Age-Specific Salivary Immunoglobulin A Response to *Streptococcus mutans* GbpB[▽]

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In a follow-up study of children infected with *Streptococcus mutans* at an early age (children previously shown to respond poorly to *S. mutans* GbpB), there was a delay in their immune response, rather than a complete inability to respond to this antigen. Epitopes in the N-terminal third of GbpB were identified as targets for naturally induced immunoglobulin A antibody in children at an early age.

Streptococcus mutans is a major dental caries pathogen in humans. Experimental immunization with *S. mutans* antigens involved in the adherence/accumulation of bacteria to tooth surfaces (AgI/II, glucosyltransferases, and GbpB) has been shown to induce antibody responses that interfere with *S. mutans* infection and caries development in animal models (for reviews, see references 7 and 8). However, the influence of the immune response in the initial establishment of *S. mutans* in children remains poorly understood. We have previously shown that children infected by *S. mutans* at an early age (mean of 17 months) tend to demonstrate a weak salivary immunoglobulin A (IgA) antibody response to GbpB, while saliva samples of uninfected children in the same population (matched for several factors of putative influence on infection and/or IgA response) often contain high levels of GbpB-reactive IgA antibody (6). Thus, natural salivary IgA antibody responses to GbpB at an early age may account, in part, for resistance to *S. mutans* infection. However, the conditions for such early IgA responses to GbpB remain unclear. GbpB was shown to be an immunodominant antigen in adults (9) and children (10), although the patterns of IgA specificities to *S. mutans* antigens vary significantly, even in siblings (10). We hypothesize that individual patterns of *S. mutans* antigen epitopes presented on major histocompatibility complex (MHC) class II receptors to Th cells might account for some of these differences. In the present study, we continued to monitor for up to 1 year the patterns of salivary antibody reactivity with *S. mutans* antigens in children who had been investigated in the original study (6) and screened for salivary IgA antibody to six putative immunodominant GbpB epitopes, selected on the basis of MHC class II human allele binding.

The study population included 119 of the 160 children enrolled in the original study (6) in which the IgA immune responses had been analyzed, beginning at the ages of 5 to 13 months (baseline; time zero [T0]) and 6 months thereafter (11

to 19 months; T6). In the present study, the levels of *S. mutans* infection and patterns of salivary antibody response to *S. mutans* and *Streptococcus mitis* (a control oral organism) in these children were analyzed for a subsequent year at 6-month intervals (T12 and T18). Thus, at the end of the study, children were between 23 and 31 months of age. Clinical and microbiological exams for diagnosis of caries lesions and levels of *S. mutans* infection, respectively, were performed as previously described (6). Briefly, oral samples collected with sterile tongue blades were inoculated onto Rodac plates containing MSB (Difco, Sparks, MD) with 0.2 U bacitracin per ml and 20% sucrose. After incubation (at 37°C in candle jars for 48 h), the number of *S. mutans*-like colonies was determined using a stereoscopic microscope on a predetermined area (1.5 cm^2) of the tongue blade impression. The levels of *S. mutans* infection were then expressed as CFU/area. To analyze the influence of IgA response on early *S. mutans* infection, a subset of 21 early *S. mutans*-infected children were matched to 21 uninfected children as described elsewhere (6). From these initial 21 pairs, 15 complete pairs remained until the end of the study. Saliva samples collected at T12 and T18 were clarified and frozen at −70°C as described before (6). Extracted proteins (16 μg) from boiled cells of *S. mutans* strain 3VF2 and *S. mitis* strain ATCC 903 were separated on sodium dodecyl sulfate-6% polyacrylamide gels and stained with Coomassie blue R250 (Bio-Rad, Hercules, CA) to check protein profiles, and the same batch of the antigen extract was subjected to Western blot assays for all the study phases (T0 to T18). All these assays were performed exactly as in the previous study (6). Interassay variability was controlled using a standard saliva sample obtained from an adult subject that was reassayed in all the study phases (T0 to T18). Thus, the results of Western blot assays performed in the two initial study phases (T0 and T6; previously published in reference 6) could be compared with those of the subsequent phases (T12 and T18).

The total levels of IgA, IgA1, and IgM were determined in capture enzyme-linked immunosorbent assays (ELISAs) using microtiter plates (Costar 3590; Corning, NY) coated for 24 h at 4°C with 2 μg/ml goat IgG anti-human IgA or 2 μg/ml goat IgG anti-human IgM in carbonate-bicarbonate buffer, pH 9.6. All antibody reagents were affinity purified and obtained from

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Zymed Laboratories (South San Francisco, CA). ELISAs were performed as previously described (6), with the difference that secondary antibodies for IgA1 and IgM were mouse IgG anti-human IgA1 (1:500 dilution) and mouse IgG anti-human IgM (1:500 dilution), respectively. For these isotypes, reactions with biotin-conjugated goat IgG anti-mouse IgG (1:10,000 dilution) were followed by overnight incubation with alkaline phosphatase-streptavidin (Sigma) (1:500 in phosphate-buffered saline [PBS], pH 7.5). All reactions were revealed by incubation with p-nitrophenyl phosphate disodium (12), and plates were read in an ELISA plate reader (VersaMax; Molecular Devices) to obtain A_{405} units. The same antibody reagents and standard dilutions were used for the samples collected in all the study phases (T0 to T18).

The patterns of reactivity of IgA antibody with *S. mutans* and *S. mitis* antigen extracts were determined in Western blot assays using saliva samples collected at T0 and T6 (previously published [6]) and T12 and T18 for the remaining subset of 15 pairs of infected and uninfected children. Western blots were performed as detailed in the previous study (6), using the ECL system (Amersham Biosciences, Little Chalfont, United Kingdom) followed by membrane exposure to X-ray films. Densitometric values of GbpB and other *S. mutans* antigens reactive with IgA were obtained by analysis of the digitized X-ray images using a scanning densitometer (Bio-Rad GS-700 imaging densitometer). Measures of intensity were expressed as arbitrary units (au), and comparisons were made between eight pairs of infected and noninfected children out of the 15 pairs analyzed (6). Seven of the 15 pairs were excluded from comparisons because the initially infected and/or noninfected child of the respective pair did not sustain their *S. mutans* infection status during the follow-up period (see below).

Sequences that have a high probability of MHC class II binding may reflect epitopes likely to be involved in the induction or regulation of immune responses (4). Thus, six GbpB-derived peptides of predicted MHC class II binding were identified (11) and assayed against saliva samples (see below) in order to identify GbpB epitopes responsible for the observed IgA antibody responses at T0 and T6. Since one or both saliva samples for three pairs of children were insufficient for assay, only 18 pairs of children, out of the initial 21 pairs, were analyzed (T0 to T6) for antiepitope activity. Saliva samples were diluted 1:20 in PBS-BN (PBS containing 1% bovine serum albumin [Sigma] and 0.05% sodium azide [pH 7.4]) with 2.0% Prionex stock (PentaPharm Ltd., Basel, Switzerland), decomplemented at 56°C for 30 min, followed by adsorption onto and then elution (by centrifugation) from Wekcell cellulose sponges to reduce mucin content. Salivary IgA reactivities with GbpB peptides were tested using a particle-based multiplex fluorescence immunoassay (Luminex Corp., Austin, TX). Fluorescently tagged microspheres were coated with GbpB purified by fast-performance liquid chromatography or with one of the following GbpB-derived peptides: SYI (113-KSNA ATSYINAIINSKSVSD-132), QGQ (52-KHKLITIQGQVSA LQTQQAG-71), VAR (92-QTLSSKIVARNESLKQQARS AQ-111), QAA (252-QAAQAQAAANNNNTQATDA-270), ANY (390-YVTGVQGGQIQVQEANYAG-409), or SIG (412-SIGNYRGWFNPGSVSYIYPN-431). Mixtures for assay contained (i) a combination of all peptide-coated microspheres or (ii) GbpB-coated microspheres. Then 50-μl portions of the
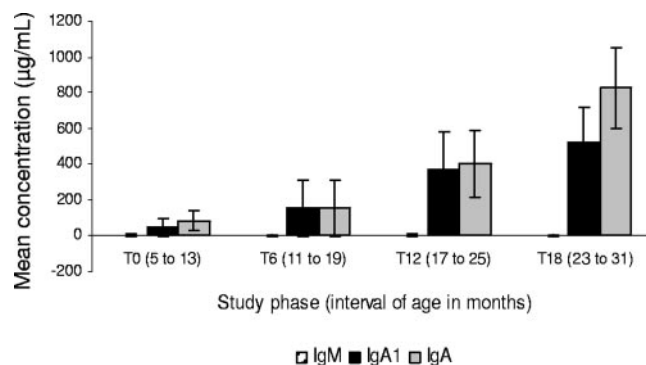

FIG. 1. Levels of salivary IgM and IgA1 and total IgA observed during a 1.5-year longitudinal study of 119 children. Immunoglobulins were measured by ELISAs at T0 (baseline) and T6, T12, and T18 for 6-, 12-, and 18-month follow-up analysis.

bead mixtures were added to the wells on 1.2-μm-pore-size filter plates (Millipore Corp., Bedford, MA). Microspheres were then washed twice with PBS-BN under vacuum and resuspended in 50 μl of the same buffer. Fifty microliters of each diluted saliva sample (1:8 in PBS-BN) was then added and incubated at 37°C with shaking for 120 min. After the buffer was drained under vacuum, microspheres were incubated with a 1:500 solution of goat anti-human IgA (Jackson Immunoresearch) in PBS-BN for 30 min at 37°C. Microspheres were then incubated with a 1:250 solution of R-phycoerythrin-conjugated donkey anti-goat IgG (Jackson Immunoresearch) for 30 min at 37°C. All incubations were performed in the dark. After the samples were washed, they were resuspended in PBS-BN and read in a Luminex 100 analyzer. Results were reported as median fluorescence intensity (FI).

Figure 1 shows changes in the mean levels of salivary IgA, subclass IgA1, and IgM in the 119 studied children. The IgA1 subclass accounted for the majority of the salivary IgA in all phases (518.76 ± 198.0 μg/ml, mean ± standard deviation at T18), but the proportion of IgA1 in relation to total IgA decreased at T18. The levels of IgM were minimal throughout the study.

The *S. mutans* infection status changed in several infected and noninfected children during the course of the study. Among the 15 children in whom *S. mutans* infection was initially detected (infected group), *S. mutans* could not be detected in five of these subjects at either T12 or T18. The mean *S. mutans* infection level of these five children at T6 was 26.5 CFU/area (±41.5), and the range of salivary IgA antibody reaction with GbpB in Western blot assays varied from undetectable to 342.0 au at T6 (median, 35.6 au). In addition, at T6, 4 of the 15 children in whom *S. mutans* was initially undetectable became detectably infected (59.3 ± 36.3 CFU/area [T12] and 30.5 ± 46.5 CFU/area [T18]). Reactivity with GbpB in these children ranged from undetectable to 399.7 au (median, 53.8) at T6. To simplify the analysis, pairs that included children whose *S. mutans* infection status changed from T0 to T6 were excluded, i.e., comparisons of the IgA reactivities were performed on the eight pairs of infected and noninfected children who sustained their status of infection during the complete study period (T0 to T18).

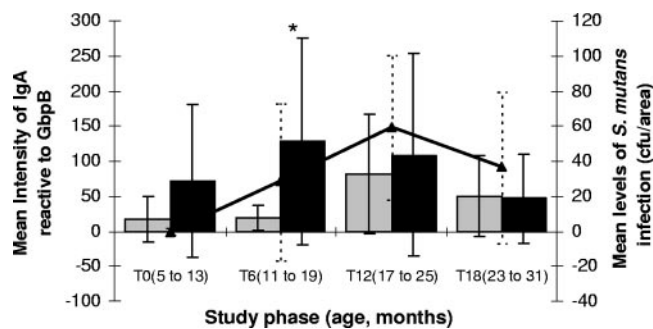Carious lesions were detected at T12 and T18 in three

FIG. 2. Comparisons of the mean intensities of Western blot reactivity of salivary IgA antibody with native GbpB between the subset of eight infected children (shaded bars) and their noninfected counterparts (black bars). The mean levels of *S. mutans* infection observed in the infected group are indicated (▲; see the right-hand *y* axis). The solid and dashed error bars show the standard deviations for the IgA intensities of the reaction and levels of *S. mutans* infection, respectively. Significant differences in median values between the infected and uninfected groups are indicated by the asterisk (Mann-Whitney U test, $P = 0.028$).
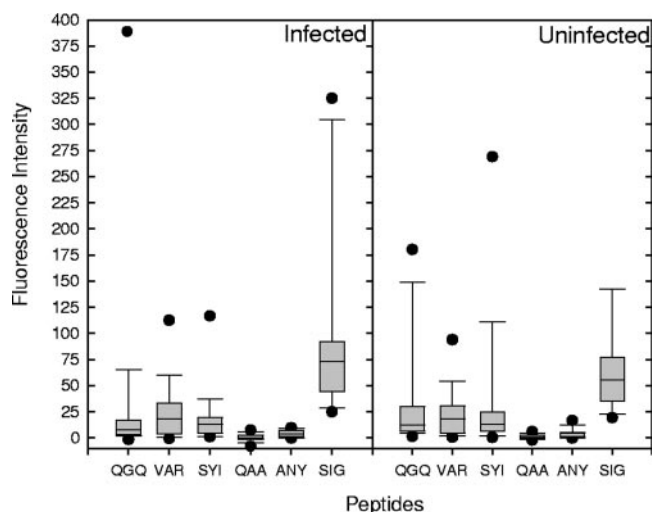

FIG. 3. Box plots of FI of salivary IgA antibody reactivity with microspheres coated with 20-mer GbpB-derived peptides. Saliva samples were obtained from *S. mutans*-infected ($n = 18$) and nondetectably infected ($n = 18$) children at T0. Median values are indicated by the horizontal lines within the boxes. The boxes span the 25th and 75th percentiles, while the error bars subsume the 90th and 10th percentiles and solid black circles indicate the 5th and 95th percentiles.

(37.5%) and four (50.0%) children, respectively, in the initially infected group. The four children affected at T18 included the same three with caries lesions at T12. The mean numbers of caries lesions were 0.6 ($\pm$1.1) and 1.4 ($\pm$1.9), respectively, at these time intervals. None of the noninfected children showed lesions during the end of the study. Both groups showed similar increases in total salivary IgA protein at T6 to T18 (infected group, 455.8 $\pm$ 173.5 and 932.0 $\pm$ 298.5 μg/ml, respectively; noninfected group, 469.9 $\pm$ 144.8 and 866.3 $\pm$ 294.7 μg/ml, respectively).

Shown in Fig. 2 are the salivary IgA antibody levels to *S. mutans* GbpB throughout the study period. The median intensities of salivary IgA antibody levels reactive with GbpB were significantly higher at T6 in the noninfected group compared with the infected group (Mann-Whitney, $P < 0.05$). No significant differences were observed, however, between the overall levels of IgA antibody reactivity with *S. mutans* and *S. mitis* antigen extracts in either group (data not shown). Interestingly, at T18, the mean level of GbpB-reactive IgA antibody had declined from the peak response in the noninfected group. The IgA antibody response to GbpB was delayed in the infected group. By T12, the saliva samples of many infected children contained detectable antibody to GbpB, although as a group this increase in intensity was not statistically significant. The levels of *S. mutans* infection in the infected group also tended to increase from T0 to T12, although no significant variability was seen in the level of infection in this time frame. At the later collection points, the distributions of anti-GbpB antibody intensities were similar in the infected and noninfected groups. Thus, the data seem to suggest that the infected group's immune response to *S. mutans* GbpB was delayed compared with that observed in the noninfected group. Reasons for this apparent delay remain to be explained, but we speculate that the GbpB-specific IgA responses that developed in the infected children at T12 were due to the increased *S. mutans* antigenic challenge, as reflected by the increasing *S. mutans* levels of infection at the same period (Fig. 2). Interestingly, after the increased IgA response to GbpB in the infected group, *S.*

mutans infection levels tended to decrease or at least to stabilize (from T12 to T18) (Fig. 2). Thus, vaccination approaches to direct patterns of IgA response to *S. mutans* protective epitopes before or at the very initial challenge for *S. mutans* infection might be very helpful to prevent infection at an early age. On the other hand, GbpB homologues have been found in other pathogens and commensal organisms of the mucosa, e.g., *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Enterococcus faecium*, and *Streptococcus sanguinis* (5, 13). Thus, it will be important to analyze how levels of IgA reactive with GbpB homologues are associated with the initial colonization by these organisms and to identify cross-reactive epitopes.

To gain insight into the GbpB epitope-specific nature of the initial mucosal immune responses to this protein, salivary IgA antibody reactive with six 20-mer GbpB peptides was measured by the Luminex fluorescence assay. Figure 3 summarizes the salivary IgA antibody reactivity to these peptides observed in saliva samples from infected and noninfected children taken at T0. Significant levels of salivary IgA antibody activity were observed for three peptides in the N-terminal third (VAR, QGQ, and SYI) and one peptide (SIG) at the C terminus of GbpB. Salivary antibody levels to the VAR and QGQ peptides were significantly correlated (Spearman rank) at T0 with antibody levels to GbpB ($r^2 = 0.404$ and $P < 0.015$ and $r^2 = 0.357$ and $P < 0.026$, respectively), strengthening their relationship to GbpB-induced responses. No obvious differences were observed, however, between salivary IgA antipeptide levels in infected versus noninfected children at T0 (Fig. 3) or T6 (Fig. 4). Although not reaching statistical significance, the median salivary IgA antibody levels to intact GbpB (measured in the multiplex assay) in noninfected children were higher (102 FI and 128 FI, respectively) at T0 and T6 than those of infected children (48 FI and 94 FI, respectively), paralleling the GbpB-reactive antibody levels observed on Western blots.
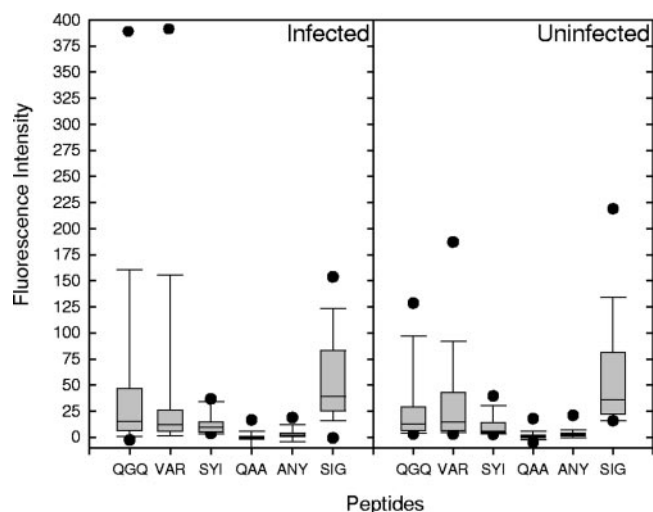
FIG. 4. Box plots of FI of salivary IgA antibody reactivity with microspheres coated with 20-mer GbpB-derived peptides. Saliva samples were obtained from *S. mutans*-infected children (*n* = 18) and children with nondetectable infection (*n* = 18) at T6. Median values are indicated by the horizontal lines within the boxes. The boxes span the 25th and 75th percentiles, while the error bars subsume the 90th and 10th percentiles and solid black circles indicate the 5th and 95th percentiles.

The oral cavity has a highly diverse indigenous microbiota that increases in complexity as teeth erupt in the mouth (1, 3, 10). The effect of the salivary IgA response on the establishment of commensal and pathogenic species could provide important insights to understanding susceptibility to infection and directing suitable immunization strategies to control pathogen infection. Previously, we have shown that the presence and intensity of salivary IgA antibody reactivity to GbpB measured on Western blots during the first 12 to 18 months of life are negatively associated with *S. mutans* infection (6). In this study we show that GbpB-specific salivary antibody responses, again measured on Western blots, continue to increase, but are self-limiting in both *S. mutans*-infected children and children with nondetectable infection. Interestingly, the decrease in salivary IgA antibody to GbpB seen in noninfected children at T18 occurred during a phase of apparent maturation of the IgA response, as indicated by a reduction in the proportion of IgA1 subclass in relation to total IgA at this time (Fig. 1) which is more typical of older children and adults (2, 12).

To investigate whether differences in the intensities of response to GbpB between infected and noninfected children would be associated with distinct patterns of GbpB epitope recognition, we selected saliva samples collected at the study phases T0 and T6 (Fig. 2) where differences in salivary IgA antibody reactive with the native GbpB protein were more striking. Although Luminex measurements of saliva samples suggested that noninfected children had somewhat higher levels of IgA antibody to GbpB N-terminal peptides than infected children, these differences at T0 and T6 were not as remarkable as those observed in the Western blot assays for GbpB (Fig. 2). It is possible that GbpB reactivity detected in the Luminex assays reflects only external hydrophilic parts of the protein or antigenic determinants dependent on the GbpB

native conformation. Since Western blots use denatured GbpB, this assay might reflect a higher/different array of reactive GbpB epitopes than are exposed when protein is attached to microspheres.

Analysis of salivary IgA antibody reactivity revealed that the region between GbpB residues 52 and 132 (peptides QGQ, VAR, and SYI) contained several linear epitopes to which children apparently responded following initial *S. mutans* challenges. Furthermore, two of these "responses" were significantly correlated with salivary IgA antibody levels to GbpB. These data indicate that subunit vaccines based on GbpB sequence should contain some or all of this sequence. Also, tracking antibody responses to epitopes in this region may correlate with early exposure to *S. mutans*, since these antibody specificities were observed in saliva samples of children in whom *S. mutans* had not yet been detectably incorporated into the oral biofilm.

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