Published in final edited form as:

J Periodontol. 2012 February ; 83(2): 228–234. doi:10.1902/jop.2011.110116.

Levels of Serum Immunoglobulin G Specific to Bacterial Surface Protein A of *Tannerella forsythia* are Related to Periodontal Status

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Abstract

Background—*Tannerella forsythia* (*Tf*) is a Gram-negative anaerobe implicated in the development of periodontal disease. Bacterial surface protein A (BspA) is a surface-expressed and -secreted protein that is recognized as an important virulence factor of *Tf*. This study was undertaken to determine whether *Tf* BspA induces an antibody response in periodontal disease. We hypothesized that serum immunoglobulin (Ig)G antibody levels against BspA correlate with the disease of patients.

Methods—Sera were obtained from 100 patients with cardiac disorders and periodontal disease and 73 patients who experienced myocardial infarction but were periodontally healthy. Sera samples were assayed for anti-BspA antibody (total IgG and IgG subtypes) by enzyme-linked immunosorbent assay (ELISA). Antibody levels were measured in ELISA units by using an arbitrary patient as a standard.

Results—A negative correlation was found with BspA-specific total IgG antibody titers and the severity of disease measured as the clinical attachment level (CAL) when healthy and diseased groups were analyzed separately (healthy group: [−0.23, correlation value] Student's *t* value [73 degrees of freedom] = 1.99; *P* = 0.05; diseased group: [−0.21] *t* [100 degrees of freedom] = 2.12; *P* = 0.03). However, there was a positive correlation ([0.18 correlation value] Student's *t* value [173 degrees of freedom] = 2.39; *P* = 0.017) when healthy and diseased groups were combined. A strong positive correlation ([0.338 correlation value] Student's *t* value [173 degrees of freedom] = 4.69; *P* < 0.0001) between the BspA-specific IgG titers and periodontal probing depth was observed when healthy and disease groups were combined.

Conclusions—Data demonstrated that antibodies to *Tf* BspA were elicited in patients with periodontal disease, and antibody levels were associated with the disease severity. Furthermore, data suggested that anti-BspA IgG might have a protective function in periodontal disease by minimizing the loss of tooth attachment tissue.

Keywords

Immunoglobulin G; periodontitis

Periodontitis is a chronic inflammatory disease that initiates the destruction of the periodontium and the tissues that surround and support teeth. If left untreated, periodontitis often leads to tooth loss.¹ Periodontitis has a microbial etiology, and inflammation triggered by a predominantly Gram-negative subgingival biofilm initiates the disease process. Specifically, a bacterial consortium that includes *Porphyromonas gingivalis* (*Pg*), *Treponema denticola*, and *Tannerella forsythia* (*Tf*) is strongly implicated in periodontitis.² However, an analysis³ of the oral microbiota suggested that there may also be other periodontal pathogens involved.

It is well documented that there is an immune response to periodontal pathogens associated with various forms of periodontal diseases.⁴⁻⁶ Elevated serum antibodies to periodontal bacteria were reported in chronic periodontitis,⁷ aggressive periodontitis,⁸ and refractory periodontitis.⁹ Despite accumulating evidence of an association of *Tf* with various forms of periodontitis,² only a couple of studies^{10,11} examined the immune responses to the whole *Tf* bacterium or its specific components in periodontitis. With respect to the specific components, the immunoglobulin (Ig)G response to the bacterium's S-layer protein¹² and detachment factor¹³ were found to be significantly elevated in periodontitis patients.

The paucity of information on the immune response to components of *Tf* is likely due to the fact that this bacterium is difficult to cultivate in the laboratory, and thus, many bacterial components have not been fully characterized. Nevertheless, *Tf* expresses a number of putative factors that are likely to play roles in pathogenesis.¹⁴ The study of Sharma et al.¹⁵ focused on the bacterium's surface as well as secreted protein bacterial surface protein A (BspA). BspA is a 98-kDa protein with leucine-rich repeat and bacterial Ig-like domains and has multiple functions. These functions include binding to fibrinogen and fibronectin¹⁵ and the induction of proinflammatory cytokine expression in host cells by activating Toll-like receptor 2.¹⁶ Moreover, BspA expression was critical for causing alveolar bone loss in a mouse model of infection-induced periodontal destruction.¹⁷ The genome sequence deposited in the Oralgen database predicts for other BspA-like proteins in *Tf* as well. A BspA homolog in *Tf* was shown to be upregulated several fold in patients with periodontitis.¹⁸ These studies suggested that BspA is an important virulence factor of *Tf*, and hence, the immune response in periodontitis to this antigen is likely to be critical for the pathogenicity of *Tf*. Understanding the relationships between the serum IgG antibody levels to bacterial components such as BspA and clinical parameters of periodontal diseases might help to elucidate the role of these humoral immune responses in periodontal disease. Moreover, the antibody levels to BspA might serve as surrogate markers in the diagnosis of the disease.

The present study was initiated to determine the extent to which serum IgG levels to the *Tf* BspA protein correlate with periodontal disease status and, therefore, may define the prognosis of periodontal disease.

MATERIALS AND METHODS

Patient Sera

Sera were obtained from 100 patients involved in the Periodontal Intervention for Cardiac Events: Pilot Trial (5U01DE 13940 to 3 Periodontal and Vascular Events [PAVE]) and 73 patients from the Periodontal Infections and the Risk for Myocardial Infarction (MI) study (5RO1 DE 12085 MI). All patients from the PAVE study had periodontal disease and cardiac disease. There were 80 males (Table 1) (mean age: 60.2 years) and 20 females (mean age: 58.1 years). Eighty-three percent of patients were white, and the ethnicity of the other 17% of patients enrolled from the PAVE study was as follows: 15% African-American, 1% Asian, and 1% unspecified. At the time of enrollment for serum collection, 28% had diabetes, and 32% were smokers.

The criteria for periodontal disease were as follows: the patient presented with 6 natural teeth, including third molars, with 3 teeth with periodontal probing depths (PDs) 4 mm; 2 teeth with interproximal clinical attachment levels (CALs) 2 mm; and 10% of sites with bleeding on probing (BOP). Samples used as controls in our study were from the control group of the MI study. These control patients had had an incident myocardial infarction but were considered periodontally healthy. A total of 98.6% of these patients were white, and the other 1.4% of these patients were African American. Eleven percent of these patients reported having diabetes, and 9.7% of these patients reported currently smoking. An approval was obtained from the University at Buffalo Institutional Review Board before collecting sera from individuals in the PAVE and MI studies. All patients provided written informed consent to participate.

Enzyme-Linked Immunosorbent Assay (ELISA) to Determine Antibodies to Whole-Cell Bacteria and BspA Protein in Patients With and Without Periodontal Disease

Sera were assayed for the anti-BspA antibody (total IgG and IgG subtypes 1 through 4) titers by ELISA. Ninety-six-well plates[‡] were coated with 5 ng recombinant BspA protein per well that was purified as previously described.¹⁶ Plates were washed five times with 0.1M Tris, pH 7.3, 0.15 M NaCl, and 0.05% Tween 20 (TBS-T) and blocked for 1 hour with 1% bovine serum albumin in TBS-T. Blocked plates were washed three times with TBS-T and incubated with various dilutions of each patient's sera (1:400 to 1:1,600) for 1 hour at room temperature. Plates were washed five times with TBS-T and incubated with goat anti-human IgG or mouse monoclonal antibodies to human IgG1, 2, 3, or 4 (clones for IgG1, 2, 3, and 4, respectively: 4E3, 31 to 7-4, HP6050, and HP6025).[§] The goat anti-human IgG was γ -chain specific and obtained by cross-absorption with human IgM and IgA and affinity purification on human IgG linked to agarose.^{||} All antibodies were conjugated with horse-radish peroxidase (HRP) and first tested by Western blotting to confirm the functionality. Last, plates were washed five times with TBS-T and treated with 3,3',5,5'-tetramethylbenzidine (TMB) microwell peroxidase substrate[¶] and subsequently read on a microtiter plate reader.[#]

[‡]Idea Scientific, Minneapolis, MN.

[§]Southern Biotech, Birmingham, AL.

^{||}Southern Biotech.

[¶]KPL, Gaithersburg, MD.

Antibody titers were calculated as follows: an arbitrary serum confirmed to have a moderate antibody level judged by Western immunoblotting was used as a standard and taken as one ELISA unit and then serially diluted on every plate to generate a standard curve. Each patient's sera was compared to the standard curve, and relative titer values were calculated appropriately.¹⁹

For assaying anti-whole-cell *Tf* total IgG titers, 96-well plates were coated with 1.0×10^6 bacterial cells overnight as described previously.¹⁷ After coating, plates were washed five times with TBS-T and blocked for 1 hour with 1% bovine serum albumin in TBS-T. Blocked plates were washed three times with TBS-T and incubated with various dilutions of each patient's sera (1:400 to 1:1,600) for 1 hour at room temperature. Plates were washed five times with TBS-T and incubated with peroxidase-conjugated goat anti-human IgG. Finally, after washing plates with TBST, plates were treated with TMB microwell peroxidase substrate and read on a microtiter plate reader, and antibody titers were calculated per the aforementioned protocol for the BspA protein.

Western Immunoblotting to Determine IgG Subclass Response to BspA

Purified full-length recombinant BspA protein prepared as previously described¹⁶ was loaded as a single-slotted well alongside a protein marker lane on 10% sodium dodecyl sulfate–polyacrylamide (1 mm thick) minigels, and separated by the method of Laemmli.²⁰ Proteins were transferred from gels to nitrocellulose membranes using a semiwet transfer system,^{**} and membranes were blocked in 5% nonfat milk prepared in TBS-T overnight at 4°C. Membranes were washed three times for 10 minutes each with TBS-T. Sera of 19 patients were simultaneously tested on each membrane by using an immunoblotting unit.^{††} Sera were incubated for 1 hour at room temperature on a rocker and washed as before, and the bound human IgG subclass antibodies were detected by incubation for 1 hour at room temperature with a 1:500 dilution of HRP-coupled respective mouse anti-human subclass antibodies (clones for IgG1 through 4 were 4E3, 31 to 7-4, HP6050, and HP6025, respectively). After washing membranes, bound antibodies were detected with HRP colorogenic substrate TMB.

Statistical Analyses

Associations between continuous variables were assessed by the Pearson product moment correlation coefficient (*r*). Analyses of differences in antibody titers between groups were determined with the non-parametric Mann-Whitney *U* test. Analyses were done using a statistical software package.^{‡‡} Testing was done at a 5% level of significance.

[#]iMark Microplate Reader, BIO-RAD, Hercules, CA.

^{**}Surfblot, Thermo Fisher Scientific, Rochester, NY.

^{††}Idea Scientific.

^{‡‡}PASW Statistics (formerly SPSS), IBM, Chicago, IL.

RESULTS

Antibodies to Whole-Cell Bacteria and BspA Protein in Patients With and Without Periodontal Disease

ELISA was performed to determine the IgG antibodies titers against whole-cell *Tf* as well as BspA protein. The results showed no statistically significant differences in the mean anti-whole-cell titers between periodontally diseased and healthy groups (Fig. 1A). Results suggested that infection by *Tf* occurred in all individuals irrespective of their disease status, and/or *Tf* expressed common antigens shared by other organisms. With regard to the BspA, the mean anti-BspA IgG titer was significantly higher in patients with periodontal disease compared to the periodontally healthy group (Fig. 1B).

Correlation Coefficients of Anti-BspA IgG Levels and Clinical Parameters in Patients With and Without Periodontitis

Correlations between anti-BspA IgG levels and clinical parameters (Table 2) of sampling sites were analyzed using the Pearson correlation coefficient. Table 3 shows correlation values between the mean periodontal data of patients (BOP, PD, and CAL) and their antibody titers. There were no correlations between BOP and antibody titers. Results showed a negative correlation with BspA-specific total IgG antibody titers and the severity of disease measured as CAL when the healthy and diseased groups were analyzed separately (healthy group: $[-0.23, \text{correlation value}]$, Student's t value [73 degrees of freedom] = 1.99; $P = 0.05$; diseased group: $[-0.21, \text{correlation value}]$, Student's t value [100 degrees of freedom] = 2.12; $P = 0.03$). However, there was a positive correlation ($[0.18, \text{correlation value}]$, Student's t value [173 degrees of freedom] = 2.39; $P = 0.017$) when healthy and diseased groups were combined. In contrast, when BspA-specific titers were correlated with disease measured as the PD, there were no significant correlations in the healthy or diseased groups. However, a strong positive correlation ($[0.338] t [173 \text{ degrees of freedom}] = 4.69$; $P < 0.0001$) between the BspA-specific IgG titers and PD was observed when healthy and diseased groups were combined. These data indicate a significant positive correlation of anti-BspA antibodies with the mean PD in pooled healthy patients and patients with disease, which support the hypothesis that the immune response to BspA is related to the severity of periodontal disease. Strikingly, when the healthy and diseased groups were analyzed separately based on CAL status, the correlation of anti-BspA IgG antibodies with the mean CAL became a statistically significant negative value.

IgG Subclass Response to BspA and Disease Severity

The specific IgG subclass responses to the BspA protein were determined by Western immunoblotting. IgG subtypes that were present in all patients were IgG1 and 4 or both, and no detectable antibodies of the IgG2 or 3 subclasses were observed (a representative blot is shown in Fig. 2). The BspA-specific antibody switching to only IgG1 and 4 subclasses is intriguing, and future studies will be required to better understand the underlying mechanism of this subtype restriction. To examine if a relationship existed between the IgG subclass response to BspA and disease severity, we performed the Pearson correlation among antibody titers of each subclass with the CAL within the diseased group. Antibody titers for

each subclass were determined by ELISA. We observed no correlation to the subtype antibody titers and disease severity (data not shown).

DISCUSSION

This study shows that there was a significant mild, negative correlation between levels of BspA-specific antibody and the severity of periodontitis measured as the CAL. When the data were combined for both patient pools, the range of observed values was greatly increased for CAL and IgG titers. This extended range swamped the mild negative association observed within each group and produced a positive correlation.

Second, IgG1 and IgG4 subtype antibodies to BspA protein were elicited in the population studied, although their levels did not correlate with the disease severity. Studies found that ethnicity, culture, and regional factors can affect the susceptibility and severity of periodontitis.¹⁰ Demographic, behavioral, and characteristics of oral and general health were strong determinants of systemic antibody responses to periodontal bacteria in a nationally representative sample of United States adults.²¹ It was reported that Hispanic patients have a statistically significant lesser amount of antibody titers against *Tf* compared to Asian Americans and African Americans.²² However, whether race plays any role in the antibody response to *Tf* or its virulence factor BspA could not be evaluated because our patient pool mainly consisted of white patients. This issue will be addressed in our future studies by analyzing patients of more diverse ethnicities. Moreover, a recent study²³ suggested that *Tf* infection is more likely to cause periodontitis in overweight women than in normal-weight women. According to another recent study,²⁴ overweight or obese individuals have an overgrowth of *Tf* compared to normal-weight individuals, thus possibly subjecting overweight and obese individuals to a higher risk of developing periodontal disease. Therefore, it would be interesting to examine antibody responses to BspA in these populations as well to define the role of the antibodies in disease pathogenesis and as predictors of periodontal status.

The role of antibodies to periodontal pathogens is still unclear and is likely complex because of the possible protective and/or destructive effects of antibodies.²⁵ It has been well documented that some periodontal bacterial antigens induce host destructive immune responses, and some induce immune responses that are protective.²⁵ High titers of antibodies specific to *Pg* antigens were found in periodontal patients, suggesting the destructive function of such antibodies.²⁵ In contrast, studies also found serum IgG antibodies to *Aggregatibacter actinomycetemcomitans* or *Pg* in periodontally healthy patients to be higher than those in patients with active disease, thereby suggesting that these antibodies have a protective role.²⁶ In addition, studies also found no statistically significant correlation between the periodontal disease severity and total IgG antibodies to *Pg* antigens, specifically the arginine-lysine specific proteinase-adhesin complex.²⁷ However, when antibody subtypes were analyzed, there was a positive correlation of the proteinase-specific IgG2 and, conversely, a negative correlation for the IgG4 subtype with disease severity.²⁷ Shelburne et al.²⁸ found that antibodies specific to *Pg* heat-shock protein homologue and clinical measurements were negatively correlated pretreatment and positively correlated post-treatment, suggesting that the immune response to this *Pg* antigen is protective. Sharma et al.¹⁷ showed that immunization with BspA protein was able to block the *Tf*-induced

alveolar bone loss in mice. Therefore, it is reasonable to assume that BspA-specific antibodies that are able to block the BspA-mediated release of proinflammatory cytokines involved in inflammatory tissue destruction could be protective in humans.

The immune responses to *Tf* or its antigens were not investigated as extensively as the other periodontal pathogen *Pg*. Persson et al.¹⁰ showed that patients with periodontitis had higher antibody titers to *Tf* than did patients with gingivitis. On the contrary, Califano et al.²⁹ did not find a difference in the antibody titers to the crude extract of *Tf* in patients with periodontitis compared to those with gingivitis. With regard to the immune response to *Tf*-specific antigens, it was shown that 270- and 230-kDa proteins of the microorganism's S-layer were highly antigenic, and antibodies to the S-layer proteins were present in sera from patients with aggressive periodontitis and chronic periodontitis.¹² Moreover, a recent study¹³ detected higher IgG titers to the *Tf* detachment factor in crevicular fluids from diseased sites compared to healthy sites.

CONCLUSIONS

Data demonstrated that antibodies to the *Tf* BspA protein were elicited in patients with periodontal disease and increased antibody levels associated with reduced periodontal CAL. Thus, the data suggests that BspA-specific antibodies may have a protective function, likely through blocking the BspA-mediated inflammatory periodontal destruction.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Dr. Karen Falkner, Research Assistant Professor, Departments of Oral Biology and Women Health Initiative, University at Buffalo, for her support in obtaining serum samples. This study was supported by the National Institutes of Health (NIH)/National Institute of Dental and Craniofacial Research, Bethesda, Maryland (grant DE014749 to Dr. Sharma). Patient-sample collections were supported by NIH (grants U01-DE13940 and DE12085 to Dr. Genco). The authors report no conflicts of interest related to this study.

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Figure 1.

A) Tf whole cell-specific serum IgG levels in periodontally healthy and diseased groups. **B)** BspA-specific serum IgG levels in periodontally healthy and diseased groups. *Standardized test statistics = 8.840 ($P = 0.0001$). Bars represent mean $\pm$ SEs. EU = ELISA units; NS = not statistically significant.

Figure 2.

Western immunoblot analyses of serum IgG subtype response to BspA. BspA protein that was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels was electroblotted onto nitrocellulose membranes and probed with patient sera using a multiblot apparatus. Tracks labeled with identical numbers were reacted with serum from the same patient and probed with mouse monoclonal antibody specific to human IgG1, 2, 3, or 4. Tracks labeled with the letter M show the migration of prestained molecular weight protein markers. Results of 10 individual patients are shown as representative data. Arrows indicate positions of the BspA band.

Table 1

Research Patient Demographics

Group	n	Male (%)	White (%)	With Diabetes (%)	Current Smokers (%)	Age (years; mean)	Age (years; SD)
PAVE	100	80.0	83.0	28.0	32.0	59.8	8.06
MI	73	63.0	98.6	11.0	9.7	56.4	8.42

Table 2

Clinical Characteristics of Patients

Characteristic	Periodontally Diseased (PAVE patients)	Periodontally Healthy (MI patients)
CAL (mm; mean $\pm$ SD)	2.84 $\pm$ 1.15	1.81 $\pm$ 1.35
PD (mm; mean $\pm$ SD)	2.64 $\pm$ 0.08	1.75 $\pm$ 0.35
BOP (%; mean $\pm$ SD)	44 $\pm$ 29	35 $\pm$ 21
Anti-BspA IgG titers (EU; mean $\pm$ SE)	43,260 $\pm$ 74	10,089 $\pm$ 10

EU = ELISA unit.

Table 3

Correlation of Serum Anti-BspA IgG Levels With Clinical Measurements

Parameter	Periodontally Diseased (n = 100)		Periodontally Healthy (n = 73)		Pooled Patients (N = 173)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
BOP	−0.15	NS	0.039	NS	0.05	NS
PD	−0.06	NS	0.02	NS	0.338	<i>P</i> <0.0001
CAL	−0.21	0.03	−0.23	0.05	0.18	0.015

NS = not statistically significant.

Correlation values (Pearson *r*) shown are for BOP, PD, and CAL against serum anti-BspA IgG titers for pooled periodontally healthy and diseased groups. *P* values for each group are also shown.