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Serodiagnosis of *Porphyromonas gingivalis* Infection by Immunoblot Analysis with Recombinant Collagenase

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The *Porphyromonas gingivalis* collagenase-specific serum immunoglobulin A (IgA), IgM, and IgG responses from 20 patients with early-onset periodontitis (EOP), 20 patients with adult periodontitis (AP), and 20 age- and sex-matched healthy controls were examined by immunoblot analysis. A recombinant collagenase antigen used for the immunoblot analysis was produced by using the plasmid pGEX-2T, which allows the fusion between the collagenase and glutathione *S*-transferase. There was no significant difference in collagenase-specific IgG antibody detection between samples from the EOP, AP, and control groups. In contrast, 85% of AP and EOP sera had collagenase-specific IgA antibodies, whereas only 20% of control sera showed collagenase-specific IgA reactivity. Plaque samples from all groups were assessed by PCR with primers complementary to the collagenase-encoding gene *prtC*. The results indicated that 90% of AP and EOP plaque samples and 10% of control samples were positive for *P. gingivalis*. All patients with collagenase-specific IgA antibodies were PCR positive. The results of the study indicate a nearly complete concordance ($k = 0.856$) between the presence of collagenase-specific IgA antibodies and PCR detection of *P. gingivalis*. By using PCR as the "gold standard," the sensitivity and specificity of the IgA immunoblot test were 94.7 and 90.9%, respectively. Therefore, the recombinant collagenase is a potential candidate for use in the serodiagnosis of periodontitis.

The gram-negative anaerobe *Porphyromonas gingivalis* is one of several bacterial species closely associated with various forms of periodontal disease (20). Detection of this organism is useful as an indicator and/or predictor for the progression of periodontal disease (18). Although the isolation of *P. gingivalis* by selective media allows for efficient recovery (17), these culture systems are still time-consuming and laborious. Therefore, additional methods have been developed, including immunological methods (23), the benzoyl-DL-arginine-naphthylamide test (11), DNA probe tests (4), and PCR (21). Diagnostic procedures that use PCR can be performed either directly (2) or after the immunomagnetic separation of *P. gingivalis* (1) from the plaque samples.

A serological test may be an alternative approach for the identification of patients infected with this organism. Periodontal patients have in their sera immunoglobulin antibodies against a variety of antigens from *P. gingivalis* (6, 14, 16, 22, 25). However, since sera from healthy controls also contain antibodies against most of these antigens (7, 8, 19, 22), it is now unclear which specific antigen should be used for the serodiagnosis of acute *P. gingivalis* infections. In the present study, immunoblot analysis in which the *prtC*-encoded collagenase (10) was used as the antigen was performed with serum from patients with periodontitis. For the production of large quantities of collagenase, a fusion protein consisting of the entire collagenase and glutathione *S*-transferase was constructed.

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MATERIALS AND METHODS

Patients. A total of 60 white subjects, 20 patients with early-onset periodontitis (EOP), 20 patients with adult periodontitis (AP), and 20 healthy controls matched for sex and age to the patients with EOP, were enrolled in the study. Diagnosis was based on clinical disease characteristics as established by the World Workshop in Clinical Periodontics in 1989 (3). Study subjects signed the informed consent form approved by the Ethics Committee of the Medical Faculty, Julius Maximilian University, Würzburg, Germany. Study subjects were recruited from the Polyclinic of Operative Dentistry and Periodontology, Julius Maximilian University, and represented a consecutive sample. Subjects with any of the following conditions were excluded from the study: professional tooth cleaning within the previous 3 months, use of systemic antibiotics in the previous 6 weeks, long-term use of nonsteroidal anti-inflammatory drugs, systemic disease, pregnancy, and antibiotic premedication required for periodontal examination. To determine the extent and severity of periodontal disease, intraoral radiographs were taken and the subjects were clinically examined. Pocket probing depths and probing attachment levels at six sites per tooth were assessed with a North Carolina periodontal probe (Hu-Friedy, Chicago, Ill.). Venous blood was obtained from all subjects by the standard venipuncture technique.

PCR amplifications and production of recombinant collagenase. PCR detection of *P. gingivalis* in plaque samples was performed as described previously (2), with minor modifications. Briefly, supragingival plaque was removed and the supragingival area was dried with sterile gauze. By using a sterile curette, subgingival plaque samples were then taken from the two deepest pockets. For PCR analysis plaque samples were suspended in 1 ml of sterile saline and were centrifuged at 19,000 × *g* for 2 min. The pellet was resuspended in 1 ml of saline, vortexed, and centrifuged again as described above. PCR with primers coll-1 and coll-2 was carried out as described previously (2). No other method was used to detect *P. gingivalis* in subgingival plaque.

The *P. gingivalis prtC* gene was amplified from type strain ATCC 33277 by PCR with primers Ex1 (5′-CCC GGA TCC ACA CTC ATG CGC TCC GTC-3′) and Ex2 (5′-GGG CCC GGG TTA TTC TTC TCT TTT GTC-3′). Primer Ex1 contains a *Bam*HI site, and primer Ex2 contains a *Sma*I site. Thirty cycles were performed, each consisting of a denaturing step of 30 s at 94°C, an annealing step of 60 s at 47°C, and a 100-s extension step at 72°C. After the last cycle the amplification products were extended for another 5 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis with 1% agarose gels, visualized by staining with ethidium bromide, and purified with GeneClean (Dianova, Hamburg, Germany). The purified DNA was digested with *Bam*HI and *Sma*I and was ligated into the pGEX-2T (Pharmacia LKB, Heidelberg, Germany) vector that had been restricted with *Bam*HI and *Sma*I. The resulting plasmid (pWK1) encoded a fusion protein containing the glutathione *S*-trans-

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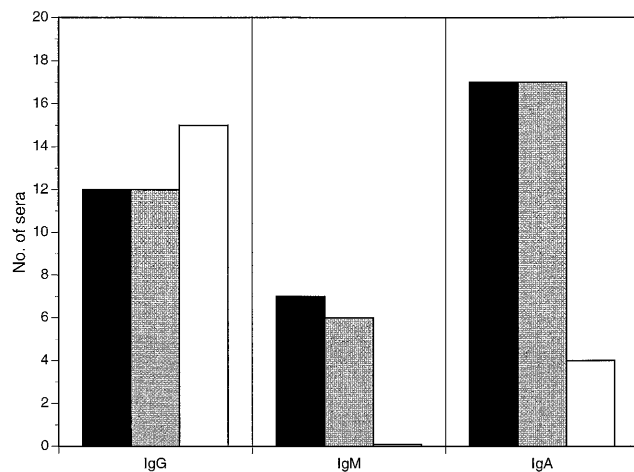

FIG. 1. Frequency of IgG, IgM, and IgA reactivity against recombinant collagenase in sera from patients with EOP (black columns), patients with AP (grey columns), and healthy controls (empty columns).

ferase at its N terminus and the complete collagenase at its C terminus. The *prtC*-containing part was completely sequenced by the *Taq* cycle sequence method. The deduced sequences were identical to the nucleotide sequence of *prtC* published by Kato et al. (10). Laboratory strain H1469 transformed with the recombinant plasmid pWK1 was grown at 37°C to an optical density of 0.8 and was then induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After growth for an additional 2 h the cells were pelleted and disrupted as described previously (9). The fusion protein had a molecular mass of approximately 65 kDa determined by analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunoblot analysis. Western blotting (immunoblotting) to nitrocellulose was carried out in a blotting chamber (Bio-Rad, Munich, Germany) as described previously (9). Two-millimeter-wide nitrocellulose strips were used for the immunoblot analysis. They were incubated overnight at room temperature with a 1:100 (vol/vol) dilution of the subject's sera in phosphate-buffered saline (PBS) containing 0.05% Tween buffer. After washing in PBS-Tween buffer three times, the nitrocellulose strips were incubated with goat anti-human immunoglobulin A (IgA) (diluted 1:1,000) or IgM or IgG (each diluted 1:3,000) (Dianova) for 2 h. The development of the strips was performed as described previously (9).

Statistical analysis. Measurement of the concordance between the immunoblot test and the PCR assay was done by determination of the concordance index as described by Cohen (5).

RESULTS

Immunoblot analysis with recombinant collagenase. The results of the immunoblot analysis with the recombinant collagenase antigen with sera from patients with AP and EOP and from healthy controls are presented in Fig. 1. The prevalence of specific IgM and IgA antibodies against collagenase was elevated in patients with AP and EOP compared with that in the controls. However, no major differences in the IgG reactivity was found between patients and controls. None of the serum samples showed reactivity to the purified glutathione *S*-transferase used as a negative control (data not shown). This indicates that the response is directed against the collagenase and not the glutathione *S*-transferase. No significant reactions against proteins other than the 65-kDa collagenase were found.

PCR detection of *P. gingivalis* and correlation with serology. By PCR, *P. gingivalis* was detected in plaque samples from 18 of 20 patients with AP, as well as patients with EOP (Table 1). As seen from the data in Table 1, all patients with collagenase-specific IgA also had a positive PCR result. Only one patient with EOP, one patient with AP, and no control subject lacking collagenase-specific IgA antibodies were PCR positive. Overall, there was nearly complete concordance ($k = 0.856$) between the results of the IgA immunoblot test and the PCR assay. By using PCR as the "gold standard," the sensitivity and specificity of the IgA immunoblot test were 94.7 and 90.9%, respectively.

DISCUSSION

P. gingivalis has been identified as a major pathogen in AP, and it also appears to play a role in the pathogenesis of other forms of periodontitis (20). Identification of *P. gingivalis* and determination of the specific host response against this organism are important to develop and assess new concepts for the prevention and treatment of periodontal diseases associated with this microorganism. In the present study, it was confirmed that *P. gingivalis* is frequently present not only in patients with AP but also in patients with EOP. Because the native collagenase is difficult to produce in large quantities, the collagenase and glutathione *S*-transferase were fused by using the plasmid vector pGEX-2T. This vector has proved to be an efficient agent in the production of other bacterial antigens (9). The collagenase-specific IgA antibody response showed a sensitivity and specificity of 94.7 and 90.9%, respectively, in detecting a patient infected with *P. gingivalis* by PCR. Therefore, the collagenase may be useful for serological diagnoses. Its sensitivity and specificity appear to be superior to those of other previously reported microbiological methods such as the DNA probe test (91 and 68%, respectively) (15), the benzoyl-DL-arginine-naphthylamide reaction (100 and 20%, respectively) (11), and the bacterial concentration fluorescence immunoassay (100 and 57%, respectively) (24) when compared with culture.

Studies with other antigens of *P. gingivalis* for comparison of the serum antibody reactivities of patients with periodontitis and healthy subjects have revealed conflicting results with respect to their value for assessing an infection with this pathogen (7, 8, 19, 22). These inconsistencies may be explained by racially diverse study populations or regional differences in the epidemiology of infection. In addition, the use of different serological tests and strains of *P. gingivalis* and different antigens such as whole cells (22), outer membrane proteins (6), lipopolysaccharides (16), fimbriae (6), and a 75-kDa protein (25) may also have caused discrepancies in the results.

Our results indicate that 75% of periodontally healthy sub-

TABLE 1. Correlation between PCR detection of *P. gingivalis* in plaque samples and collagenase-specific serum IgA reactivity in patients with periodontitis and controls

P. gingivalis PCR result	No. of serum samples with collagenase-specific IgA in:					
	Controls		Patients with AP		Patients with EOP	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	2	0	17	1	17	1
Negative	2	16	0	2	0	2

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jects possess collagenase-specific IgG antibodies. This may indicate a past infection sufficient to provoke an immune response but unable to induce clinical manifestations. On the other hand, 85% of patients with EOP and AP but only 20% of the healthy controls had serum IgA antibodies. It can be assumed that the presence of IgA antibodies indicates an acute infection. As of yet, there is no conclusive evidence in humans that *P. gingivalis*-specific serum antibodies are protective, and this could be due to the fact that *P. gingivalis* resides intracellularly (12).

It is notable that 15% of patients with EOP and AP failed to mount a collagenase-specific IgA antibody response. In these patients collagenase-specific IgM and IgG were also absent. Failure to mount a response against *P. gingivalis* has also been observed by other investigators using outer membrane proteins as the antigen source, assuming that seronegative periodontitis patients were not infected with *P. gingivalis* (13, 22). In two of our seronegative patients we were able to show evidence of *P. gingivalis* infection. In future studies, it will be interesting to determine whether failure to produce collagenase-specific antibodies is a risk factor for the progression of periodontal disease.

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