Host Responses to Recombinant Hemagglutinin B of Porphyromonas gingivalis in an Experimental Rat Model

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Porphyromonas gingivalis, a gram-negative, black-pigmented anaerobe, is among the microorganisms implicated in the etiology of adult periodontal disease. This bacterium possesses a number of factors, including hemagglutinins, of potential importance in virulence. Several hemagglutinin genes have been identified, cloned, and expressed in Escherichia coli. The purpose of this study was to characterize host responses to purified recombinant hemagglutinin B (rHag B), using the conventional Fischer rat as the experimental animal model. The effectiveness of immunization with rHag B on protection against experimental periodontal bone loss following infection with P. gingivalis was also evaluated. Groups of rats were immunized by the subcutaneous route with rHag B in complete Freund’s adjuvant, immunized with rHag B and orally infected with P. gingivalis, nonimmunized and noninfected, or orally infected with P. gingivalis only. Serum and saliva samples were collected throughout the experiment and evaluated for serum immunoglobulin G (IgG) and IgM and salivary IgA antibody responses by enzyme-linked immunosorbent assay. No salivary IgA anti-Hag B activity was detected in any of the groups of rats. A slight serum IgM response similar to that seen in preimmune samples was observed. Serum IgG antibody activity to Hag B was detected only in samples from rats immunized with rHag B. This response was primarily of the IgG1 and IgG2a subclasses, followed by IgG2b and low levels of IgG2c.

Supernatants from rHag B-stimulated splenic lymphoid cells from immunized rats contained high levels of gamma interferon, followed by interleukin-2 (IL-2), IL-10, and then IL-4. These results are consistent with the induction of T helper type 1 (Th1)-and Th2-like responses. Western blot analysis of sera derived from rHag B-immunized rats reacted with trichloroacetic acid (TCA) precipitates of P. gingivalis 33277, 381, A7A1-28, and W50, revealing a 50-kDa band reflective of Hag B. However, sera derived from rats immunized with P. gingivalis whole cells or from rats infected with P. gingivalis only did not react with rHag B but did react with TCA precipitates of P. gingivalis strains. Finally, radiographic measurements of periodontal bone loss indicated that rats immunized with rHag B had less bone loss than those infected with P. gingivalis only. These results demonstrate the effectiveness of purified rHag B in inducing a protective immune response and support the potential usefulness of this component of P. gingivalis in the development of a vaccine against adult periodontitis.

Human adult periodontitis is an infectious disease initiated and perpetuated by specific gram-negative bacteria. Among these, the oral, black-pigmented anaerobe Porphyromonas gingivalis has been accepted as an etiological factor and thus implicated in the pathogenesis of the disease (10, 38, 39). This microbial pathogen has been commonly isolated from periodontal diseased sites (26, 39, 40), and specific antibodies to this bacterium have been found in patients with periodontitis (29, 30, 45).

P. gingivalis possesses a number of potential virulence factors thought to be important in the disease process (13, 28, 31, 33, 42). Among these are the hemagglutinins, nonfimbrial adhesins expressed on the surface of P. gingivalis. Hemagglutinins have been implicated in virulence due to their ability to agglutinate erythrocytes, which suggests that they may have a role in adhesion to host tissues (12, 34). It is not yet known how many different P. gingivalis hemagglutinins exist, and there is no evidence as to their specific function. Early studies by Inoshita et al. (14) reported the presence of three major proteins in affinity-purified hemagglutinin preparations. Using monospecific polyclonal and monoclonal anti-hemagglutinin antisera, Mouton et al. (28) detected two major protein antigens in immunoblots of cell surface extracts of P. gingivalis. Extensive studies by Progulske-Fox et al. (34, 35) and Lepine et al. (24, 25) described the cloning of four P. gingivalis genes and their expression in Escherichia coli. These genes encode for the proteins Hag A, B, C, and D. Hag A and D have about 73.8% identity, whereas Hag B and C are 98.6% homologous. However, neither shows significant homology to Hag A. There are several genes encoding for hemagglutinin molecules, which may be an indication of their importance in virulence. Currently, Hag A and B have been more extensively characterized than Hag C or D. Furthermore, there has been a great deal of interest in the potential utilization of Hag B in vaccine development. For instance, Dusek et al. (6, 7) successfully expressed the hagB gene in an avirulent strain of S. typhimurium. With the mouse as the experimental animal model, humoral immune responses were evaluated following the intragastric administration of the attenuated Salmonella strain. Both systemic and mucosal antibody responses to Hag B were induced, thus demonstrating its immunogenicity. In a later study, it was shown that the subsequent administration of the Salmonella strain to mice resulted in a recall response to Hag B in both serum and secretions (23). However, in these studies, the involvement of the immune response in protection from periodontal disease was not determined. Thus, although the utilization of Hag B in vaccine development seems promising, an evaluation of its role in disease protection is essential.

Previous studies in our laboratory have shown that induction
of anti-P. gingivalis antibodies was associated with less bone loss in an experimental rat model, suggesting a role for specific antibodies in periodontal disease protection (18, 19). However, the specificity of the protective antibody was not established.

The present investigation evaluated the humoral immune response induced following subcutaneous (s.c.) immunization with recombinant Hag B (rHag B) in an experimental rat model. Levels of serum and salivary anti-Hag B antibodies were determined following immunization and/or infection with P. gingivalis. We also assessed the immunoglobulin G (IgG) subclass of the anti-Hag B antibodies and the production of T helper (Th) cytokines induced in rHag B-stimulated splenic lymphoid cell cultures. Last, protection was evaluated by radiographic assessment of periodontal bone loss.

MATERIALS AND METHODS

Animals. Conventional Fischer CD F(344) rats used in this study were bred and maintained in Trexler isolators and in horizontal laminar flow units at the University of Alabama at Birmingham (UAB). Animals were fed sterile diet L-085 (Harlan Teklad, Madison, Wis.) and were given water ad libitum throughout the experimental period. All experiments were approved by the UAB Institutional Animal Care and Use Committee.

Bacteria. P. gingivalis 33277, ATCC 33277, and ATCC 53977, and W50 were used in these studies. The bacteria were cultured and maintained on enriched Trypticase soy plates consisting of Trypticase soy agar supplemented with yeast extract (1%), 5% defibrinated sheep blood, hemin (5 mg/liter), and menadione (1 mg/liter) at 37°C in an anaerobic atmosphere of 10% H2, 5% CO2, and 85% N2 (43, 44). For the preparation of P. gingivalis for various purposes including infection and for use as whole-cell (WC) coating antigen in the enzyme-linked immunosorbent assay (ELISA), cultures were grown in basal anaerobic broth (44) at 37°C under anaerobic conditions (17, 18). The bacteria were harvested, washed in sterile phosphate-buffered saline (PBS) (6,000 g for 20 min), and resuspended in PBS. The estimated number of bacteria in the suspension was determined by reading the optical density at 580 nm and extrapolating to a standard curve. For oral challenge, the bacteria were used at the time of challenge.

The reactivity of sera from rats s.c.-immunized with P. gingivalis strain WC was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (19, 21). Briefly, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The reactivity of sera from rats s.c.-immunized with P. gingivalis strain WC was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (19, 21).

Cytokine assay. The reactivity of sera from rats s.c.-immunized with P. gingivalis strain WC was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (19, 21).
Antibody responses. Antibody activities to rHag B and P. gingivalis WC were assessed by a previously described ELISA (16, 18, 19, 37). Briefly, individual wells of flat-bottom 96-well plates (Nalge Nunc International, Rochester, N.Y.) were coated with rHag B (50 ng/ml) or P. gingivalis WC proteins in bicarbonate-carbonate buffer (pH 9.6; 100 µl; 1 µg of rHag B per ml or 5 × 10^8 cells/ml) or with anti-rat α, μ, or ω heavy-chain antibody (UAAB Immunochemical Core Facility) or affinity purified anti-rat IgG1, IgG2a, IgG2b, or IgG2c (The Binding Site Inc., San Diego, Calif.) in bovine-serum albumin (pH 8.2). Nonspecific binding sites were blocked with PBS containing 5% FCS (Gemini Bioproducts, Calabasas, Calif.) and 0.05% Tween 20 (pH 7.4) (Fisher Scientific, Fair Lawn, N.J.) for 2 h at room temperature. From a starting dilution of serum (1:100 for IgG1 and IgG2a; 1:50 for IgG2b or IgG2c) or saliva (1:15 for IgA) prepared in PBS containing 1% FCS and 0.1% Tween 20, five twofold dilutions were added in duplicate to individual wells. Samples of serum and saliva collected from individual rats throughout the experiment were assayed for antibody activity, and all assays were repeated two or three times. The levels of total salivary IgA and of salivary IgA and serum IgM and anti-Hag B or anti-P. gingivalis antibody activities were determined by using standard curves generated with a calibrated pool of Fischer rat sera with known amounts of the corresponding immunoglobulins and wells coated with anti-rat α, μ, or ω heavy-chain antibody (37) and are shown as geometric means ×/− standard deviation (SD). To quantitate IgG subclass antibody activities in the experimental serum samples, a calibrated pool of Fischer rat sera (The Binding Site Inc.) with known amounts of various IgG subclasses was used to generate standard curves. After incubation (2 h at 37°C) and washing of plates, biotin-conjugated anti-rat IgA, IgG, or IgM (UAAB Immunochemical Core Facility) or HRP-conjugated anti-rat IgG subclass (The Binding Site Inc.) antibody was added to appropriate wells. After overnight incubation (4°C), plates with biotinylated anti-rat antibodies were washed and streptavidin-HRP (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was added for 30 min at room temperature. All plates were washed, peroxidase substrate [2'-2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] in citrate buffer (0.3 mg/ml, pH 4.0) containing 0.003% hydrogen peroxide was added, and color development was recorded at 414 nm. The concentrations (nanograms per milliliter) of specific anti-Hag B and anti-P. gingivalis antibody activities were determined by assessing the samples simultaneously with the calibrated pool of Fischer rat sera and interpolating from a standard curve by using a four-parameter logistic algorithm.

Evaluation of periodontal bone destruction. The amount of periodontal bone loss was assessed by radiographic analysis as previously described (18, 19). At the termination of the experiment period, periodontal bone loss was assessed in rats infected with P. gingivalis or in rats immunized s.c. (rHag B) and infected in order to determine the effect of anti-Hag B antibodies on protection against P. gingivalis infection. The radiographic method assesses vertical bone loss, which is a linear measurement from the cementoenamel junction to the crest of the alveolar bone. Each mandible, with the lingual side down, was placed on top of an infraoral film resting on a styrofoam platform. The radiation source (Trophy model SU47 portable dental unit; Trophy Radiologie, Vincennes, France) was positioned 25 cm from the film platform. A 10-mm stainless steel dimensional reference was incorporated into each radiographic image to ensure consistent positioning and to allow the digital analysis to be expressed in millimeters of reference. The radiographic results are expressed as millimeters of bone loss between the experimental and control groups. Further analysis of the IgG response to rHag B indicated that the primary subclasses of antibodies induced were IgG1 and IgG2a (Fig. 2A) followed by IgG2b and low levels of IgG2c (Fig. 2B). The IgG1, IgG2a, and IgG2b responses appeared to peak earlier in the group B rats than in the group A rats; however, no significant difference in the IgG subclass responses between the two groups of rats was seen.

Antigen-induced proliferative responses and cytokine production. Proliferative responses to rHag B were seen in splenic lymphoid cell cultures from rats in groups A and B (Fig. 3A). Optimal stimulation to rHag B was seen with a dose of 5 µg/ml in cultures from both groups of rats. Cell cultures derived from rats immunized and infected showed a slightly higher SI to all doses of rHag B tested than those derived from immunized-only rats, although the difference was not significant. Little or no proliferative response was seen when the cells from either group of rats were stimulated with P. gingivalis WC (Fig. 3B). A slight stimulation was seen with 8 × 10^8 bacterial WC in cell cultures derived from immunized and infected rats. Cell cultures from both groups of rats responded to ConA in a dose-dependent manner (Fig. 3C). The response of cells from immunized rats was higher than (but not significantly different from) that seen with cells from immunized and infected animals.

Supernatants from lymphoid cells stimulated with rHag B and assayed for the presence and level of cytokines revealed significantly higher (P < 0.05) levels of IFN-γ in the CS of cells derived from immunized and infected rats than in CS from cells derived from immunized-only animals (Fig. 4A). The levels of IL-2 in the CS from the immunized and infected group were also significantly higher (P < 0.05) than those seen in the CS of the immunized-only rats (Fig. 4B). IL-4 was detected in CS, but in low amounts (Fig. 4C). The levels of IL-4 in supernatants of rHag B-stimulated cell cultures from immunized rats were slightly higher than (but not significantly different from) those seen in cultures from immunized and infected animals. Finally, the levels of IL-10 detected in the CS of rHag B-stimulated cells derived from immunized and infected rats were higher than but not significantly different from those seen in cultures from the immunized-only group (Fig. 4D).

Immune response to P. gingivalis WC. To determine if the anti-Hag B antibodies could react with P. gingivalis, which...
could help determine the potential protective effect of these antibodies against infection with this pathogen, serum samples from rats immunized with rHag B were tested for reactivity against *P. gingivalis* WC by ELISA. The reactivity to WC of serum samples from group A rats was 10- to 100-fold lower than that seen to rHag B (Fig. 5). Some antibody activity was seen in serum samples when WC suspended in PBS were used as coating antigen, whereas no increased in activity above baseline (day 0 sample) was seen with WC treated with formalin. Serum samples from rats immunized by the s.c. route with *P. gingivalis* WC had high levels of antibody activity to formalin-treated *P. gingivalis* WC and essentially no anti-Hag B antibody activity (Fig. 6). Similar results were obtained when untreated *P. gingivalis* WC were used as coating antigen (data not shown).

Good serum IgG antibody responses to *P. gingivalis* WC were also seen in sera from rats in group D (data not shown).

**Western blot analysis of antibody responses.** Serum from rats immunized with rHag B primarily reacted with a protein of the approximate size of 50 kDa in the rHag B preparation and with the TCA precipitates of *P. gingivalis* 33277, 381, A7A1-28, and W50 (Fig. 7A), indicating that Hag B is a membrane component of these *P. gingivalis* strains. Sera from rats immunized with *P. gingivalis* WC (Fig. 7B) or infected with *P. gingivalis* only (data not shown) did not react with rHag B but did react with TCA precipitates of *P. gingivalis* 33277, 381, A7A1-28, and W50.

**Assessment of experimental periodontal bone loss.** At the end of the experiment (days 70 to 80), the mandibles from
individual rats were removed and the amount of bone loss was quantified by the radiographic technique (Fig. 8). Bone loss in rats infected with *P. gingivalis* was significantly greater (*P*, 0.05) than that in the animals which were immunized with rHag B and then infected with *P. gingivalis*.

**DISCUSSION**

The purpose of this study was to characterize the immune response induced following systemic immunization with purified rHag B and to determine the effectiveness of the response in protection against periodontal bone loss in an experimental rat model. When serum samples from rats immunized by s.c. administration of rHag B in CFA were assessed for antibody activity, a low level of IgM anti-Hag B activity was detected. The level of activity was similar to that seen in preimmune serum samples and in serum samples from nonimmunized, noninfected animals, suggesting the presence of Hag B determinants which have cross-reactivity with components of the indigenous microorganisms in the rat. This finding was similar to our previous observations on IgM anti-*P. gingivalis* activity in the rat (19) and to what has been observed in periodontally involved and control individuals (5). In contrast, a good serum IgG anti-Hag B response was seen in rats immunized with the purified protein, demonstrating the immunogenicity of this protein.
molecule in the rat model. These results extend the findings of Dusek et al. (6, 7) and Kohler et al. (23), who reported the induction of a systemic and mucosal response to this protein in mice following oral administration of a Salmonella typhimurium strain expressing the cloned Hag B.

Analysis of the IgG subclass responses to rHag B in immunized or immunized and infected rats indicated that IgG1 and IgG2a were the primary IgG subclasses of antibodies induced, followed by IgG2b and a very low level of IgG2c. In the murine system, Th2-like cells produce IL-4, IL-5, and IL-10 and support an IgG1 subclass response, whereas Th1-like cells produce IFN-γ and IL-2 and are involved in isotype switching to IgG2a (9, 27, 41). The correlate of murine IgG1 in the rat is IgG1 and IgG2a, while murine IgG2a is homologous to rat IgG2b (1). Therefore, the IgG subclass response pattern observed in both groups of experimental animals would suggest a mixed Th1 and Th2 response to systemically administered rHag B. The production of IFN-γ, IL-2, IL-10, and some IL-4 by antigen-stimulated splenic lymphoid cells from these rats supports the above observation. At the time of cytokine assessment, cells from immunized and infected rats produced significantly higher levels of IL-2 and IFN-γ than cells from immunized-only rats. This finding suggests that infection with P. gingivalis of immunized rats promoted the Th1-like response pattern. The apparent earlier peak in the IgG2b response in the immunized and infected rats compared to the immunized-only group supports this possibility. Furthermore, previous studies in our laboratory have shown that immunization with P. gingivalis WC induced predominantly IgG2b antibodies, therefore indicating the involvement of Th1-like cells (18). It is unclear why only low levels of IL-4 were detected in the immunized animals since they had high levels of IgG1 and IgG2a anti-Hag B antibodies reflective of a Th2-like response pattern. Others have also reported the induction of a dominant IgG1 antibody response in mice following immunization with an antigen and adjuvant, as well as high IFN-γ and low IL-4 production in vitro (46). It is possible that in our study the cytokine profile induced by rHag B in the splenic lymphoid cell cultures would have revealed a higher level of IL-4 if cells were assessed shortly, instead of 70 to 80 days, after immunization. It is also possible that the amount of antigen induced IL-4 was sufficient to promote a Th2-like response. Nevertheless, the finding of higher levels of IL-4 in the immunized-only group than in the immunized and infected rats supports the notion of a shift toward a Th1-like response upon P. gingivalis infection.

Kohler et al. (23), who analyzed the serum IgG subclass distribution in mice following oral administration of a Salmonella strain expressing the cloned Hag B, reported a predominant IgG2a anti-Hag B response. This predominant Th1-like response pattern could have been influenced by the Salmonella vector, as suggested by other investigators (32, 47) who reported systemic Th1-like responses to cloned antigens.
expressed by *S. typhimurium*. Conversely, our laboratory reported a mixed Th1- and Th2-like response pattern to a cloned antigen of *Streptococcus mutans* when expressed by *S. typhimurium* (11). In this latter study, responses to the purified *S. mutans* antigen were primarily Th2-like. This finding suggests that the property of the cloned antigen can influence the host immune response and that the nature of the response is not entirely determined by the *Salmonella* vector. This possibility gained support from our findings which revealed a mixed Th1- and Th2-like response to purified rHag B even when the antigen was administered in CFA, a condition which would favor a Th1-like response. Further studies will be required to define properties of cloned antigens and immunization regimens important in influencing the nature of the specific host responses.

To determine the relevance of anti-Hag B antibodies in protection against *P. gingivalis* pathogenesis, we next examined the ability of these antibodies to react with the bacteria. The amount of reactivity to freshly harvested *P. gingivalis* was more than 10-fold lower than that seen to rHag B. This finding suggests that Hag B antigenic epitopes are minimally expressed on the surface of *P. gingivalis* cells for induction of immune responses or that differences may exist in the antigenicity of native and rHag B. The former possibility gains support from the finding that serum from rats immunized s.c. with freshly harvested *P. gingivalis* contained a high level of antibodies reactive with WC but essentially no reactivity to Hag B. These serum samples, as well as those from rats infected with *P. gingivalis*, also reacted with TCA precipitates of the various *P. gingivalis* strains, exhibiting a number of bands as has been previously shown (3, 20). Western blot analysis of serum samples, as well as those from rats infected with WC and ascribed this effect to a difference in the ability of the antigens to react with WC. However, it has also been suggested that formalin treatment has been used to inactivate bacterial toxins or to stabilize bacterial proteins for vaccine preparations (4, 36). Formalin treatment has been shown to alter epitopes and influence the nature of the immune response and antigen-induced cytokine production by splenic lymphoid cell cultures from immunized animals. Finally, rats immunized with rHag B were protected against experimental periodontal bone loss following infection with *P. gingivalis*. These results support the potential use of rHag B as a candidate antigen for the development of a vaccine against periodontal disease.

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**REFERENCES**


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