-10 were induced by HmuY than by other antigens derived from the wild-type *P. gingivalis* strains. In contrast, total antigens derived from the *hmuY*-deletion mutant strain induced the production of significantly higher levels of IL-8 and significantly lower levels of IL-1β.

Conclusion: Our data suggest that *P. gingivalis* HmuY may be considered an immunogenic protein associated with host–pathogen interactions.

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Porphyromonas gingivalis is an important etiologic agent of chronic periodontitis (CP). The bacterium produces a number of virulence/antigenic factors such as capsule, lipopolysaccharide (LPS), fimbriae and outer-membrane proteins that increase humoral and cellular host responses, which can eventually lead to connective tissue destruction and alveolar bone loss (1,2). *P. gingivalis* LPS (*Pg*LPS) evokes atypical pattern-recognition receptors for the innate host defense system: signaling occurs through toll-like receptor (TLR)-2 or between TLR-2 and TLR-6 (3). The pathway involved in this signaling leads to an anti-inflammatory profile of

immune responses. *Pg*LPS is able to induce the up-regulation of B_1 cell receptors through mechanisms involving tumor necrosis factor-α release and neutrophil influx, which are largely sensitive to resolvin E1 (4).

Considerable effort has been made to study cytokines and chemokines released by different host cells when exposed to components of periodontopathogenic bacteria. It has been shown that the levels of interleukin (IL)-1β, IL-10 and IL-8 in the crevicular gingival fluid from periodontitis patients are associated with periodontal status (5,6). Up-regulation of IL-1β leads to a large number of significant changes, consistent with a pathologic role of this cytokine in periodontitis, including enhancement of inflammatory cytokines, chemokines, transcription factors, MMPs, adhesion molecules and nuclear factor-κB-dependent anti-apoptotic genes. In addition, cells cultured with *P. gingivalis* produce increased levels of IL-10, and low levels of cytokines related to the Th1 cell profile (e.g. interferon-γ and tumor necrosis factor-β) are also produced (7). In periodontitis, antibody production is also a major feature of the adaptive host response to bacteria and bacterial products. Several studies have demonstrated high levels of total immunoglobulin (Ig)G raised against *P. gingivalis* in patients with periodontitis (8,9).

Host–pathogen cross-talk involves many immunogenic molecules, including lipoproteins. One *P. gingivalis* lipoprotein, HmuY, is expressed constitutively at low levels, with the highest levels found in *P. gingivalis* grown under low-iron/heme conditions (10), typical of the oral biofilm. HmuY is a surface-exposed heme-binding protein, which may be released into the host environment in the form of both outer-membrane vesicles and soluble protein (10,11), and therefore would be available for recognition by the host immune response during CP.

As a result of the importance of cellular and humoral responses induced by *P. gingivalis* in CP, the objectives of the present study were to measure the serum levels of IgG, IgA and IgG subclasses (1, 2, 3 and 4) specific for *P. gingivalis* HmuY protein and to evaluate the production of selected cytokines by human peripheral blood mononuclear cells (PBMC) upon stimulation with *P. gingivalis* antigens. We chose representative cytokines related to periodontal status: IL-1β and IL-8, which are involved in the upregulation of inflammatory reactions; and IL-10, which is involved in the downregulation of inflammatory reactions.

Material and methods

Characterization of the sample

Fifty-nine systemically healthy Brazilian subjects, including 29 patients with CP and 30 nonperiodontitis (NP) control subjects, who attended the Dental Ambulatory of Feira de Santana State University, participated in this study between August 2008 and November 2009. Informed and written consent was obtained from each participant. The study design was approved by the Feira de Santana State University Ethics Committee (Protocol no. 013/2002). Exclusion criteria included diabetes, cardiovascular disease and any other systemic disease that could alter the course of periodontal disease. Former smoking, pregnancy at the time of the examination, previous consumption of systemic antimicrobial or anti-inflammatory drugs (6 or 2 mo, respectively, before the start of the study) and periodontal therapy during the last 12 mo were also exclusion criteria.

Disease classification

The periodontal examination was made in six sites per tooth, and all teeth were evaluated, excluding third molars. Clinical definition of patients with periodontitis was the presence of four or more teeth showing one or more sites with a probing depth of ≥ 4 mm, clinical attachment loss of ≥ 3 mm and bleeding on probing at the same site (12). The chronic character of the disease was based on the American Academy of Periodontology criteria (13).

Blood sampling

Peripheral venous blood was drawn from the cubital fossa of each individual. Five milliliters was collected into tubes containing a clot activator, the tube was centrifuged for 5 min at 3461 *g* and the supernatant was removed and stored at −20°C. In addition, 10 mL of peripheral venous blood was collected into heparin-containing tubes for the isolation of PBMC (described later).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting

Purified HmuY (5 μg per well) was separated electrophoretically in 12% sodium dodecyl sulfate–polyacrylamide gels under reducing conditions, as described by Laemmli (14). Transfer of proteins onto nitrocellulose membranes (Millipore, Billerica, MA, USA) was carried out according to Towbin *et al*. (15). Membranes were blocked overnight at 4°C with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and 1% bovine serum albumin (BSA), then incubated for 1 h at 37°C with pooled sera diluted 1 : 100 in PBS-T containing 0.5% BSA. After washing in PBS-T, the membranes were incubated for 1 h at 37°C with rabbit anti-human IgG–horseradish peroxidase (HRP; Sigma-Aldrich, St. Louis, MO, USA) conjugate. The reaction was developed using 4-chloro-1-naphtol (Sigma-Aldrich) and H_2O_2.

Cell culture and cytokine quantification

PBMC were obtained from peripheral blood by density centrifugation (Sep-Cell; StemCell Technologies Inc., Tukwila, WA, USA) and washed twice in Roswell Park Memorial Institute (RPMI) culture medium (LGCBio, São Paulo, SP, Brazil). Before culture, cells were counted using optical microscopy. Their viability was examined by Trypan Blue staining. For each experiment, 1×10^6 cells per well were used. PBMC were grown in 24-well flat-bottom culture plates for 48 h at 37°C, under 5% CO_2 and humidified air. A pilot study was carried out to

identify the optimal mitogen and antigen concentrations for use in the assays. Thereafter, 1×10^6 cells were added to each well with pokeweed mitogen (PWM; 2.5 μg/mL; Sigma-Aldrich), LPS from *Escherichia coli* (*Ec*LPS; 2.5 μg/mL; Sigma-Aldrich), or the following antigens from *P. gingivalis*: *Pg*LPS (2.5 μg/mL; InvivoGen, San Diego, CA, USA), extracts from *P. gingivalis* wild-type ATCC33277 (*Pg*ATCC33277; 0.5 μg/mL) and A7436 (*Pg*A7436; 5.0 μg/mL) strains and from the *hmuY*-deletion mutant strain (*Pg*TO4; 5.0 μg/mL), and purified HmuY (2.5 μg/mL). *Pg*ATCC33277 is a well-known, widely used avirulent wild-type strain of *P. gingivalis*, whereas *Pg*A7436, highly similar to the W83 virulent wild-type strain, was used previously to characterize HmuY protein and to construct the *hmuY*-deletion mutant strain (*Pg*TO4) (10,16). Our studies demonstrated that similar levels of HmuY are produced by *P. gingivalis* ATCC33277, W83 and A7436 wild-type strains (10, and data not shown). Cells with no antigen added served as a negative control. The concentrations of IL-1β, IL-8 and IL-10 in the culture supernatants were quantified using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA).

Culture of *P. gingivalis* and preparation of extracts were performed as previously described (9,16). Truncated *P. gingivalis* HmuY, lacking the first 25 residues (NCBI accession no. CAM 31898), was overexpressed by pHmuY11 plasmid in *E. coli* ER2566 cells (New England Biolabs,Boston, MA, USA) and purified from a soluble fraction of *E. coli* lysate, as previously reported (16). Putative contaminating endotoxins were removed from HmuY samples using Detoxi-Gel Endotoxin Removing Columns (Thermo Scientific, Waltham, MA, USA).

Serum antibody analysis

The levels of HmuY-specific antibodies in serum samples was measured by ELISA. Briefly, 5 μg/mL of HmuY was used to coat 96-well polystyrene microtiter plates (Costar, Corning, IL, USA) overnight at 4°C. After blocking at 37°C for 2 h, with PBS containing 2% BSA, the microtiter plates were washed. Then, serum samples were diluted (1 : 1000, 1 : 500, 1 : 100, 1 : 50 or 1 : 100 for IgG, IgG1, IgG2, IgG3 or IgG4 assays, respectively) in PBS containing 0.05% BSA, and incubated at 37°C for 1 h. Then, the microtiter plates were washed and incubated at 37°C for 1 h with 100 μL of rabbit anti-human IgG (diluted 1 : 25,000) or anti-human IgA (diluted 1 : 50,000) conjugated to HRP (Sigma-Aldrich). For immunoglobulin subclass analysis, incubation was carried out with mouse anti-human IgG1, IgG3 (diluted 1 : 2000), IgG2 or IgG4 (diluted 1 : 1000) conjugated to biotin (Southern Biotech, Birmingham, AL, USA), followed by washing and incubation for 45 min with HRP-conjugated streptavidin (Bio-Rad, Atlanta, GA, USA). All reactions were developed with tetramethylbenzidine (TMB) and the absorbance at 450/630 nm was measured using a microplate reader (Bio-Rad).

Statistical analysis

The Student's *t*-test was used to compare clinical features between groups. Median values were used as a measure of general tendency because the immunological data were not normally distributed. Differences in serum antibody and cytokine levels between paired groups were assessed using the Mann–Whitney *U*-test. Differences in cytokine induction among different *P. gingivalis* antigens were evaluated using the Kruskal–Wallis test, and the alpha values were corrected using the Bonferroni *post-hoc* test.

Results

Table 1 summarizes the clinical findings for the subjects, such as age, number of teeth, bleeding on probing, probing depth and clinical attachment levels. There was no statistically significant difference in age between patients with CP and NP controls. As expected, the periodontal status between the groups showed statistically significant differences.

IL-1β

Production of IL-1β by PBMC varied depending on the antigens used as stimuli. Purified HmuY, as well as extracts from the strains expressing Hmu protein, induced IL-1β production, albeit at different levels. Purified HmuY induced the highest levels of IL-1β, and *Pg*ATCC33277 and *Pg*A7436 extracts induced the production of slightly lower levels of IL-1β ($p < 0.001$). In contrast, the extract derived from *Pg*TO4, an *hmuY*-deletion mutant strain, was not able to induce the production of IL-1β, suggesting that HmuY may play an important role in the induction of IL-1β (Fig. 1). It seems that *Pg*LPS does not

Table 1. Clinical features of the nonperiodontitis (NP) control subjects and patients with chronic periodontitis (CP)

Parameter	NP (n = 30)	CP (n = 29)
Number of women/men	16/14	20/9
Age (years)	34.52 ± 9.85	36.61 ± 10.9
Number of teeth	27.93 ± 2.11	20.41 ± 6.43
BOP	10.07 ± 6.23	21.41 ± 6.17
Percentage of sites with PD ≥ 4 mm	0.0 ± 0.0	24.42 ± 17.19
Percentage of sites with CAL ≥ 3 mm	0.0 ± 0.0	71.35 ± 16.88
Percentage of sites with CAL ≥ 5 mm	0.0 ± 0.0	31.76 ± 22.65
Percentage of sites with CAL ≥ 3 mm + PD 4 mm + BOP	0.0 ± 0.0	17.14 ± 12.01

Values are expressed as mean ± standard deviation, unless indicated otherwise. BOP, bleeding on probing; CAL, clinical attachment level; PD, probing depth.

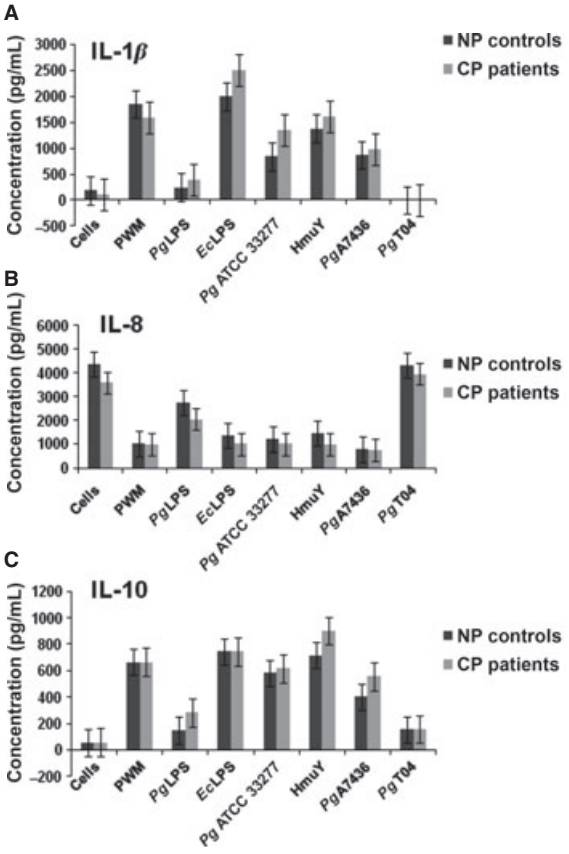

Fig. 1. Production of interleukin (IL)-1β (A), IL-8 (B) and IL-10 (C) by peripheral blood mononuclear cells (PBMC) derived from patients with chronic periodontitis (CP patients) ($n = 29$) and from nonperiodontitis control subjects (NP controls) ($n = 30$) upon stimulation for 48 h with *Porphyromonas gingivalis* (*Pg*) antigens, pokeweed mitogen (PWM) and *Escherichia coli* lipopolysaccharide (*Ec*LPS). Cells, unstimulated cells. Each experiment was carried out at least twice in duplicate.

contribute significantly to IL-1β production as the results obtained with this antigen were very similar to those observed for unstimulated cells.

IL-8

Production of IL-8 by PBMC was inhibited by HmuY and total *P. gingivalis* antigens, except for antigens derived from *Pg*TO4 (Fig. 1). Lower levels of IL-8 were produced by stimulated cells compared with unstimulated cells ($p < 0.001$). In contrast, antigens derived from *Pg*TO4 showed a lower ability to inhibit IL-8 compared with *Pg*LPS ($p = 0.01$), *Ec*LPS, antigens derived from *Pg*ATCC33277 and *Pg*A7436, and HmuY ($p < 0.001$). *Pg*LPS exhibited a lower ability to inhibit IL-8 than *Ec*LPS, antigens derived from *Pg*ATCC33277 and *Pg*A7436, and HmuY. There was no significant difference between CP and NP groups regarding IL-8 production.

IL-10

Purified HmuY induced the production of higher levels of IL-10 than did extracts from *Pg*ATCC33277 and *Pg*A7436 ($p < 0.05$). The levels induced were significantly reduced when the *Pg*TO4 extract, which does not contain HmuY, was used ($p < 0.001$). *Pg*LPS also induced the production of this cytokine, but at lower levels. Also, PBMC from individuals with CP produced higher levels of IL-10 when stimulated with *Pg*LPS compared with PBMC obtained from the NP controls ($p < 0.05$) (Fig. 1).

Humoral response

Analysis of the humoral response to *P. gingivalis* HmuY demonstrated that HmuY was recognized by pooled sera from patients with CP (Fig. 2). In contrast, NP control subjects did not produce specific antibodies against HmuY. Patients with periodontitis showed higher levels of total serum IgG ($p < 0.001$) and IgG1 ($p < 0.005$) specific for HmuY compared with NP control subjects, as determined by ELISA (Fig. 3). There was no statistically significant difference between the CP patients and NP controls when IgA, IgG2, IgG3 and IgG4 were evaluated (data not shown).

Discussion

Secreted and structural components of *P. gingivalis* play a crucial role in the induction of innate immune responses, including cytokine production by epithelial cells and by localized or circulating macrophages. However, the specific components of *P. gingivalis* that initiate and stimulate progression of the disease process, and the precise mechanisms by which the bacterium activates host cells, have not been clearly identified. Therefore, in the present study we examined the production of IL-1β, IL-8 and IL-10 by PBMC after stimulation with *P. gingivalis* antigens.

IL-1β has been correlated with periodontal tissue destruction, and higher concentrations of this cytokine have been found within diseased sites and in the gingival crevicular fluid of patients with CP (6,17). In contrast, IL-10 is an anti-inflammatory cytokine that suppresses bone loss (18). IL-10 may also affect bacterial clearance, as it was shown, in a *P. gingivalis*-induced abscess model, that IL-10-deficient mice are resistant to tissue destruction (19). Our data demonstrated that purified HmuY protein, total antigens derived from *P. gingivalis* and *Pg*LPS induce PBMC to produce higher levels of IL-1β and IL-10. It is also worth noting that the relative quantity of anti-inflammatory IL-10 produced was lower compared with the quantity of proinflammatory IL-1β produced. The

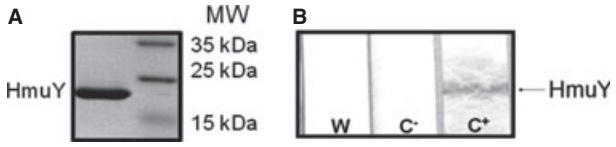

Fig. 2. Western blotting profile of *Porphyromonas gingivalis* HmuY. The protein was overexpressed, purified from *Escherichia coli* cell lysate, analyzed for purity using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining (A), and then detected by probing with serum antibodies from controls and patients (B). A pool of sera from nonperiodontitis control subjects was used as a negative control (C^-) and a pool of sera from patients with chronic periodontitis was used as a positive control (C^+). The white strip (W) was incubated with 1% bovine serum albumin only. This experiment was carried out three times and representative data are shown.

finding that *Pg*LPS induces higher levels of IL-10 in subjects with periodontitis is in agreement with the fact that this particular molecule participates in the signaling of an anti-inflammatory pathway. In contrast, stimulation with antigens derived from a strain of *P. gingivalis* that does not produce HmuY results in lower levels of IL-1β and IL-10, suggesting that HmuY is crucial for their production.

IL-8, a proinflammatory chemokine, is considered to be responsible for the migration and accumulation of neutrophils, which are the predominant line of defense in the gingival crevice (20). The presence of mediators, such as IL-8, contributes to the remarkable ability of the host to limit periodontal bacterial growth. However, contradictory results have been published regarding the levels of this cytokine present within diseased sites and in the gingival crevicular fluid of periodontitis patients (5,21). It has also been shown that *P. gingivalis* prevents IL-8 accumulation when gingival epithelial cells are exposed to mixtures containing this bacterium and other subgingival plaque bacteria (22). In this study we found that, in contrast to IL-1β and IL-10, HmuY inhibited the production of IL-8 by PBMC, as unstimulated cells or cells stimulated with *Pg*TO4 antigens produced higher levels of this cytokine. This suggests that HmuY can play an important role in the inhibition of IL-8 production.

The pattern of cytokine induction/inhibition presented in this study suggests that HmuY may participate in the evasion mechanisms of *P. gingivalis*, once neutrophil recruitment is impaired, while IL-10 modulates the inflammatory response necessary for the elimination of the bacteria. Our results demonstrate that the production of the cytokines assessed in this study, after stimulation with HmuY, may be similar to that observed after stimulation of dendritic cells with *P. gingivalis* hemagglutinin B (HemB), the latter occurring through TLR-4 activation (23). To determine the immune-response profile induced by HmuY, other cytokines need to be analyzed, such as IL-12, interferon-γ, IL-4, IL-13, IL-17 and IL-23.

In periodontitis, the antibody levels in serum may influence the initiation and progression of periodontal tissue loss (24–26). It has been shown that subjects with CP exhibit higher levels of total IgG that react with a 40-kDa outer-membrane protein or gingipains (27,28). Recently, we demonstrated that *P. gingivalis* HmuY elicits high titers of IgG in rabbits (10), suggesting that this antigen would be available for recognition by the immune response during CP. Indeed, in the present study we demonstrated increased levels of anti-HmuY IgG and IgG1 in patients with CP. In a recent report (10) we showed that HmuY may be a unique

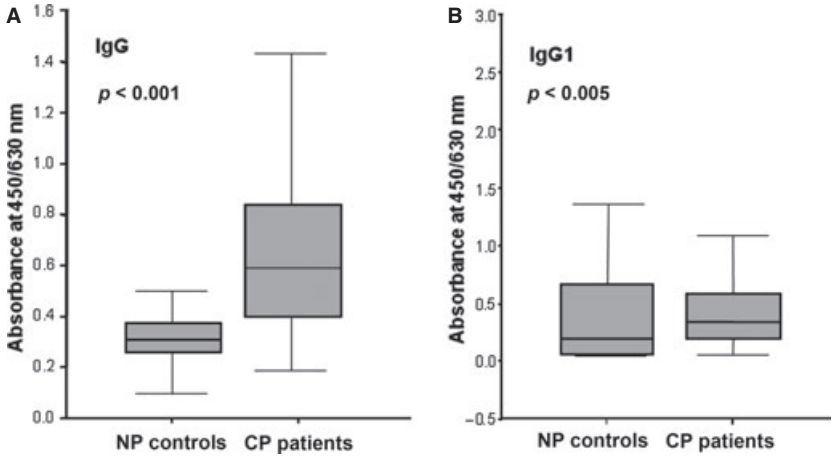

Fig. 3. Concentrations of total immunoglobulin (Ig)G (A) and IgG1 (B), in sera from patients with chronic periodontitis (CP patients; $n = 29$) and nonperiodontitis control subjects (NP controls; $n = 30$), which reacted with *Porphyromonas gingivalis* recombinant HmuY. The results were obtained using ELISAs. Box-plots indicate medians (central line), interquartiles (box limits), and 10th and 90th percentiles (whisker limits) of the concentrations of serum IgG (A) and IgG1 (B) in CP patients and in NP controls. Each experiment was carried out at least twice, in duplicate.

P. gingivalis protein because very low homology has been found between HmuY and proteins identified in other periodontopathogens. The most similar homologues of HmuY have been found within *Bacteroides* species, but our experimental data demonstrated that antibodies to HmuY did not cross-react with proteins derived from a *B. fragilis* strain (10).

Conclusions

Here we show, for the first time, that *P. gingivalis* HmuY may be considered an immunogenic protein associated with host–pathogen interactions. Although our findings demonstrate that HmuY shows potential for use in assessment of immunogenicity in periodontitis, further studies need to be performed to examine a host-protective effect of antibodies to HmuY.

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