Characterization of Salivary Immunoglobulin A Responses in Children Heavily Exposed to the Oral Bacterium *Streptococcus mutans*: Influence of Specific Antigen Recognition in Infection

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The initial infection of children by *Streptococcus mutans*, the main pathogen of dental caries, depends on the ability of *S. mutans* to adhere and accumulate on tooth surfaces. These processes involve the adhesin antigen I/II (AgI/II), glucosyltransferases (GTF) and glucan-binding protein B (GbpB), each a target for anticaries vaccines. The salivary immunoglobulin A (IgA) antibody responses to *S. mutans* antigens (Ags) were characterized in 21 pairs of 5- to 13-month-old children. Pairs were constructed with one early *S. mutans*-infected and one noninfected child matched by age, racial background, number of teeth, and salivary levels of IgA. Specific salivary IgA antibody response and *S. mutans* infection levels were then measured during a 1-year follow-up. Robust responses to *S. mutans* were detected from 6 months of age. Salivary IgA antibody to AgI/II and GTF was commonly detected in salivas of all 42 children. However, GbpB-specific IgA antibody was seldom detected in the subset of infected children (38.1% at baseline). In contrast, most of the subset of noninfected children (76.2%) showed GbpB-reactive IgA antibody during the same period. Frequencies of GbpB responses increased with age, but differences in intensities of GbpB-IgA antibody reactions were sustained between the subsets. At baseline, GbpB-reactive IgA antibody accounted for at least half of the total salivary IgA *S. mutans*-reactive antibody in 33.3 and 9.5% of noninfected and infected children, respectively. This study provides evidence that a robust natural response to *S. mutans* Ags can be achieved by 1 year of age and that IgA antibody specificities may be critical in modulating initial *S. mutans* infection.

*Streptococcus mutans* is considered to be pathogenic for dental caries, because of its ability to adhere and accumulate in the dental biofilm in the presence of sucrose and to produce and tolerate high concentrations of acids, which promote tooth demineralization. Three main cell-associated antigens (Ags) of *S. mutans* have been shown to be involved in the capacity of these microorganisms to adhere and accumulate in the dental biofilm. These antigens include an adhesin (AgI/II), glucosyltransferases (GTF) which synthesize glucan from sucrose, and glucan-binding protein B (GbpB) (21, 38, 39, 42). The biological role of these Ags in virulence has been demonstrated in animal models (3, 32, 35, 42) or in vitro studies of biofilm formation (24). Several studies have also demonstrated that induction of specific antibodies against each antigen can confer protection from dental caries development in animal models (16, 18, 39). The studies formed the basis for the use of these antigens in phase one clinical trials of caries vaccines in adults (16, 18, 39).

Although *S. mutans* can be detected in caries-free subjects, high proportions of these organisms in the dental biofilm are consistently associated with high caries activity (39). The earlier children become infected with *S. mutans*, the more likely they are to develop dental caries (19). Initial establishment of

*S. mutans* seems to be associated with the eruption of primary molars, which normally takes place between 19 to 30 months of age. It is thought that these teeth provide noncolonized and retentive surfaces for *S. mutans* biofilm formation (6). This period has been defined as a window of infectivity because after about 30 months of age there is a decreasing risk for *S. mutans* acquisition (6). It has been argued that this reduced risk of infection results from the establishment of a competitive commensal microbiota on tooth surfaces (6, 7). However, the influence of the maturation of the host immune system on this process is unclear. Although high sucrose intake can promote heavy *S. mutans* infection associated with severe caries (40), differences in sucrose consumption do not always result in different *S. mutans* infection levels and caries development. For example, within a high-sucrose-exposed population of nursery children, we have observed a small subset of heavily infected children from 24 to 30 months of age who did not develop the disease (23). In fact, in this subset, *S. mutans* levels were often subsequently reduced during a 1-year follow-up period (23). High fluctuations in *S. mutans* levels had also been observed in a population at low risk for caries after 30 months of age (31). Variations in immunological status and the virulence of *S. mutans* infecting genotypes may account for these observations.

Previous studies have indicated a high diversity of patterns of salivary immunoglobulin A (IgA) response to *S. mutans* Ags in children and adults (4, 5, 31). However, there has been no consistent evidence that differences in patterns of salivary IgA
specificities or intensity of response influence the susceptibility to *S. mutans* infection and caries development.

We hypothesize that the capacity to mount salivary IgA antibody responses to virulence-associated antigens early in life may influence the ability of *S. mutans* to infect or to accumulate to significant levels in the oral cavity. To address this hypothesis, we have characterized the intensity and specificity of salivary IgA levels to *S. mutans* antigens in a 1-year prospective study of 5- to 13-month-old children at high risk of infection. Subjects were drawn from a population with low socioeconomic status, high sucrose intake, and heavy exposure to *S. mutans* (23, 27). Specific patterns of IgA antibody response to *S. mutans* and *Streptococcus mitis* antigens were compared between 21 matched pairs of children who were either infected or not infected at an early age with *S. mutans*. The results suggest that early robust responses to *S. mutans* GbpB are associated with initial resistance to infection in this population.

**MATERIALS AND METHODS**

**Study population and design.** The study population included all the 5- to 13-month-old children who attended the 26 public nursery schools in the city of Piracicaba, São Paulo, Brazil (Escolas Municipais de Ensino Infantil [EMEIs]). Thus, at the time of the initial visits, a total of 160 children were enrolled at baseline. Because these institutions follow the same policy for care provision, children are provided with a nearly homogeneous sucrose-rich diet during the 10-h period that they stay in the EMEIs. The dietary schedules at the EMEIs consists of five meals provided at 2- to 3-h intervals, four of which include beverages and/or solid food with high concentrations of sucrose (for details, see reference 23). Exposure to fluoride and habits of oral hygiene are also quite similar between the EMEIs (23). Mothers of all children enrolled in the study have previously signed informed consents (approved by the Ethical Committee of the University of Campinas-School of Dentistry of Piracicaba, São Paulo, Brazil, proc. 110). These children are part of a prospective study with a 1.5-year follow-up, starting at the age of 5.5 months. At each visit to the EMEIs (every 3 months) until the age of 42 months, a total of 164 children remained in the study. Examination of these 141 children and collection of salivary and/or microbiological samples were performed at baseline (time zero [T₀]), at 6 months (T₆), and at 12 months (T₁₂) after baseline measurements. To analyze the influence of the salivary immune response on infection, a subset of 42 children consisted of 21 pairs of children in which one child was colonized at T₀ and another child was not colonized at T₀. The *S. mutans*-infected group included all the 21 children with detectable levels of *S. mutans* at T₀ and T₆. Noninfected children were sorted by several variables of putative influence on *S. mutans* infection levels to assemble the 21 pairs. Thus, children were matched by gender, age, number of erupted teeth, total levels of salivary IgA, and sucrose intake at home. If more than one noninfected child matched the respective *S. mutans*-infected subject, only one was selected randomly.

**Saliva sampling.** Samples of unstimulated whole saliva were collected from the floor of the mouth during the child’s daily residence at the EMEI, using sterile polypropylene graduated transfer pipettes. Collections were performed at least 1 h after feeding to avoid contamination with nonsalivary components. Approximately 200 to 500 μl of saliva was transferred to 1.5-ml tubes to which 10 μl of 250 mM EDTA had been added. Samples were placed on ice and processed within 1 h of collection. Salivas were centrifuged at 13,000 × g at 4°C for 10 min, and the supernatants were collected and frozen at −70°C until laboratory analysis. Total concentration of protein in salivas was determined by the method of Bradford to check for variations in salivary flow (Sigma, St. Louis, MO). Samples from children with mucosal breaks were excluded from the analysis.

**Determination of *S. mutans* and antigen preparation.** Oral samples were collected with sterile tongue blades and inoculated onto Rodac plates containing MSB (mitis salivarius agar supplemented with 0.2 U of bacitracin per ml and 20% sucrose; Difco, Sparks, MD) using a method previously described (20), with modifications (23). Brieﬂy, a sterile tongue blade was introduced in the mouth and rotated until humidified with saliva. Tongue blades were pressed by both sides of the dorsum of the tongue to remove excess saliva and were immediately pressed against the convex surface of the MSB agar. Plates were transported to the laboratory within a maximum of 1 h and incubated at 37°C in candle jars for 48 h to 72 h. The number of *S. mutans*-like colonies was determined using a stereoscopic microscope in a predetermined area (1.5 cm²) of the tongue blade impression. A total of eight isolates was picked from each plate, unless a lower number of colonies was obtained. Thus, one to eight colonies per child were selected. This number of isolates was selected based on previous studies that showed that testing more than eight isolates did not signiﬁcantly increase the likelihood of additional genotype detection (22). Attempts were made to select the colonies that were representative of the colonial morphologies observed. Isolates were picked from each plate, regrown, and stored at −80°C as previously described (20). The genotypic identity of the *S. mutans* isolates was determined by arbitrarily primed polymerase chain reaction (AP-PCR), also as previously described (25).

For antigen preparation, protein extracts were obtained from one *S. mutans* isolate representative of each AP-PCR genotype identiﬁed in each infected child. For this purpose, colonies from fresh cultures in Todd Hewitt agar were inoculated into tubes with 3 ml of Todd Hewitt broth (Difco) and incubated in candle jars for 18 h. Bacterial cells were then harvested from 1 ml of cultures previously adjusted to an A₅₆₀ of 1.0. Cell pellets were then boiled in Laemmli buffer for 5 min, and protein extracts were separated by centrifugation at 4°C (10,000 × g for 4 min). Protein concentrations were determined by the method of Bradford. A total of 16 μg of protein extract was separated in sodium dodecyl sulfate–6% polyacrylamide gels and stained with Coomassie blue R 250 (Bio-Rad, Hercules, CA) for evaluation of the protein profiles. The same procedures were performed with two strains of *S. mitis*, the clinical isolate 54EL1 and strain ATCC 903. The latter was selected as a control *S. mitis* standard applied in all immunoblotting experiments because it showed the highest number of protein bands.

**Determination of salivary immunoglobulin by ELISA.** Total levels of IgA were determined in capture enzyme-linked immunosorbent assays (ELISA) using microtiter plates (Costar 3590, Corning, NY) coated for 24 h at 4°C with 2 μg/ml of a polyclonal goat anti-human IgA in carbonate-bicarbonate buffer, pH 9.6, at 37°C. All antibody reagents were affinity purified and obtained from Zymed Laboratory (South San Francisco, CA). After being coated, plates were washed and blocked for 1 h at room temperature with bovine serum albumin (0.1%) in phosphate-buffered saline (PBS), pH 7.5. Diluted salivary samples (1:200 in PBS) were applied in triplicate, and plates were incubated for 2 h at room temperature. All experiments included serial dilutions (1:0, 0.5, 0.25, and 0.125 μg/ml) of a standard sample of human IgA antibody purified from serum (Sigma) and a standard sample of pools of saliva collected from one adult subject. The secondary antibody was biotin-conjugated goat IgA anti-human IgA (Sigma) at a dilution of 1:14,500. After incubation with a solution of streptavidin, conjugated with alkaline phosphatase (Sigma) (1:500 in PBS, pH 7.5), antibody reactions were revealed by incubation with the substrate p-nitrophenyl phosphate (Sigma) (1 mg/ml). After the reactions were revealed, plates were read (UV Max, Molecular Devices). Negative controls included uncoated, no saliva, and no primary antibody wells. For determination of IgA concentrations, absorbance values were plotted against the standard curve obtained for the serial dilutions of the purified human IgA within a linear range.

**Western blot analysis of salivary antibody to *S. mitis* and *S. mutans* antigens.** To analyze the influence of patterns of specificity of IgA response to *S. mitis* Ags in the levels of infection, Western blot assays were performed using saliva samples collected at T₀ and T₆. Salivas of both children in each pair were tested against Ags extracted from one isolate representative of each AP-PCR profile identified in the infected child of the pair. Ag extracts from a standard *S. mutans* strain (3VF2) were also included in all assays. Thus, the salivary response to *S. mitis* Ags was determined for at least two distinct genotypes. Salivas collected from six children infected by two to three isolates with distinct AP-PCR profiles were tested against Ags extracted from all genotypes identified. As a control for the maturation of immune response, Western blot assays were also performed to check the complexity of salivary IgA antibody response to *S. mitis* strain ATCC 903.

For Western blotting, a total 16 μg of protein of the Ag extracts was loaded per lane, separated by sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (26). After being stained with Red Ponceau (Sigma), membranes were washed, blocked overnight at 4°C (in Tris-buffered saline–Tween, pH 7.5, 5% nonfat milk). Incubations with salivas diluted 1:100 were performed at room temperature for 2 h. As negative controls, membranes were incubated only with blocking buffer, and as positive controls, membranes were incubated with a standard saliva sample obtained from an adult.
subject whose pattern of reaction with *S. mutans* and *S. mitis* antigen extracts had been previously measured. The secondary antibody was goat IgG anti-human IgA conjugated with horseradish peroxidase (1:40,000 dilution). Antibody reactions were developed using an ECL system (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). For this purpose, immunoblots were incubated with ECL detection solution and then exposed to the same X-ray film for 5 min. The developed X-ray films were scanned in a scanning densitometer (Bio-Rad GS-700 Imaging Densitometer) to analyze patterns of antigen recognition, including the number and intensity of reactive bands. Migration positions of AgI/II, GtfC, and GbpB in all Ag extracts were determined in parallel Western blot assays performed with rabbit antiserum specific to AgI/II (kindly provided by Michael W. Russell from the State University of New York at Buffalo, NY), monoclonal antibody against GtfC (kindly provided by Kazuo Fukushima from the University of Matsudo at Chiba, Japan), and specific polyclonal rat antisera to GbpB (38). To further strengthen the specificities of salivary IgA to GbpB, fast protein liquid chromatography-purified GbpB, obtained as previously described (30), was applied in blots and probed with representative saliva samples that have shown strong GbpB reactivity patterns in assays with Ag extracts. Similar experiments with purified GTP or AgI/II were not performed, because no significant differences in recognition of these Ags were detected in Ag extracts between the subsets of children, as later described, and because there was insufficient saliva volume for these assays.

**Statistical analysis.** Comparisons of the frequencies of children with distinct IgA antibody specificities were tested by a chi-square test. Differences in the densitometry values of reactive Ags between the subsets of infected and noninfected children were analyzed by a Mann-Whitney U test. To compare patterns of Ag recognition between distinct *S. mutans* genotypes, the number of coincident IgA-reactive bands between all the genotypes tested was determined. These values were divided by the number of the total IgA-reactive bands observed for the genotype that showed the highest number of IgA-reactive bands. The mean number of IgA-reactive bands in Ag extracts from *S. mitis* was also determined and compared between the subsets of the *S. mutans*-infected and noninfected children and with the mean number of reactive bands in Ag extracts from *S. mutans* genotypes.

**RESULTS**

**Salivary levels of IgA.** Figure 1 shows the fluctuations in the levels of salivary immunoglobulins observed at baseline (*T₀*) and at a 6-month follow-up (*T₆*). High variability in the concentration of total salivary IgA was observed among the 141 children from whom the 21 pairs of children were selected, even when concentration values were normalized by the total concentration of protein (Fig. 1). The mean value of IgA concentration at baseline was 97.5 μg/ml (standard deviation [SD], 91.5). A significant increase in IgA concentration was detected at *T₆* (mean, 473.6 μg/ml; SD, 195.7). Ratios of values of IgA normalized by protein concentration also increased from *T₀* (mean ratio, 0.15; SD, 0.15) to *T₆* (mean ratio, 0.50; SD, 0.30). Among the subset of 21 *S. mutans*-infected children, median levels of IgA were 79.7 and 498.8 μg/ml at *T₀* and *T₆*, respectively. These levels did not significantly differ from median levels of their noninfected pairs in both phases of the study (85.3 and 484.2 μg/ml at *T₀* and *T₆*, respectively).

**Mutans streptococcal infection and caries experience in the 21-pair subset.** At baseline, *S. mutans* was recovered from five (3.1%) children. After a 6-month follow-up (*T₀*), a total of 21 children (14.9%) presented detectable levels of *S. mutans*. The median age of infection was 17 months. In this subset of 21 children, *S. mutans* infection ranged from low levels (1 CFU) to heavy levels (>100 CFU) median, 40 CFU per predetermined area). Mean levels of *S. mutans* infection at *T₀* was 44.5 CFU/area (SD, 40.6). After the 1-year follow-up (*T₁₂*), six children from the infected group who previously carried 1 to 50 CFU of *S. mutans* showed no detectable levels of *S. mutans* (pairs 1, 2, 3, 4, 17, and 21) (Table 1). Also at *T₁₂*, five children from the noninfected group showed detectable levels of *S. mutans* (pairs 2, 3, 13, 14, and 21) (Table 1). At this time, *S. mutans* levels ranged from 23 to >100 CFU mutans streptococci, and the mean level of infection was 31.5 CFU/area (SD, 37.4). Percentages of *S. mutans*-infected children (*n* = 21) with caries lesions were 4.8 (*n* = 1), 23.8 (*n* = 5), and 61.9% (*n* = 13) at *T₀*, *T₆*, and *T₁₂*, respectively. Caries scores at *T₁₂* ranged from 1 to 7 cavities (mean, 0.70 ± 1.62) and 1 to 16 white spot lesions (mean, 2.14 ± 4.10). Within the noninfected group, no children presented signs of caries at *T₀*. At *T₀*, two (9.5%) children (numbers 7 and 13) presented with white spot lesions. At *T₁₂*, white spot lesions were detected only in children numbers 13 and 14, because lesions detected at *T₀* in child 7 had...
regressed. From this same group, child number 13 also presented with one cavity. At T\(_{12}\), children had a median of 16 erupted teeth; all 20 teeth were erupted in only six children (three from each subgroup).

**Complexity of IgA response to S. mutans and S. mitis antigens.** Significant complexity in IgA antibody responses to S. mutans Ags, as defined by the number of IgA-reactive bands, was identified in salivas of children as young as 6 months of age. The level of complexity varied among the children studied. The complexities of response at T\(_0\) or T\(_6\) were not associated with age nor with levels of mutans streptococci (Pearson’s r, \(-0.21\) to \(-0.10\); \(P > 0.40\), for the 3VF2 strain Ag extract). The number of IgA-reactive S. mutans 3VF2 bands ranged from 0 to 13 (mean, 7.2 ± 3.8) and 0 to 14 (mean, 6.3 ± 3.8) at T\(_0\) and T\(_6\), respectively. There were no significant differences in the mean number of reactive Ags between the subsets of infected and noninfected children at T\(_0\) and T\(_6\) (Mann-Whitney U test, \(P > 0.6\)).

**TABLE 1.** Total levels of salivary IgA, intensities of GbpB-specific IgA reactions, and percentage of intensities of IgA reactive to GbpB to the sum of intensities of total S. mutans-reactive IgA bands in pairs of S. mutans-infected and noninfected children at baseline and after a 6-month follow-up

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<th>IgA titer (µg/ml) at:</th>
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Noninfected pair

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\(a\) Children with non detectable levels of SM after 1 year.

\(b\) Children with detectable levels of S. mutans after 1 year.

\(c\) Intensities were determined by the densitometry of reactive bands.

\(d\) Densitometric values of IgA-reactive GbpB bands were divided by the sum of the densitometric values measured in all IgA reactive bands and expressed as percentage values.

\(e\) Second exam (T\(_6\)) was performed 8 months after baseline examination.
The number of IgA-reactive *S. mitis* bands ranged from 0 to 17 (mean, 7.2 ± 4.7), and from 1 to 19 (mean, 8.2 ± 4.2) at T₀ and T₆, respectively. The degree of complexity of response to *S. mutans* was associated with the complexity of response to *S. mitis* both at T₀ (Pearson’s r, 0.37; P < 0.02) and at T₆ (Pearson’s r, 0.40; P < 0.02). Comparison of patterns developed with salivary IgA antibody to *S. mutans* Ags and *S. mitis* Ags at T₀ and T₆ revealed that specificities to these two oral species were not related, except for a single *S. mitis* Ag of approximately 44.9 kDa that was frequently recognized when GbpB-reactive IgA was detected in the same saliva (Fig. 2). This reactivity with the 44.9-kDa *S. mitis* component was not due to cross-reactive antibodies to *S. mutans* GbpB, because polyclonal antibodies to GbpB did not recognize the smaller *S. mitis* Ag (Fig. 2D).

**Antigenic variations among *S. mutans* genotypes.** Potential differences in salivary IgA antibody reactivity with Ags from indigenous versus stock strains of *S. mutans* were analyzed comparing the numbers of IgA antibody-reactive bands of the same size between each infecting genotype (isolated from the infected child) and the standard 3VF2 strain. Median percentage values of coincident bands to total number of bands identified were 77.5 and 67.0% for T₀ and T₆, respectively. Examples of immunoblots comparing the standard and one infecting *S. mutans* strain per infected subject are shown in Fig. 2. In addition, patterns of response were compared between strains representative of each infecting *S. mutans* genotype in the six children who were infected by two to four distinct genotypes, as identified by AP-PCR (Fig. 3). Although some differences in patterns were observed between genotypes, reactions to Ags AgI/II, GtfC, and GbpB were very similar in frequency and intensity, even when slight differences in migration occurred, as, for example, the GbpB from genotype *S. mutans* 7 recovered from child number 17 (Fig. 3). Also the total numbers of reactive bands of the standard and infecting genotypes were highly associated both at T₀ and at T₆ (Pearson’s r, 0.81 [P < 0.001] and 0.69 [P < 0.001], respectively).

**Specific antibody levels to AgI/II, GtfC, and GbpB.** Identities of AgI/II, GtfC, and GbpB in Ag extracts from all the *S. mutans* genotypes tested were confirmed in parallel Western blot assays using the respective specific antibodies. Representative
immunoblots where these Ags were probed with specific antibodies are shown in Fig. 4. Variation in the migration patterns was higher for AgI/II (Fig. 4A). Sizes of this Ag were between 176.6 to 200.5 kDa, as calculated on the basis of standard proteins. GtfC migrations were less variable, with approximate sizes of 158.9 kDa, while GbpB sizes varied from 62.9 to 58.5 kDa. Besides differences in migration, variations in the intensity of reactivity were also observed.

FIG. 4. Representative gels for localization of AgI/II, GtfC, and GbpB in protein extracts from distinct S. mutans genotypes. A total of 16 µg of protein was loaded per lane, and membranes were probed with rabbit antiserum specific to AgI/II (A), monoclonal antibody P32 anti-GtfC (B), or rat antiserum specific to GbpB (C). Each lane represents a distinct genotype of S. mutans. Molecular sizes (kDa) are indicated at left. AgI/II was the most variable in migration pattern, with approximate sizes ranging from 180.3 to 200.5 kDa; the most common size was 194 kDa. GtfC proteins show approximate sizes of 158.9 kDa. GbpB was about 60 kDa, although in the protein extract at lane 9 it has migrated to a position of approximately 58.5 kDa.

GtfC proteins were approximately 158.9 kDa. GbpB proteins were of approximately 60 kDa. Note that GbpB from genotype S. mutans 7 migrated to a position calculated as 58.5 kDa.
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TABLE 2. Comparisons of the frequencies of children with positive salivary IgA responses to Ags from distinct S. mutans genotypes and to S. mitis at baseline (T₀) and after a 6-month follow-up (T₁₂) between S. mutans-infected and their respective noninfected matches and mean number of Ags recognized by each group at phases T₀ and T₁₂.

<table>
<thead>
<tr>
<th>Strain and antigen extract</th>
<th>No. (%) of children with positive salivary IgA response at T₀ (n = 21)</th>
<th>No. (%) of children with positive salivary IgA response at T₁₂ (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Noninfected</td>
</tr>
<tr>
<td>S. mutans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag I/II</td>
<td>19 (90.4)</td>
<td>17 (81.0)</td>
</tr>
<tr>
<td>Gfts</td>
<td>17 (81.0)</td>
<td>17 (81.0)</td>
</tr>
<tr>
<td>GbpB</td>
<td>8 (38.1)α</td>
<td>16 (76.2)α</td>
</tr>
<tr>
<td>Mean number of reactive Ags ± SD</td>
<td>7.6 ± 3.6</td>
<td>6.9 ± 4.0</td>
</tr>
<tr>
<td>Infecting genotypeb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag I/II</td>
<td>18 (85.7)</td>
<td>16 (76.2)</td>
</tr>
<tr>
<td>Gfts</td>
<td>18 (85.7)</td>
<td>16 (76.2)</td>
</tr>
<tr>
<td>GbpB</td>
<td>8 (38.1)α</td>
<td>16 (76.2)α</td>
</tr>
<tr>
<td>Mean number of reactive Ags ± SD</td>
<td>6.4 ± 2.8</td>
<td>6.5 ± 3.9</td>
</tr>
<tr>
<td>S. mitis</td>
<td>44.9 kDa Ag</td>
<td>12 (57.1)</td>
</tr>
<tr>
<td>Mean number of reactive Ags ± SD</td>
<td>6.7 ± 4.7</td>
<td>7.6 ± 4.7</td>
</tr>
</tbody>
</table>

α Differences between infected and noninfected groups: chi-square = 4.76, P < 0.02
β Only the genotype with highest number of IgA-reactive bands was selected for the analysis among the two or more infecting genotypes tested in children infected by multiple genotypes.

DISCUSSION

S. mutans is transmitted to children through contact with saliva from infected individuals. These bacteria can be detected using culture methods by 19 to 30 months of age, with a median age of approximately 26 months (6, 31). In populations at high risk for caries, S. mutans can be detected in high frequency by the end of the first year of life (23, 27) or even in preBondet-5-6-month-old children (28). In severe cases of caries development, mutans streptococci may comprise up to 60% of the total cultivable organisms in the dental biofilm (40). In this study, we analyzed a population defined as heavily exposed to S. mutans, because of previous studies showing a high prevalence of early and heavy infection associated with caries incidence (23, 27) and earlier data indicating early S. mutans transmission from maternal (17) and nonmaternal (25) sources. Additionally, a sucrose-rich diet was provided in the nurseries during the 10-h period of day care (23). Caries lesions were detected at a very young age in the early S. mutans-infected group; 62% of children presented 1 to 17 caries lesions by T₁₂, within an age range where teeth had not
completed or had nearly completed eruption. The median age of
the initial S. mutans detection in the infected group was 17
months, which is lower than that reported in other populations.
Except for the first five children of the infected group (from
pairs 1 to 5) (Table 1) whose median age of infection was 11
months, all other S. mutans detections occurred at the second
exam (T2) at a median age of 17.5 months. This enabled us to
analyze specific IgA antibody responses in the earliest phases
of S. mutans challenge that potentially would lead to infection.

The establishment of competitive commensal bacteria on teeth
has been associated with decreased risk for S. mutans coloni-
zation (7) and may be part of the reason for the low rate of
initial acquisition of S. mutans after 2 to 2.5 years of age (6, 19,
23, 31). Reduced risk for acquisition of gram-positive and
gram-negative pathogens in other mucosal areas, e.g., Strepto-
coccus pneumoniae, Haemophilus influenzae, and Moraxella ca-
tarrhalis, has been reported also to occur by the age of 2 years
(12, 13, 15). Maturation in mucosal immune responses during
this period was suggested to influence the colonization of these
pathogens (12, 15). Significant increases in IgA levels observed
from T0 to T2 (Fig. 1) were likely due to physiological matu-
rature of the mucosal immune system.

Studies in older children and adults revealed a tendency for
higher intensity and more complex patterns of IgA reaction to
S. mutans Ags among low-infected and/or low-caries-active
subjects in comparison to caries-active ones (4, 5). However,
no specific pattern of IgA response was associated with sus-
ceptibility to infection. Because the degree of Ag challenge can
influence the development of adaptive immune responses,
variations in the intensity and duration of exposure to S. mu-
tans in adults might interfere, in part, in the analysis of the
independent influence of the immune response on infection in
adult subjects. The intensity of the IgA antibody response to
every common colonizers of the oral cavity has been shown to
increase from birth to 4 years of age (37), which may reflect the
increasing extent of cumulative antigenic challenge (37). On
the other hand, IgA antibody reactivity with several oral bac-
teria species in childhood is reported in the absence of detect-
able levels of the respective species (1, 11, 31). Several hypoth-
eses were raised to address this observation, including cross-
reactive antibodies, anti-idiotypic induction, and antigenic
expression due to colonization of other mucosal sites, as well as
the occurrence of transitory infection and variations in culture
techniques (11, 37). Studies of salivary IgA antibody responses
to known species-specific Ags involved in virulence and colo-
nization may help to simplify associations between antibody
response and S. mutans challenge and infection.

Three main groups of S. mutans surface Ags (AgI/II, GTF,
and GbpB) have been shown to induce protective immunity
against dental caries in animal models (16, 18, 39), but whether
such responses can interfere with S. mutans infection or patho-
genesis in humans is unresolved. In the present study, we show
that an intense and complex pattern of salivary IgA responses
to S. mutans Ags can be naturally achieved before 1 year of age
(Fig. 2 and Table 1) in children who are under heavy challenge
with S. mutans. We also observed that occurrence of salivary
IgA antibody specific to S. mutans Ags, including AgI/II, GTF,
and GbpB, is independent of detectable infectious levels (Ta-
ble 2). The patterns of IgA antibody reactivity with S. mutans
Ags were robust and comparable to the intensity of reactions
to Ag extracts from S. mitis, a pioneer colonizer of the oral
cavity (33). These findings differ from a previous study per-
formed in children in whom median S. mutans initial acqui-
sition occurred at 27.5 months of age (31). In that study, S. mu-
tans-specific IgA responses achieved a significant degree
of complexity (at least six distinct reactive bands) generally by
28 to 34 months of age, although salivary IgA responses to S.
mitis Ags were well developed within the 13 to 23 months of
life. The absolute levels of IgA in the saliva tested were some-
what higher than observed in other reports (9). This may be a
consequence of the use of a monomeric serum IgA (this study)
rather than a dimeric secretory IgA standard. The much earlier
and more intense challenge with S. mutans seen in the present
study may partially explain the significantly earlier develop-
mant of salivary IgA responses. Apart from these population
differences, both studies suggest that persistently low levels of
infectious challenge beneath the sensitivity of the bacterial culture
techniques are sufficient to induce an S. mutans-specific secre-
tory IgA antibody response, since both studies described com-
plex S. mutans-specific IgA patterns in subjects whose infec-
tions were nondetectable. The sensitivity thresholds of sampling
and culturing methods may limit detection of transitory or
low-level S. mutans genotypes. It should also be noted that the
application of a highly sensitive chemiluminescent method for
antibody detection may have improved our ability to identify
more intense and complex patterns of S. mutans-specific IgA.

An important insight provided by this study of matched
infected and noninfected children is that the degree of S.
mutans initial infection is not associated with intensity and
complexity patterns of response but, rather, with the specific-
ities of IgA to antigens relevant to S. mutans establishment in
the dental biofilm. In this regard, GbpB-specific IgA appears to
have a unique role in modulating the level of infection both in
frequency and in intensity of response (Tables 1 and 2). In
several children GbpB-specific IgA antibody accounted for
more than 50% of the total S. mutans reactive IgA (Table 2),
a trait that was more frequently detected in the noninfected
group. GbpB was previously described as an immunodominant
protein in children from 28 to 51 months of age (31) in adults
(30) from the United States and in adults from Taiwan (where
this protein is referred to as secreted antigen A) (8). Express-
ion levels of GbpB are associated with the capacities of dis-
tinct S. mutans genotypes to grow as biofilms (24). Several
polymorphisms have been described in GbpB (24) as well as in
GTF genes (26), which might reflect antigenic variations that
would facilitate infecting S. mutans genotypes to evade specific
immune responses. However, significant differences in the pat-
terns of specificity and intensity of IgA response when Ag
extracts from distinct S. mutans genotypes were compared were
not frequent (Table 2 and Fig. 3). No homologues of GbpB were
identified in the commensal species of the oral cavity sequenced so far (e.g., Streptococcus sanguinis, Strepto-
coccus gordonii, and S. mitis), or in the Streptococcus sobrinus
species. On the other hand, GbpB homologues were identified
in several pathogenic gram-positive species, e.g., S. pneu-
moniae, group B streptococcus, and Enterococcus faecium (24),
and it would be interesting to analyze how the response to
GbpB may interfere with infection by these other pathogens.

Children from populations that are modestly challenged
with S. mutans display salivary IgA antibody specific for GTF
S. mutans. We also observed that occurrence of salivary
IgA antibody specific to S. mutans Ags, including AgI/II, GTF,
and GbpB, is independent of detectable infectious levels (Ta-
ble 2). The patterns of IgA antibody reactivity with S. mutans
Ags were robust and comparable to the intensity of reactions

purified from S. mutans (14, 31). However, several commensal streptococci of the oral cavity produce variable numbers of GTF isotypes with distinct degrees of sequence similarity to the three GTF produced by S. mutans (GtfB, GtfC, and GtfD) (2). Differences in levels of S. sanguinis and S. mutans GTF-reactive antibodies within children indicate, however, that modest cross-reaction occurs at least between the counterparts produced by these two species (14). In the present study, close to 90% of the 42 studied children displayed salivary IgA antibody reactive to GTF. The lack of association between infection and the GTF-specific antibody response might be because the naturally induced GTF antibodies might have specificity for epitopes irrelevant for enzymatic activities. It has been shown that functionally relevant GTF peptides used in subunit anticaries vaccines are only modestly recognized by naturally induced human salivary IgA (35). We cannot exclude the possibility that IgA-reactive bands may include antibody reactions to separate proteins with equal migration patterns, especially with GtfC and AglII, for which we did not test representative salivas against the purified forms of these Ags. AglII was frequently recognized by the salivary IgA antibody of our study population despite the presence of infection. Some epitopes of AglII have been shown to be cross-reactive to human IgG domains, and several commensal species of the oral cavity, e.g., S. sanguinis and Streptococcus oralis, produce homologues that share 57 to 73% of identity to AglII.

Associations between the specificity of secreted IgA to initial acquisition of bacterial pathogens of the mucosa suggest the role of mucosal immunity in regulating the persistence of pathogens of the nasopharynx (12, 13, 41). For example, a peak of immune response to a potential vaccine target of H. influenzae, the outer membrane protein P6 (29), was detected early in life (1 to 24 months) among children with short persistence of colonization, while low response to P6 occurred in children colonized for several distinct strains during extended periods (12). Persistent and recurrent colonization by non-typeable H. influenzae strains in otitis-prone children was also associated to a poor local immune response to P6 (41). The reasons for the differences in patterns of early response to GbpB remain to be determined, and one hypothesis is that distinct haplotypes of major histocompatibility complex class II may be related to variations in the presentation of functionally important and immunogenic GbpB epitopes to Th cells. Bioinformatic analysis of GbpB sequence has revealed several peptides of putative high binding affinity to distinct human major histocompatibility complex class II alleles, and at least one of these peptides has shown remarkable protective effects in caries vaccine experiments in animal models (32).

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