Humoral Immune Response to an Antigen from Porphyromonas gingivalis 381 in Periodontal Disease

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The humoral immune responses of patients with periodontitis were evaluated to characterize the host response to Porphyromonas gingivalis. A sonic extract of P. gingivalis 381 from whole cells was fractionated by gel chromatography and ion-exchange chromatography. The fractionated extracts were evaluated by Western blot (immunoblot) analyses with individual sera. A dominant antigen was identified from the sonic extract with an apparent molecular mass of 53 kDa. The 53-kDa protein antigen (Ag53) was purified by affinity chromatography by using a monoclonal antibody. Ag53 was detected on the vesicle surface of P. gingivalis 381 by immunoelectron microscopy by using the monoclonal antibody and was detected as a major protein in the outer membrane and in vesicles by Western blot analysis. Monoclonal antibody cross-reactivity to Ag53 in the sonic extracts of P. gingivalis ATCC 33277, P. gingivalis 1021, and Porphyromonas endodontalis ATCC 35406 was revealed. Seventy-seven patients with periodontitis were examined for their responses to Ag53. Serum immunoglobulin G (IgG) from 54 patients reacted strongly to Ag53; however, serum IgG from the remaining 23 patients did not exhibit detectable reactivity at all to Ag53, even though the patients had high serum IgG titers to the sonic extract. Ag53 is a new marker that represents an interesting aspect of the humoral immune response to P. gingivalis in patients with periodontitis.

Porphyromonas gingivalis, a periodontopathic bacterium, has been isolated frequently from periodontal pockets (15, 20, 29–31, 35, 38) and exhibits numerous virulence factors (2, 4, 5, 7, 9, 11, 14, 28, 32, 34, 36) related to the destruction of periodontal tissues. Many components of P. gingivalis are antigenic. The structural components, i.e., capsule (16, 17, 23) and fimbriae (25, 37), and the materials that have biological activity, i.e., lipopolysaccharide (23, 25, 27), hemagglutinin (23), and trypsinlike protease (10), have all been detected as antigenic determinants in sera from patients with periodontitis. Recently, several antigens of P. gingivalis have been purified (1, 19). The isolated antigens vary in chemical composition and biological properties. However, the immunoglobulin G (IgG) antibody titers to all of the isolated antigens were reported to be elevated in sera of patients with periodontitis. These results make the relationship between P. gingivalis and the development, progression, and activity of periodontal disease difficult to interpret. The focus of these studies was the antibody titer in individual serum samples. Little attention was paid to intersubject variability. Because the host immune response in periodontitis is complex and the key factors that alter the course of periodontal disease have not been identified, it is appropriate to identify bacterial antigens that are important to host immune response and, potentially, to the progression of disease, in order to clarify the relationship between specific P. gingivalis antigens and serum antibody titer. Further, the relationship between serum antibody titer and periodontal disease status may begin to give a clearer picture of the variation in host response that relates to disease susceptibility.

In this study, we isolated an antigen from P. gingivalis 381 that showed strong antigenicity and clear differences in the humoral immune response among patients with periodontitis. We also characterized physical properties of the antigen.

MATERIALS AND METHODS

Bacteria and antigen preparation. P. gingivalis 381, 1021, and ATCC 33277, P. endodontalis ATCC 35406, Porphyromonas asaccharolyticus ATCC 27260, Prevotella intermedia ATCC 25611, Prevotella loescheii ATCC 13930, Prevotella denticola ATCC 33547, and Prevotella melanogenica ATCC 33185 were grown and sonic extracts were prepared as described previously (21). Except where noted, studies were done with P. gingivalis 381.

Outer membrane preparation. The outer-membrane-enriched fraction was prepared from P. gingivalis 381 as previously described (12).

Antigen fractionation. The sonic extract (100 mg [dry weight]) was fractionated by using a Toyopearl HW-60F (Tosoh, Tokyo, Japan) column chromatograph (2.6 by 85 cm; equilibrated with 50 mM carbonate-bicarbonate buffer [pH 9.6]; flow rate, 0.5 ml/min) and a DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column chromatograph (1.6 by 10 cm; equilibrated with 10 mM Tris HCl buffer [pH 7.5]; flow rate, 0.7 ml/min). The binding antigens were eluted by use of a linear gradient of NaCl, as follows: 0 to 0.5 M (475 ml) and 0.5 to 2.0 M (100 ml). An aliquot of each fraction was assayed for antigen reactivity by Western blot (immunoblot) analysis.

Subjects. Seventy-seven patients with elevated serum antibody titers (above 300 enzyme-linked immunosorbent assay [ELISA] units [21]) to the sonic extract were selected for this study from the periodontitis patient pool of Okayama

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University Dental Hospital. Serum samples were obtained from the 77 patients (49 female and 28 male) and from 10 periodontally healthy subjects (5 female and 5 male). All patients and controls were in good general health. The patients with periodontitis were classified into three groups, i.e., those with adult periodontitis (AP), those with rapidly progressive periodontitis (RPP), and those with juvenile periodontitis (JP), by use of the criteria presented by Page and Schroeder (26) with modifications by Murayama et al. (21). The serum IgG antibody titers to the sonic extract from P. gingivalis 381 were determined by the ELISA as described previously (21).

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with a 7.5 to 15% linear-gradient acrylamide gel for separation, was performed in a discontinuous buffer system (13) with a Mini protein II cell (Bio-Rad Laboratories, Richmond, Calif.). Samples were dissolved in a sample buffer (1% 2-mercaptoethanol, 2% SDS, and 10% glycerol in 0.125 M Tris HCl buffer [pH 6.8]) and treated at 100°C for 5 min. The molecular-weight-standard proteins (and their molecular weights) were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and a-lactalbumin (14,400). The proteins in the gel were stained with Coomassie brilliant blue R-250.

**Western blot.** Western blot analyses were performed as described previously (12). Sera from the patients with periodontitis and the healthy subjects were used at a final dilution of 1:100 with 5% (wt/vol) skim milk in Tris-buffered saline (10 mM Tris HCl buffer [pH 7.5], 0.9% NaCl) (M-TBS). Horseradish peroxidase-conjugated goat anti-human IgG antibodies (1:500 dilution with M-TBS; ICN Biochemicals, Costa Mesa, Calif.) or horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:500 dilution with M-TBS; ICN) were used for detection.

**Monoclonal antibodies.** For immunization, a fraction (partially purified antigen) rich with 53-kDa protein antigen (Ag53) in DEAE-Sepharose CL-6B chromatography was further separated by SDS-PAGE. The acrylamide gel containing Ag53 was cut, hashed by passing through a 27-gauge hypodermic needle, and mixed with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The prepared antigen (100 μl per mouse) was subcutaneously injected into BALB/c mice (male, 4 weeks old; Charles River Japan, Kanagawa, Japan). The animals were boosted intraperitoneally with 150 μl of partially purified antigen (1 mg/ml) 3 weeks after primary immunization. Spleen cells and SP2/0-Ag14 BALB/c myeloma cells were fused under polyethylene glycol 4000 (E. Merck, Darmstadt, Germany) at 37°C. Hybrid cells were cultured selectively in HAT medium (Dulbecco’s modified Eagle medium containing 2% hypoxanthine-aminopterin-thymidine solution [Flow Laboratories, McLean, Va.] and 10% fetal calf serum). Supernatant fluid from growing clones was screened for antibody production by Western blot by using a Sreenner Blotter (Sanplatech, Osaka, Japan). Positive clones were subjected to two limiting dilutions to assure monoclonality. The monoclonal antibody was obtained as ascites fluid from BALB/c mice injected with hybridoma cells and was purified with 50% saturated ammonium sulfate and DEAE-Sepharose CL-6B ion-exchange chromatography.

**Purification of Ag53.** A 20-ml (dry weight) amount of sonic extract was dissolved in 20 ml of binding buffer (10 mM Tris HCl buffer [pH 7.5]) containing 2 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Mo.) and applied to an affinity chromatography column (1.0 by 3.5 cm) made from CNBr-activated Sepharose-4B (Pharmacia) gel combined with monoclonal antibodies (MABgE). The antigen-binding step was repeated 10 times. The column was washed with 70 ml of binding buffer containing 0.5 M NaCl, the bound antigen was eluted with 0.1 M glycine HCl buffer (pH 2.5) containing 1 M NaCl, and elution was monitored at OD280. The eluate was collected in volumes of 1 ml per fraction in tubes containing 50 μl of 1 M Tris HCl buffer (pH 9.0).

**Immunoelectron microscopy.** Immunoelectron microscopy was performed as previously described (12). P. gingivalis 381 cells were incubated with monoclonal antibody (1:50 dilution with phosphate-buffered saline) or BALB/c mouse serum, as the negative control, and were labeled with goat anti-mouse IgG conjugated to 10-nm-diameter colloidal gold spheres (Auro probe EM BAR G10; Janssen Lige Science Products, Olen, Belgium).

**Isolation of vesicles.** Vesicles of P. gingivalis 381 were isolated by the method described by Grenier and Mayrand (6).

**RESULTS**

**Antigen screening with patient sera.** A dominant Ag53 (eluted at 0.15 M NaCl by DEAE-Sepharose CL-6B chromatography) in the sonic extract was detected in the sera of many patients and clearly showed differences in immunoreactivity among patients. The Ag53 reacted strongly to the serum IgG antibody from some patients with periodontitis (Fig. 1, lanes 4 to 8 and 11 to 17) and reacted weakly or not at all to the serum IgG antibodies from the other patients with periodontitis (Fig. 1, lanes 1 to 3, 9, and 10). Nevertheless, all of these patients showed extremely high serum IgG titers to the sonic extract from P. gingivalis 381. We were interested in the differences among the patients with periodontitis in their humoral immune response to the Ag53. The Ag53 was purified to confirm the response of the patients with periodontitis.

**Purification of Ag53.** Six hybrid cell lines (HCBgA, HCBgE, HCBgF, HCBgG, HCBgH, and HCBgI) that produced monoclonal antibodies were established to obtain monoclonal antibodies to Ag53. All monoclonal antibodies
from the six cell lines formed a single immunoreactive blot at 53 kDa when Western blot analysis was used. MABgE, a monoclonal antibody produced from HCBgE, was used in further studies. The affinity chromatography profile of MABgE is shown in Fig. 2A. Purified antigen revealed a single band at 53 kDa when analyzed by SDS-PAGE (Fig. 2B, lane 2).

**Properties of Ag53.** (i) **Humoral immune response to Ag53.** Serum IgG antibodies to purified Ag53 from the 77 patients with periodontitis and 10 healthy subjects were determined by Western blotting (Table 1). Serum IgG from 54 of the patients with periodontitis reacted with Ag53; serum IgG from the remaining 23 patients and 5 of the healthy subjects did not react with Ag53 at all. Serum IgG from the other five healthy subjects reacted very weakly. More patients with early-onset periodontitis, RPP and JP, did not react to Ag53 than patients with AP (*P = 0.08* between AP and JP; *P = 0.09* between AP and early-onset periodontitis; *chi-square* test). These results are shown in part in Fig. 3.

(ii) **Location of Ag53 in *P. gingivalis* 381.** To clarify the localization of Ag53, *P. gingivalis* 381 treated with MABgE gold was observed by electron microscopy. Gold particles were detected bound to vesicles but were not detected elsewhere on *P. gingivalis* 381 cells (Fig. 4A). The existence of Ag53 in vesicle components and in the outer membrane was confirmed by SDS-PAGE and Western blotting. In SDS-PAGE analysis of the vesicles and the outer membrane as well as the sonic extract, bands clearly stained with Coomassie brilliant blue were observed at the position of 53 kDa, and these bands were further detected by MABgE on Western blot analysis (Fig. 4C).

(iii) **Ag53 in other Porphyromonas and Prevotella species.** Sonic extracts from *P. gingivalis* 1021, *P. gingivalis* ATCC 33277, *P. endodontalis* ATCC 35406, *P. asaccharolyticus* ATCC 27260, *P. intermedia* ATCC 25611, *P. loescheii* ATCC 15930, *P. corporis* ATCC 33547, and *P. denticola* ATCC 33185 were analyzed by Western blot by using monoclonal antibodies for detection. There was cross-reactivity only in the 67-kDa antigens from *P. gingivalis* ATCC 33277 and 1021 and in the 53-kDa antigen from *P. endodontalis* ATCC 35406. These immunoreactive bands were more weakly stained than the immunoreactive blot in *P. gingivalis* 381.

**DISCUSSION**

The Ag53 purified in this study is unique in that the patients with periodontitis and with elevated serum IgG antibody levels to crude antigen preparations from *P. gingivalis* 381 were clearly divided into two groups on the basis of their humoral immune responses to Ag53.

The patients in this study who had an elevated serum IgG antibody titer to the sonic extract from *P. gingivalis* 381 probably had been previously infected with *P. gingivalis*.
However, it was not clear whether they had been infected by\textit{P. gingivalis} with Ag53 or a common epitope. This obvious difference in response to Ag53 may simply reflect the difference in predominance of \textit{P. gingivalis} with Ag53. Umemoto et al. (33) reported that the distribution of \textit{P. gingivalis} serotype in patients with periodontitis was simple, that is, no patients harbored more than one serogroup of \textit{P. gingivalis}.

The factors that play important roles in the process of antibody production are genetically controlled. Thus, the capacity for antibody production varies with the subject. Therefore, it is possible that some patients with periodontitis simply will not react to Ag53, even if the homologous isolate expresses the antigen. A mother and daughter previously reported with a history of early-onset periodontitis (24) were subjects of this study as well. Their serum IgG antibody titers against \textit{P. gingivalis} 381 were high, as follows: the mother, with RPP, had a titer of 3,939 ELISA units, and the daughter, with JP, had a titer of 4,936 ELISA units. However, neither responded to Ag53 (Fig. 3, lane 25, results with the mother; Fig. 3, lane 36, results with the daughter). On the other hand, it is noteworthy that some healthy subjects with low serum IgG titers (under 100 ELISA units) to the sonic extract from \textit{P. gingivalis} 381 also responded to Ag53, although their responses were weak. Evaluation of Ag53 in clinical isolates and serum IgG antibody to Ag53 from the same patient will be helpful in resolving this issue. Furthermore, analysis of antibody-secreting cells in localized tissue, which was done with fimbriae and lipopolysaccharide from \textit{P. gingivalis} (25), will be necessary.

In a comparison of antibody response to Ag53 with clinical diagnosis, more patients with JP and RPP did not respond to Ag53 than patients with JP. Classification based on response to Ag53 or other disease markers will be helpful in developing patient categories that do not depend entirely on clinical diagnosis. The group of patients that did not respond to Ag53 had almost the same clinical findings as the group that responded to Ag53. Because many factors influence the clinical findings of periodontal disease, it is difficult to construct a simple relationship between humoral immune response to periodontopathic bacteria and the crude clinical measures used to describe periodontitis.

Immunoelectron microscopy showed MABgE bound to the surface of the vesicles around the bacterial cells and not on the surface of the bacterial cells themselves. However, Ag53 was detected not only in the vesicle components but also in the outer membrane components. The difference between MABgE binding to the vesicles and that to cell surfaces may be influenced by the difference in their surface structures (8). The similarities between vesicles and the outer membrane demonstrated by SDS-PAGE analysis were reported previously (3, 6). Ag53 is shown as a major protein that exists commonly in vesicles and the outer membrane of \textit{P. gingivalis}. Whether or not patients with periodontitis have antibodies against Ag53 may affect the demonstration of biological activities of the vesicles (6, 18) in local lesions.

\textit{P. gingivalis} ATCC 33277 and 1021 and \textit{P. endodontalis} ATCC 35406 have antigens that have an epitope in common with Ag53. These proteins have been seen as major proteins in the SDS-PAGE study of outer membrane from \textit{P. gingivalis} (22). These antigens, of 53 and 67 kDa, may comprise a new category in the taxonomy of \textit{Porphyromonas} spp. and be helpful to the ecological study in subgingival flora.

Ag53 is an antigen that appears on the surface of vesicles and is one of main proteins in the outer membrane of \textit{P. gingivalis}. This antigen represents a specific marker of \textit{P. gingivalis} infection and an interesting aspect of the varied humoral immune response observed among patients with periodontitis.

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