Modified Immunogenicity of a Mucosally Administered Antigen

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Streptococcus mutans is present in the saliva of most individuals and is modified by salivary components bound to the cells. These saliva-bound S. mutans are swallowed, exposed to high levels of acidity in the stomach, and presented to the common mucosal immune system. Much effort has been directed to identifying the specific S. mutans antigens that the mucosal immune responses are directed against. However, little is known about the host-antigen determinants that the mucosal immune system recognizes. The immunogenicity of gastrically intubated untreated S. mutans cells, cells coated with whole human saliva, cells treated with HCl (pH 2.0), and saliva-coated and acid-treated cells in mice was investigated. Saliva and serum samples were assayed for naturally occurring antibody against the untreated or treated S. mutans cells. In general, the levels of salivary IgA and serum IgG antibodies to the antigen against which the mice were immunized were significantly higher (P ≤ 0.05). In addition, human saliva and serum samples from 12 subjects were assayed for naturally occurring antibody against the untreated or treated S. mutans cells. In every case, significantly higher reactivity was directed against the saliva-coated and acid-treated cells followed by the saliva-coated S. mutans. These results provide evidence for the altered immunogenicity of swallowed S. mutans in humans by coating native S. mutans antigens with salivary components and/or denaturing surface S. mutans antigens in the acidic environment of the stomach, which would lead to an immune response to modified S. mutans determinants and not to native S. mutans antigens.

Demineralization of enameled tooth surfaces is a bacterially induced disease in which Streptococcus mutans has been implicated as the major etiological agent (13). Numerous studies have examined the levels of naturally occurring salivary immunoglobulin A (IgA) and serum IgG antibodies to S. mutans antigens in dental caries-free and caries-active subjects (1, 2, 4, 8, 11, 20). Other studies demonstrated increased antibody levels and reduced S. mutans colonization in humans and experimental animals following immunization (7, 9, 10, 14, 15, 21, 22).

Secretory IgA (sIgA) antibodies are induced following mucosal administration of antigen (reviewed in reference 16). The best-studied route of sIgA induction is oral immunization. The natural process of stimulating an immune response to S. mutans is similar to that of oral immunization. S. mutans is present in virtually every mouth and is present in numbers expected to be immunogenic (13). Before salivary S. mutans is swallowed, the bacterial cells are exposed to saliva, and salivary components coat the streptococci (3, 5, 6, 17, 23). The saliva-coated S. mutans must then pass through the stomach and are exposed to stomach acids as low as pH 2.0. The saliva-coated and acid-treated bacterial cells are then passed into the small intestine, where the well-characterized mucosal immune response is generated (reviewed in reference 16). Briefly, microfolding (M) cells lining the dome of the Peyer’s patch sample the foreign antigen in the lumen of the small intestine. M cells serve to transfer the antigen to underlying antigen-presenting cells, which then present the antigen to B and T lymphocytes in the Peyer’s patch. Since S. mutans is a natural inhabitant of the oral cavity of virtually everyone and is ultimately swallowed, M cells regularly sample and process saliva-coated and acid-treated S. mutans antigens in the small intestine. Once presented with antigenic determinants, the B and T lymphocytes leave the Peyer’s patch, travel through the circulation and lymphatics, and migrate to the mucosal sites of the body, including the linings of the gastrointestinal, respiratory, and genital tracts and the salivary, mammary, and lacrimal glands. There the B lymphocytes differentiate into plasma cells and synthesize and secrete into the mucosal fluids specific IgA antibody to the foreign antigen originally encountered in the small intestine (i.e., saliva-coated and acid-treated S. mutans cells). Therefore, naturally occurring sIgA antibodies to many normal flora microorganisms as well as to pathogens are found in all secretions (2). The protective modes of action of IgA antibodies include neutralization of microbial enzymes, toxins, and viruses; agglutination of microorganisms; inhibition of attachment; colonization and penetration of antigen into mucosa; opsonization; and activation of the alternate complement pathway (12, 19). The mechanism of induction of serum IgG antibodies to S. mutans is unclear, although some stimulation undoubtedly occurs, in part, by minor breaks in the oral mucosa, the subsequent dislodgment of S. mutans cells into the circulation, and uptake by antigen-presenting cells.

Typically, antibody responses to mucosal pathogens are assessed using untreated bacterial cells without modeling the effects of the mucosal environment. Significant effort has been directed to establishing the specific S. mutans antigenic determinants with which sIgA antibodies react. However, little is known about the host-modified antigenic determinants of S. mutans that the mucosal immune system recognizes. Therefore, it was hypothesized that the major immune responses to S. mutans antigens are directed primarily at saliva- and acid-
modified antigens and that would cause weaker responses. Mice that were gastrically immunized with saliva-coated and/or acid-treated S. mutans cells had higher levels of specific salivary IgA and serum IgG antibodies than mice exposed to untreated determinants, and naturally occurring levels of human antibody to the treated cells were higher than levels of antibody to the untreated antigen. These results indicate that immune responses to S. mutans are primarily directed to saliva- and acid-modified determinants.

**MATERIALS AND METHODS**

**Preparation of treated S. mutans cells.** Untreated viable S. mutans TH16 cells (18) for the immunization of mice were prepared by overnight growth in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) at 37°C in 5% CO₂. Cells were washed with sterile saline three times and diluted with saline to an absorbance of 0.5 at 540 nm. Cells to be coated with saliva were diluted to an absorbance of 1.0 and then further diluted 1:2 with fresh pooled human saliva to be equivalent to the untreated cell preparation. Pooled saliva was obtained by mixing equal volumes from four subjects (no active carious lesions) and clarified by centrifugation (10,000 × g, 10 min). Cells to be treated with acid were diluted to an absorbance of 0.5; the pH was lowered to 2.0 by dropwise addition of 1 N HCl and incubation for 30 min at 37°C. The cell suspension was then readjusted to pH 7.0 with 1 N NaOH. The total volume of HCl and NaOH added was less than 1% of the total volume. Saliva-coated and acid-treated cells were prepared by coating with saliva as described above and then treating with acid as described above. The relative numbers of S. mutans cells were similar in all four groups due to identical dilutions and as demonstrated by absorbances of 0.5 after treatment. Untreated and treated cells for enzyme-linked immunosorbent assay were diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6, in place of saline.

**Mouse studies:** (i) Immunization of animals. Four groups of adult BALB/c mice (50 to 60 days old, six animals/group) were gastrically intubated with 50 µl of untreated, saliva-coated, acid-treated, or saliva-coated and acid-treated S. mutans cells in saline at weekly intervals for 3 weeks, and saliva and serum samples were collected 1 week later.

(ii) Collection of saliva and serum samples. Whole saliva and serum samples from immunized mice were obtained. Whole saliva samples were collected following pilocarpine stimulation. Blood samples were obtained by puncture of the retro-orbital plexus. Serum was separated from the clot by centrifugation (5,000 × g, 10 min). The saliva and serum samples were stored at −20°C until used for antibody analysis.

**Human studies:** (i) Immunoassays. Mice and human saliva and serum samples were assayed for IgA and IgG antibody activity, respectively, against the untreated, saliva-coated, acid-treated, or saliva-coated and acid-treated S. mutans cells using a modification of a previously described enzyme-linked immunosorbent assay (11). Polystyrene microtiter plates (enzyme immunoassay, Linbro; Flow Laboratories, Inc., McLean, Va.) were coated (100 µl/well) with the untreated or treated S. mutans cells (diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6) and incubated at 37°C for 3 h. Coated plates were washed three times in Tween saline (0.09% NaCl containing 0.05% Tween 20) to remove unbound antigen. Free sites on the plates were blocked by reaction with 200 µl of a solution containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) for mouse samples or 10 µg of globulin-free human serum albumin (Sigma/ml) for human sera at 25°C for 1 h. Saliva (diluted 1:4 in Tween saline) and serum (1:100) samples from mice or humans, in triplicate, were added to the wells (100 µl/well) and incubated for 2 h at 37°C. The plates were washed three times with Tween saline and incubated for 3 h at 37°C with 100 µl of horseradish peroxidase-labeled anti-mouse or anti-human IgA (for saliva samples) or IgG (for serum samples) heavy-chain-specific reagents (1: 1,000; Sigma). After washing of the plates three times with Tween saline, orthophenylenediamine dihydrochloride in citrate buffer containing H₂O₂ was added (100 µl/well). Color development was stopped after 30 min using 2 N H₂SO₄.

**DISCUSSION**

Studies were conducted in order to establish that mucosal immunization of mice with saliva-coated and/or acid-treated S. mutans cells induces stronger salivary IgA and serum IgG antibody responses than immunization with untreated cells. Some of the response to the saliva-coated preparation may be directed against the human saliva components; however, animals immunized with acid-treated cells reacted significantly more strongly to the homologous preparation than to the saliva-coated S. mutans. Furthermore, mice immunized with either the saliva-coated or saliva-coated and acid-treated preparations reacted equally as well as (salivary IgA) or significantly better than (serum IgG) mice immunized with acid-treated determinants. This implies that the bulk of the strong response to the saliva-coated and acid-treated preparation was attributed to acid modification of S. mutans cell surface determinants. In addition, naturally occurring levels of antibody to the saliva-coated and acid-treated antigen were stronger than levels of antibody to the untreated S. mutans antigens in human volunteers. The results from these experiments suggest an association between the composition of the immunogen and the specificity of the immune response generated. This was initially established in a murine model in which different S. mutans preparations were provided orally by...
gastric intubation to the animals. After the salivary and serum responses of mice were assessed, samples were collected from 12 laboratory volunteers, and naturally occurring immune responses against the preparations were determined. The ability to direct a specific immune response to an immunogen is critical for the host to provide protection against mucosal pathogens. It was hypothesized that the major immune responses against *S. mutans* in humans were directed not at untreated cell determinants but at *S. mutans* that had undergone modifications in the antigenic determinants expressed on the cell surface. Since *S. mutans* resides only in the oral cavity, it is constantly exposed to saliva and is coated with salivary components, such as mucins, specific IgA antibody, proline-rich polypeptides, statherin, amylase, and possibly other molecules (5, 6, 17, 23). This occurs before the bacterial cells are swallowed. In addition, proteolytic digestion of the bacteria may occur prior to their presentation to the M cells, and this may have occurred with the mice intubated with *S. mutans* cells in these studies. The major route of elimination of all oral bacteria is swallowing and subsequent exposure to acid in the

FIG. 1. Salivary IgA (A) and serum IgG (B) antibody responses to untreated, acid-treated, saliva-coated, or saliva-coated and acid-treated *S. mutans* cells in mice, gastrically intubated with these preparations. Asterisks indicate significantly greater (*P* < 0.05) antibody responses to stated antigens than to untreated *S. mutans* cells in each group. ELISA, enzyme-linked immunosorbent assay.
stomach. Since stomach acid routinely measures as low as pH 2.0, the acid-treated cells used here were exposed to this pH for 30 min at 37°C to mimic the exposure of swallowed *S. mutans* cells to the harsh environment of the stomach. This may either alter the antigenic determinants of the bacteria or possibly strip off salivary components and/or bacterial antigens. Following passage of swallowed *S. mutans* through the stomach, the resulting saliva-coated and acid-treated antigen is presented to the specialized immune tissues in the small intestine and a mucosal IgA immune response is generated to the specific antigen encountered.

Because much research is conducted on immune responses to mucosal pathogens and the normal mode of presentation may involve passage through an environment that is capable of modifying the antigenic determinants, it is important to assess the major determinants presented to the immune system. This is important when assessing naturally occurring immune responses to pathogens, but it is also critical in assessing the
protection resulting from vaccination. It is likely that immune responses generated by common vaccines will be directed against the native antigens on the pathogen, but they may not be strongly directed to the determinants on the pathogen where they are present on mucosal surfaces in the infected host (i.e., coated with salivary, respiratory, or genitourinary fluid components). These results provide evidence that immune responses to \textit{S. mutans} are primarily directed to modified antigenic determinants and support consideration of using determinants treated as they would be in situ in both mucosal vaccines and antigens used for assessing immune responses.

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REFERENCES


