Immunization with Porphyromonas gingivalis Capsular Polysaccharide Prevents P. gingivalis-Elicited Oral Bone Loss in a Murine Model

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The capsular polysaccharide (CPS) of the periodontal pathogen Porphyromonas gingivalis is an important virulence factor for this organism. We purified P. gingivalis CPS, immunized mice with this antigen, and assessed the vaccine potential of P. gingivalis CPS by using the murine oral challenge model. Animals immunized with P. gingivalis CPS developed elevated levels of immunoglobulin M (IgM) and IgG in serum that reacted with whole P. gingivalis organisms. The mice immunized with P. gingivalis CPS were protected from P. gingivalis-elicted oral bone loss. These data demonstrate that P. gingivalis CPS is a vaccine candidate for prevention of P. gingivalis-elicted oral bone loss.

Porphyromonas gingivalis has emerged as a leading pathogen implicated in generalized, aggressive periodontal disease, a chronic inflammatory disease of the supporting tissues of the teeth (16, 17, 23). The mechanism by which this gram-negative encapsulated anaerobe initiates periodontal disease is not fully known; however, bacterial and host factors are both critical to this process (12, 15). P. gingivalis possesses a broad array of virulence factors that allow this organism to cause disease, including fimbriae, gingipains, hemagglutinins, lipopolysaccharide (LPS), and others, such as capsular polysaccharide (CPS) (15). Using a murine model, van Winkelhoff et al. (34) found that encapsulated P. gingivalis caused serious forms of infection. This observation was confirmed by Laine et al. (21), who demonstrated that mice challenged with encapsulated P. gingivalis developed more severe infections than those challenged with unencapsulated strains. Compositional analysis of the LPS and CPS of several P. gingivalis strains has revealed the complexity of these antigens. Bramanti et al. (3) reported that the polysaccharide component of P. gingivalis LPS consists of rhamnose, glucose, galactose, mannose, glucosamine, and galactosamine. Schifferele et al. (30) reported that P. gingivalis CPS consists of glucose, glucosamine, galactosamine, and galactosaminuronic acid. Recently, Farquarson et al. (10) reported that the saccharide structure of P. gingivalis LPS O-side chain is a tetrasaccharide repeating unit comprised of 4-linked-α-L-rhamnopyranosyl, 6-linked-α-D-glucopyranosyl, 3-linked-α-D-galactopyranosyl, and 4-linked-2-acetamido-2-deoxy-β-D-glucopyranosyl and that the P. gingivalis CPS consists of mannuronic acid, glucuronic acid, galactose, and 2-acetamido-2-deoxy-β-D-glucose. Additionally, Paramonov et al. (27) reported that the O polysaccharide of P. gingivalis CPS consists of the tetrasaccharide repeating unit -6)α-D-Glc p-(1-4)α-L-Rha p-(1-3)β-D-GalNAc-(1-3)α-D-Gal p-(1-6). To date, 6 P. gingivalis capsule serotypes have been defined; however, serologic assessments of periodontitis patients suggest that additional P. gingivalis CPS-specific serotypes exist (4, 20, 32).

Adult periodontal disease is one of the most common chronic infectious diseases of humans (24). Despite this, there are no vaccines in use for the prevention of adult periodontitis. Because P. gingivalis is a significant periodontal pathogen, investigators have assessed the potential of several P. gingivalis antigens to function as vaccine candidates, including killed whole P. gingivalis organisms and specific P. gingivalis antigens such as fimbriae, fimbrillin peptides, gingipains, hemagglutinins, and others (7, 9, 11, 13, 22, 29, 31). A conjugate vaccine consisting of P. gingivalis CPS and P. gingivalis fimbriae was shown to prevent P. gingivalis infection when a murine subcutaneous challenge model was used (6). However, the use of P. gingivalis CPS as a vaccine candidate has not been described. Moreover, it is not known whether immunization with P. gingivalis CPS, either in pure form or as part of a conjugate vaccine, provides protection from P. gingivalis-ellicted oral bone loss. In this report, we present evidence to demonstrate the usefulness of P. gingivalis CPS as a vaccine candidate for prevention of P. gingivalis-ellicted oral bone loss.

P. gingivalis strain A7436 was used throughout the course of these studies and was cultivated on anaerobic blood agar plates (Becton Dickinson, Cockeysville, Md.) for 3 to 4 days at 37°C in an anaerobic environment. Plate-grown bacteria were harvested and used to seed brain heart infusion-yeast extract broth (pH 7.4) supplemented with 5% fetal bovine serum, l-cysteine (0.75 g/liter; Sigma, St. Louis, Mo.), hemin (5 mg/liter; Sigma), and vitamin K (1 mg/liter; Sigma). Overnight growth was harvested and employed in murine studies, used to coat enzyme-linked immunosorbent assay (ELISA) plates, or used to isolate CPS. For animal studies, P. gingivalis organisms were washed three times with pyrogen-free saline, adjusted to an optical density at 660 nm of 3.0 (approximately 1010 CFU/ml), and either heat killed for immunization or mixed with 2% carboxymethyl cellulose for oral challenge (13). Similarly diluted P. gingivalis organisms were fixed with 3% buffered formaldehyde. 

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at 4°C for ELISAs. Additionally, *P. gingivalis* CPS was purified by using a combination of the methodologies of Schifferle et al. (30) and Pantosti et al. (26). Briefly, *P. gingivalis* organisms grown in a 5-liter batch culture were collected by centrifugation, rinsed with saline, suspended in water, and subjected to hot phenol-water extraction. The aqueous phase was collected, extracted with ether, dialyzed against sterile filtered water, and stored at −80°C. When needed, an aliquot was thawed, adjusted to pH 5.5, and digested overnight with a nuclease cocktail consisting of DNase I and RNase A (Sigma). After a second nuclease digestion step, the pH was adjusted to neutrality and proteinase K (1 mg/ml; Sigma) was added to the sample, which was incubated overnight at 37°C with gentle shaking. Then a second proteinase K digestion was performed on all CPS preparations. The enzymatically treated aqueous phase was concentrated through a 10,000-molecular-weight-cutoff membrane, and crude CPS was precipitated with cold ethanol, suspended in deoxycholate buffer, and separated through an S-400 gel filtration column (Pharmacia, Uppsala, Sweden). Double immunodiffusion (25) was performed on 3-μl samples of each column fraction with *P. gingivalis* A7436-specific immunoglobulin G (IgG) applied to the central well. Following overnight incubation at 4°C, the resultant precipitin bands were observed (data not shown). Additionally, 20-μl samples of each fraction were separated through duplicate sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis (SDS-PAGE) gels. One set of gels was silver stained, while the second set of gels was blotted onto polyvinylidene difluoride membranes. These membranes were dried, blocked with 5% skim milk, probed with rabbit anti-*P. gingivalis* A7436 whole-organism-specific IgG, and incubated with a goat anti-rabbit IgG–alkaline phosphatase conjugate. A substrate was added to develop the blots. Immunoprecipitation, silver stain, and immunoprobe profiles showed that the fractions containing only high-molecular-mass CPS were pooled separately from the fractions containing only low-molecular-mass LPS. Those fractions that contained both antigens were discarded. The pooled fractions were concentrated, precipitated, dialyzed, and lyophilized and yielded approximately 30 mg of purified *P. gingivalis* CPS from a 5-liter broth culture. By using this procedure, we were able to obtain *P. gingivalis* A7436 CPS that was free of *P. gingivalis* LPS (Fig. 1). *P. gingivalis* CPS purity was determined by using a 1-mg/ml sample of each CPS preparation in sterile, pyrogen-free water. All *P. gingivalis* CPS preparations were free of nucleic acid according to *A*260 determinations. They possessed ~0.03% protein, which was determined by using *A*260, a bicinchoninic acid assay, and semiquantitative, silver-stained SDS-PAGE gels (with bovine serum albumin used as a protein standard), and were essentially free of endotoxin, as determined by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod Inc., Falmouth, Mass.) (data not shown).

Immunization and oral challenge studies were performed as described previously (13). In brief, 6-week-old, female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were housed and cared for in accordance with National Institutes of Health (NIH) and Boston University protocols for the care and use of laboratory animals and were separated into four groups (five mice/group). Tail bleeds were performed with all animals prior to immunization. Two groups of mice were immunized subcutaneously two times a week for 3 weeks with either 0.1 ml of heat-killed *P. gingivalis* A7436 (equivalent to 10⁶ CFU/ml) or 0.1 ml of *P. gingivalis* CPS (1 mg/ml) in sterile pyrogen-free saline. All mice were then given oral antibiotics for 10 days and allowed to rest for 3 days. Three groups of animals (including both of the immunized groups and one additional group) were challenged orally with 0.1 ml of a slurry of *P. gingivalis* A7436 in 2% carboxymethyl cellulose (approximately 10¹⁰ CFU/ml) three times in 1 week (Fig. 2). The remaining group of unchallenged mice served as age-matched controls. The animals were rested for 6 weeks, and a final serum sample was collected from...
each animal immediately prior to sacrifice. All animals were monitored daily until sacrifice and were healthy throughout the course of these studies. Levels of \( P. \text{gingivalis} \) whole-organism-specific IgG in serum were determined by ELISA as indicated previously (13). In brief, 96-well Immulon 4Hbx ELISA plates (Dynex, Chantilly, Va.) were coated with formaldehyde-fixed \( P. \text{gingivalis} \) A7436 and blocked with 2% bovine serum albumin. Serial twofold dilutions of serum from each mouse were applied to the plates in triplicate, and these plates were incubated at room temperature. Either goat anti-mouse IgM- or goat anti-mouse IgG–alkaline phosphatase conjugates (Sigma) were added to each well. The wells were washed, substrate was added, and, following a 1-h reaction, absorbance at 405 nm was read. Preimmune levels of \( P. \text{gingivalis} \)-specific IgM and IgG were low (data not shown). As anticipated, animals immunized with heat-killed \( P. \text{gingivalis} \) A7436 developed potent IgM and IgG responses (Fig. 3A and B, respectively). Interestingly, the mice immunized with \( P. \text{gingivalis} \) CPS developed significant \( P. \text{gingivalis} \) A7436-specific IgM and IgG titers. The animals immunized with heat-killed \( P. \text{gingivalis} \) possessed a \( P. \text{gingivalis} \) whole-organism-specific IgG ELISA titer that was significantly greater than that elicited by \( P. \text{gingivalis} \) CPS \((P < 0.05)\) by the Student \( t \) test. The mice that were challenged only orally with \( P. \text{gingivalis} \) developed very low IgM and IgG titers (Fig. 3).

Oral bone loss was assessed at defined landmark sites on the maxillary molars of each mouse, as previously described (13). Using a stereomicroscope, an observer blinded to the identity of the groups obtained linear measurements (14 sites) of each animal from the cementum-enamel junction (CEJ) to the alveolar bone crest (ABC) (2). Measurements were obtained with a digital camera linked to a personal computer, and on-screen measurements were converted to micrometers by using ImagePro (Media Cybernetics, Silver Spring, Md.). Measurements obtained from the CEJ to the ABC of age-matched, control BALB/c mice served as normal values. As anticipated, the maxillary molar CEJ-to-ABC measurements of the mice challenged orally with \( P. \text{gingivalis} \) A7436 were significantly larger than those of the control animals, which confirmed that the oral challenge of mice with \( P. \text{gingivalis} \) elicited oral bone loss (Fig. 4). The mice immunized with heat-killed \( P. \text{gingivalis} \) were protected from homologous oral challenge \((P < 0.05)\) by the Student \( t \) test versus results for the \( P. \text{gingivalis} \) oral challenge group), and results for them resembled those for unchallenged mice \((P > 0.3)\). Interestingly, the mice immunized with \( P. \text{gingivalis} \) CPS were also protected from subsequent \( P. \text{gingivalis} \)-elicited oral bone loss \((P < 0.05)\) by the Student \( t \) test versus results for the \( P. \text{gingivalis} \) oral challenge group) and results for them resembled those for unchallenged mice (Fig. 4).

Humans with periodontal disease produce \( P. \text{gingivalis} \) CPS-specific IgG (4, 32). Despite reports of elevated levels of \( P.
gingivalis CPS-specific antibodies, these patients are apparently unable to clear *P. gingivalis* effectively and still present with periodontitis. We previously reported that the immunization of mice with heat-killed *P. gingivalis* prevented *P. gingivalis*-elicited oral bone loss (13). Because bacterial CPSs are refractory to heating at neutral pH and make excellent vaccine candidates, we were interested in determining if *P. gingivalis* CPS was able to stimulate protection from *P. gingivalis*-elicited oral bone loss. In this study we have determined that immunization of mice with *P. gingivalis* CPS elicited IgM. Interestingly, this antigen also elicited a potent IgG response, and the animals immunized with *P. gingivalis* CPS were protected from *P. gingivalis*-elicited oral bone loss. To date, several *P. gingivalis* vaccine candidates have been reported to prevent *P. gingivalis*-elicited oral bone loss (8, 9, 13, 18, 19, 22, 28) while other *P. gingivalis* antigens, such as LPS, do not appear to provide protection from subsequent *P. gingivalis* infection (5). Unexpectedly, we observed that mice immunized with *P. gingivalis* CPS develop an IgG response that recognizes *P. gingivalis* whole organisms. It is well established that some bacterial CPSs, when used as vaccines, are capable of eliciting protective antibodies via T-cell-independent mechanisms. When the IgG response to bacterial capsules is present, IgG2 usually predominates (33), and these antibodies are relatively poor opsonins that weakly activate the complement cascade. To enhance the host antibody response and to switch the IgG subtype response to these antigens, CPSs have been conjugated to carrier molecules (1, 14). Previously, Choi et al. (6) reported that a *P. gingivalis* CPS-*P. gingivalis* fimbriulin conjugate was effective in preventing *P. gingivalis* infection when a murine abscess model was used. Although their study was compelling, Choi et al. (6) did not determine if *P. gingivalis* CPS alone could function as a vaccine candidate for prevention of *P. gingivalis*-elicited oral bone loss. Why patients with periodontal disease develop a *P. gingivalis* CPS-specific IgG response during infection and fail to resolve the disease while immunization with *P. gingivalis* CPS provides protection against infection remains to be resolved. Future studies should provide insight into the important differences in the host-adaptive immune response to *P. gingivalis* CPS.

In conclusion, the data presented in this article demonstrate that immunization of mice with purified *P. gingivalis* CPS stimulates a significant IgG response that reacts with *P. gingivalis* whole organisms. Furthermore, vaccination with *P. gingivalis* CPS prevents oral bone loss elicited by a *P. gingivalis* oral challenge in the murine model. Continued studies will further define the efficacy of *P. gingivalis* CPS as a vaccine candidate for use in the prevention of *P. gingivalis*-elicited oral bone loss.

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