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

Dario Gonzalez, Arthur O. Tzianabos, Caroline A. Genco, and Frank C. Gibson III

Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Section of Infectious Diseases, Department of Medicine, Evian’s Biomedical Research Center, and Department of Microbiology.

Boston University Medical Center, and Channing Laboratory, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts

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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*-elicited oral bone loss. These data demonstrate that *P. gingivalis* CPS is a vaccine candidate for prevention of *P. gingivalis*-elicited 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. Schifferle 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-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-. 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, fimbrijin 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*-elicited 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*-elicited 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 of 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.
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 polyvinyldene 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_{280}, 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 10^{8} 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 10^{10} 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 mouse.
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. gingivalis whole-organism-specific IgM 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. 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. gingivalis–specific IgM and IgG were low (data not shown). As anticipated, animals immunized with heat-killed P. gingivalis A7436 developed potent IgM and IgG responses (Fig. 3A and B, respectively). Interestingly, the mice immunized with P. gingivalis CPS developed significant P. gingivalis A7436-specific IgM and IgG titers. The animals immunized with heat-killed P. gingivalis possessed a P. gingivalis whole-organism-specific IgG ELISA titer that was significantly greater than that elicited by P. gingivalis CPS (P < 0.05 by the Student t test). The mice that were challenged only orally with P. 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. gingivalis A7436 were significantly larger than those of the control animals, which confirmed that the oral challenge of mice with P. gingivalis elicited oral bone loss (Fig. 4). The mice immunized with heat-killed P. gingivalis were protected from homologous oral challenge (P < 0.05 by the Student t test versus results for the P. gingivalis oral challenge group), and results for them resembled those for unchallenged mice (P > 0.3). Interestingly, the mice immunized with P. gingivalis CPS were also protected from subsequent P. gingivalis–elicited oral bone loss (P < 0.05 by the Student t test versus results for the P. gingivalis oral challenge group) and results for them resembled those for unchallenged mice.

Humans with periodontal disease produce P. gingivalis CPS-specific IgG (4, 32). Despite reports of elevated levels of P. gingivalis A7436; *, P < 0.05 versus results for animals immunized with heat-killed P. gingivalis A7436, as determined by the Student t test.

FIG. 2. Immunization and oral challenge regimen. Six-week-old BALB/c mice were immunized subcutaneously two times per week for 3 weeks (circles) with either heat-killed P. gingivalis whole organisms or P. gingivalis CPS. Sera were collected prior to immunization and immediately prior to sacrifice (arrows). Following a 10-day regimen of oral trimethoprim-sulfamethoxazole and a 3-day rest period, the animals were administered P. gingivalis A7436 by oral gavage three times during a 1-week period (G1 to G3). After 6 weeks of rest, the mice were sacrificed and oral bone loss was determined.

FIG. 3. Titers of P. gingivalis A7436-specific IgM and IgG elicited by immunization. Sera collected from BALB/c mice immediately prior to sacrifice were serially diluted twofold and applied to wells of P. gingivalis A7436-coated ELISA plates in triplicate. The ELISA titer was determined as the last dilution that possessed an absorbance reading that was two times greater than that of the control animals (no immunization, no oral challenge) for IgM (A) or IgG (B). Data are presented as the means ± standard deviations of the results for five mice per group. None, not performed; Pg, P. gingivalis A7436; HK-Pg, heat-killed P. gingivalis A7436; Pg CPS, P. gingivalis A7436 CPS; ND, no statistical analysis determined; NS, no statistically significant difference versus results for animals immunized with heat-killed P. gingivalis A7436; **, P < 0.05 versus results for animals challenged only orally with P. gingivalis; *, P < 0.05 versus results for animals immunized with heat-killed P. gingivalis A7436, as determined by the Student t test.
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 fimbrillin 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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