Human Serum Antibody Response against *Streptococcus mutans* Antigens

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Antigens from *Streptococcus mutans* were examined to identify specific polypeptides that may have stimulated antibody responses and possibly play some role in caries immunity. A group of 10 adult human subjects was screened for serum antibodies reactive with antigens from *S. mutans*. Extracellular and cellular protein preparations from *S. mutans* LM7 (Brathall serotype c) and V403 (biotype c) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western electrophoretic transfer and immunoblotting analysis. Antibodies reactive with polypeptides ranging from 34 to 400 kilodaltons in apparent molecular mass were detected by these means. Radioimmunoassay competition experiments revealed that the cellular and extracellular antigens did not compete with each other for serum antibodies. Preabsorption of sera with extracellular proteins from other oral streptococcal species prior to immunoblotting indicated that the antigens unique to *S. mutans* have molecular masses greater than 100 kilodaltons, and each individual produced antibodies against different antigens of high molecular mass. Examination of sera from young children also indicated heterogeneous responses against *S. mutans* LM7 antigens.

Dental caries continues to be a major health problem in the world today (8). Animal models have implicated *Streptococcus mutans* as the primary etiological agent (5, 6, 11). Since more than 99% of individuals in western nations develop caries, innate immune mechanisms are apparently ineffective. Though dental caries is prevalent in children, most adult humans have low incidences of dental caries. This suggests that some type of immunity might be acquired by early adulthood. Significant antibody titers in saliva and serum are present in most adult humans and have been shown to be induced by oral immunization with *S. mutans* (7, 17). Animal model systems have been used extensively to investigate the immunogenicity of *S. mutans*. For example, oral immunization with killed *S. mutans* has been shown to induce secretory immunoglobulin A (IgA) and caries immunity in rodents (18). Serum antibodies (IgG, IgM, and IgA classes) have also been demonstrated in response to whole cells, crude cell wall preparations, and extracellular proteins including glucosyltransferase in monkeys and rats (14, 21, 24). In some instances, these elevations in antibody titers have been associated with effective immunity (19, 23). Specific surface antigens which have been associated with immunity in animals include the spaA protein (R. Curtiss III, R. Goldschmidt, J. Barret, W. Jacobs, R. Pastian, M. Lyons, S. M. Michalek, and J. Mestecky, in S. Hamada, L. Menaker, H. Kiyono, J. R. McGhee, and S. M. Michalek, ed., Cellular, Molecular, and Clinical Aspects of *Streptococcus mutans*, in press) and antigen A (R. R. B. Russell, E. Abdulla, M. L. Gilpin, and K. Smith, in S. Hamada et al., ed., Cellular, Molecular, and Clinical Aspects of *Streptococcus mutans*, in press).

The purpose of this study was to identify specific antigens from *S. mutans* which have stimulated a potentially protective antibody response in the sera of an adult human populations. We were able to detect antibodies to cellular and extracellular antigens of *S. mutans* by Western blot analyses. These antigens were checked for shared immunodeterminants with extracellular proteins from other oral streptococci. We contrasted early antibody responses in humans by using the sera of young children to compare with profiles of adults. The results described here indicate heterogeneity in the immune responses of both young children and adults.

**MATERIALS AND METHODS**

**Antisera.** Human sera were obtained by venipuncture from nine adults with a normal array of carious lesions and from one caries-free adult. Two adult New Zealand White rabbits were inoculated intramuscularly with 800 μg of *S. mutans* LM7 extracellular protein emulsified in an equal volume of Freund complete adjuvant. The rabbits were boosted 1 month later with the same amount of protein without adjuvant and bled 2 weeks after the booster.

**Bacterial strains and medium.** *S. mutans* LM7 (Brathall serotype c) and V403 (serotype c) and *S. sanguis* V288 have been previously described (20). *S. mitis* 118 and *S. salivarius* I1 were clinical isolates obtained from D. LeBlanc (9). *S. faecalis* JH2-2 was obtained from D. Clewell. Streptococci were grown in chemically defined FMC medium (25).

**Preparation of streptococcal protein fractions.** Streptococcal protein fractions were obtained as previously described (20). Strains were grown aerobically for 24 to 36 h at 37°C in 1 liter of chemically defined medium (25; glucose was the carbon source) supplemented with 10 mM sodium bicarbonate. Cells were harvested by centrifugation at 9,000 × g for 20 min in an Ivan Sorvall, Inc. (Norwalk, Conn.) GSA rotor. The washed cells were suspended in 10 ml of 10 mM sodium phosphate buffer (pH 6.5) and mechanically broken in a Bead-Beater (Biospec Products, Inc., Bartlesville, Okla.). This was followed by centrifugation of unbroken cells and cell debris at 300 × g and retention of the supernatants. These constituted the cellular fractions. The extracellular fractions consisted of ammonium sulfate (60% saturated) precipitations of the 1-liter culture supernatants at 4°C. The precipitates were suspended in 10 ml of 10 mM sodium phosphate buffer (pH 6.5) and dialyzed against the same buffer. Further concentration was achieved by lyophilization and suspension in 1 ml of sodium phosphate buffer. Protein

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Intracellular proteins; while

were transferred to visualized and boiled above. Paper trocellulose reticularly transferred.

FIG. 1. Proteins were subjected to SDS-PAGE with a 10% acrylamide slab gel and stained with Coomassie blue. Lanes: A, S. mutans V403 extracellular proteins; B, S. mutans LM7 extracellular proteins; C, S. mutans V403 cellular proteins; D, S. mutans LM7 cellular proteins; E, molecular mass markers. Intracellular protein lanes represent about 200 µg of total protein, while extracellular protein lanes represent about 30 µg of total protein.

was estimated by the method of Lowry et al. (15). Fractions were aliquoted and stored frozen at −20°C.

SDS-PAGE. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12) on a 10% polyacrylamide slab gel with a 3% stacking gel and visualized by Coomassie blue staining. Proteins were boiled for 5 min in SDS sample buffer prior to electrophoresis. Prestained molecular mass standards were purchased from Bethesda Research Laboratories, Gaithersburg, Md.

Immunoblotting. Samples were run on SDS-PAGE gels as described above. Separated proteins were then electrophoretically transferred to nitrocellulose paper (28) overnight at 90 mA in a Transphor TE50 apparatus ( Hoeffer Scientific Instruments, San Francisco, Calif.). After transfer, the nitrocellulose paper was preincubated in Tris-buffered saline (50 mM Tris hydrochloride, 150 mM NaCl [pH 7.5]) with 0.05% Tween 20 (TTBS) and containing 3% gelatin to block sites of nonspecific protein binding. After 1 h, the paper was transferred to a solution of TTBS containing either a 1:1,000 dilution of hyperimmune rabbit serum, a 1:1,000 dilution of preimmune rabbit serum, or a 1:100 dilution of human serum. Incubation proceeded at room temperature for 4 h. After two washes with TTBS, the nitrocellulose paper was incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio-Rad Laboratories, Richmond, Calif.) or horseradish peroxidase-conjugated goat anti-human antibody (Cooper Biomedical, Inc., Malvern, Pa.) in TTBS for 1 h. After a distilled water rinse and two TTBS washes, the paper was submersed in a color development solution consisting of 0.05% 4-chloro-1-naphthol and 0.015% hydrogen peroxide in Tris-buffered saline–methanol (5:1 [vol/vol]). Band molecular masses were estimated by comparison with prestained molecular standards (Bethesda).

Antigen competition experiments. A radioimmunoassay was used to detect and quantitate specific antibody as described previously (26). Antigen was diluted in coating buffer (0.1 M NaCO3 [pH 9.6]) and then allowed to coat the wells of a microtiter plate. Each of the competing antigens was mixed with equivalent dilutions of human serum (1:800) and allowed to incubate overnight. The concentrations of the competing antigens are indicated on the abscissas of the plots. The antigen-antibody mixtures then were allowed to incubate in the microtiter wells containing the target antigens. After washing, 125I-labeled goat anti-human IgG (Fc specific) was added at approximately 3 x 104 cpm per well and incubated overnight. Supernatants were aspirated and, after extensive washing, individual wells were counted in an LKB Instruments, Inc. (Rockville, Md.) 1282 Compugamma gamma counter to 10% or better accuracy.

Immunoblot detection of cross-reactive proteins. Human serum was incubated overnight at 4°C with extracellular proteins of S. mutans, S. sanguis, S. mitis, or S. salivarius at concentrations of approximately 10 µg/ml. The antibody-antigen mixtures then were centrifuged at 12,000 x g in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.) for 5 min to remove immune complexes and any other insoluble material. These preadsorbed sera then were used to provide the first antibodies in immunoblot experiments as described above.

RESULTS

Identification of immunogenic S. mutans extracellular proteins. S. mutans LM7 (Bratthall serotype e) and V403 (serotype c) cellular and extracellular proteins were resolved on SDS-polyacrylamide gels. Figure 1 shows the resulting protein profiles visualized by Coomassie blue staining. Several proteins in both fractions had apparent molecular masses of greater than 100 kilodaltons. This size is in the range reported for glucosyltransferases from several S. mutans serotypes (3). Extracellular proteins from S. mutans LM7 (Fig. 1, lane B) were used to immunize rabbits in an attempt to determine which of these antigens could elicit an antibody response. Immune serum from these rabbits was tested via immunoblotting (27). S. mutans LM7 extracellular proteins were subjected to SDS-PAGE and transferred by Western blotting (28) to nitrocellulose paper. The nitrocellulose paper then was immunoblotted with hyperimmune rabbit serum as the first antibody and goat anti-rabbit antibody coupled to horseradish peroxidase as the second antibody. The resulting immunoreactive antigens are displayed in Fig. 2. Owing to the sensitivity of the immunological detection system, more bands became visible than by Coomassie blue staining of the proteins. Lane A contains extracellular proteins from LM7, and approximately 25 immunoreactive bands could be detected. These ranged from about 24 to 320 kilodaltons. Lane B contains extracellular proteins from V403, and approxi-
caries-free individual (Fig. 3, panel 3) and the other nine
be differences in immunoreactive polypeptides of higher
reactivity between the cellular and extracellular LM7 protein was mixed with equivalent
salivarius, and S. mitis, as well as S.
protein preparations was
examined to determine if there were any
unique to S. mutans but might have
proteins recognized by the human sera
and extracellular antigens of S. mutans LM7 and V403 were
lized target antigens. LM7 cellular antigens
were allowed to coat wells of a microtiter plate. Each of the
target antigen, either cellular or extracellular LM7 protein
was added to the target antigens, S.
mobileness present in other
immunodeterminants among these proteins. This was done by
a competition experiment (Fig. 4) by which the indicated
target antigen, either cellular or extracellular LM7 protein
was allowed to coat wells of a microtiter plate. Each of the
two potential competing antigens against either cellular or
extracellular LM7 protein was mixed with equivalent
amounts of human serum from a subject picked at random.
Formation of antibody-antigen complexes should effectively
reduce antibody available for interaction with the immobi-
lized target antigens. LM7 cellular antigens successfully
competed with immobilized LM7 cellular antigens, while
LM7 extracellular antigens were unable to exhibit a compara-
ble effect against cellular antigens (Fig. 4). In the reverse
situation, in which the LM7 target antigens were extracellu-
lar, no competition was observed from LM7 cellular anti-
gens. These data indicated that there was little cross-
reactivity between the cellular and extracellular antigens of
S. mutans LM7. Similar results were obtained with S.
mutans V403 (data not shown).
Cross-reactivity of bacterial antigens. The immunoreactive
proteins recognized by the human sera may not have been
unique to S. mutans but might have represented im-
munodeterminants present in other streptococcal species.
To investigate this possibility, we obtained extracellular
preparations of three other oral streptococci, S. sanguis, S.
salivarius, and S. mitis, as well as S. faecalis. Each of these
protein preparations was incubated at various concentra-
tions with human serum overnight. The absorbed sera were
then added to the target antigens, S. mutans LM7 extracel-
lar proteins, and serum antibody binding was determined
with \( ^{125}I \)-labeled goat anti-human antibody. Binding of
human serum antibodies to S. mutans LM7 extracellular anti-
gens was affected in three ways (Fig. 5). First, there was
little or no inhibition of binding, with S. faecalis protein
(except at the high dose range) as an example. Second, there
was partial inhibition of binding when S. mitis antigens were
used in preincubation. Finally, almost total inhibition of
binding could be achieved by using S. mutans extracellular
proteins in preincubation.

The immunoblots (Fig. 6) further illustrate the cross-
reactivity of certain antigens among the oral streptococci.
Incubation of human serum with either S. mutans or S.
sanguis extracellular proteins (lanes E and B, respectively)
greatly reduced the antibody available to bind to immobi-
lized S. mutans LM7 extracellular proteins. Only two
immunoreactive bands remained after inoculation with S.
sanguis; no bands remained when S. mutans was used. S.
salivarius seemed to share the fewest immunodeterminants
with S. mutans LM7, since antibody still remained available
to bind to most of the S. mutans extracellular proteins (lane

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FIG. 2. Immunoreactive S. mutans extracellular proteins as de-
tected by hyperimmune rabbit serum. Immunoblots of S. mutans
LM7 extracellular proteins (lane A) and S. mutans V403 extracel-
lar proteins (lane B). Each represents about 30 \( \mu \)g of total protein.
Proteins were subjected to SDS-PAGE with a 10% acrylamide slab
gel followed by electrophoretic transfer to nitrocellulose paper. The
paper was reacted with serum from a rabbit immunized with an S.
mobileness LM7 extracellular antigen preparation. Goat anti-rabbit
antibody coupled to horseradish peroxidase was then added, fol-
lowed by immersion in color development solution to visualize
immunoreactive bands. Major bands for LM7 were 56, 79, 86, 125,
140, and 323 kilodaltons; for V403 they were 58, 82, 133, and 154
kilodaltons.
HUMAN SERUM ANTIBODY RESPONSE TO S. MUTANS ANTIGENS

FIG. 3. Adult human serum antibody against S. mutans antigens. Panels represent immunoblots of adult subjects 1 to 6. Lanes: a, S. mutans LM7 cellular proteins; b, S. mutans V403 cellular proteins; c, S. mutans LM7 extracellular proteins; d, S. mutans V403 extracellular proteins. Asterisks indicate 61,000- and 59,000-dalton common intracellular proteins. Small arrows indicate common 57,000-dalton extracellular protein. Large arrowheads indicate 100,000-dalton molecular mass points. Panel 3 represents a caries-free individual. Intracellular protein lanes represent about 200 μg of total protein, while extracellular protein lanes represent about 30 μg of total protein.

D). Only three or four bands disappeared in the immunoblot profile. Finally, S. mitis appears to share some common immunodeterminants, since antibody remained available to bind to several (five or six) of the S. mutans LM7 proteins after preincubation with S. mitis extracellular proteins (lane C). This result agrees with the partial inhibition shown by S. mitis in Fig. 5.

Serum antibody response against S. mutans antigens in young children. Serum was obtained from five young children aged 8, 10, 10, 24, and 26 months and examined for

FIG. 4. Cellular versus extracellular competition assay. (A) S. mutans extracellular antigens were used as target antigens to coat microtiter plate wells. Each of the competing antigens was incubated with equivalent amounts of human serum and added to the coated wells. 125I-labeled anti-human IgG then was added, bound radioactivity was determined, and percent inhibition was calculated. Competing antigens: ○, S. mutans LM7 extracellular; Δ, S. mutans LM7 cellular. (B) S. mutans LM7 cellular antigens were used as target antigens. Competing antigens: ○, S. mutans LM7 extracellular; Δ, S. mutans LM7 cellular.
FIG. 5. Species specificity competition assay. *S. mutans* extracellular antigens were used as target antigens to coat microtiter wells. Each of the competing antigens was incubated with equivalent amounts of human serum and added to the coated wells. $^{125}$I-labeled antihuman IgG then was added, bound radioactivity was determined, and percent inhibition was calculated. Competing antigens: □, *S. mutans* LM7 extracellular; Δ, *S. mutans* V403 extracellular; ●, *S. mitis* extracellular; ○, *S. faecalis* extracellular.

antibodies reactive with *S. mutans* LM7 extracellular antigens. The resulting immunoblots show differing responses among the five individuals. The 8-month-old child possessed antibodies reactive with only five *S. mutans* antigens, includ-

FIG. 6. Species specificity of antibodies to *S. mutans* LM7 extracellular proteins. Immunoblots of *S. mutans* LM7 extracellular proteins. Proteins were subjected to SDS-PAGE with a 10% acrylamide slab gel followed by electrophoretic transfer to nitrocellulose paper. Human serum was incubated with the following extracellular protein preparations at 10 μg/ml before incubation with the nitrocellulose paper followed by addition of goat anti-rabbit antibody coupled to horseradish peroxidase and immersion in color development solution for immunoreactive band visualization. Lanes: A, control; B, *S. sanguis*; C, *S. mitis*; D, *S. salivarius*; E, *S. mutans* LM7. Numbers at the left indicate molecular mass markers in kilodaltons. Each lane represents about 60 μg of total protein.

FIG. 7. Serum antibody response against *S. mutans* extracellular antigens in young children. Immunoblot of *S. mutans* LM7 extracellular proteins. Proteins were subjected to SDS-PAGE with a 10% acrylamide slab gel followed by electrophoretic transfer to nitrocellulose paper. Serum from children of the following ages were incubated with the nitrocellulose paper followed by addition of goat anti-rabbit antibody coupled to horseradish peroxidase and immersion in color development solution for immunoreactive band visualization. Lanes: A, 8 months; B, 10 months; C, 10 months; D, 24 months; E, 26 months. Numbers at the left indicate molecular mass markers in kilodaltons. Each lane represents about 60 μg of total protein.
ing the major 57-kilodalton extracellular protein (Fig. 7, lane A). One 10-month-old displayed antibodies reactive with at least 16 S. mutans antigens (lane B), while another 10-month-old appeared to have no detectable antibodies reactive with these antigens. The 24- and 26-month-old children (lanes D and E) also possessed antibodies reactive with many S. mutans extracellular antigens. All except the 10-month-old (lane C) had antibodies against antigens of greater than 100,000 daltons molecular mass.

**DISCUSSION**

The work described here indicates that there are a variety of S. mutans antigens to which serum antibodies are produced in humans. The humoral immune system is probably exposed to S. mutans antigens through minor cuts and abrasions in gingival tissue which can occur during dental procedures and probably during routine brushing and cleaning of teeth. At least three antigens appeared to be common in each of the 10 adult subjects in this study. These included cellular antigens of 59,000 and 61,000 daltons and an extracellular antigen of about 57,000 daltons. The cellular antigens are in the size range of the natural antigen recently reported (Russell et al., in press) as a wall protein which cross-reacts with other oral bacteria besides S. mutans. Although the predominant antibodies were directed against similar-molecular-mass antigens below 100,000 daltons in each of the adult serum samples examined, differences appeared in responses to antigens greater than 100,000 daltons in molecular mass. This latter observation may have significance in caries development, since most of the glucosyltransferases studied thus far have molecular masses above 100,000 daltons (3), and it has been demonstrated in animals that antibodies against glucosyltransferases can confer protection against the development of dental caries. The carries-free adult subject showed no obvious differences in the immunoblot profiles except in the area above 100,000 kilodaltons molecular mass, where individuals appear to display immunoreactivity against a variety of polypeptides (Fig. 3).

There seem to be immunologically distinct antibodies against extracellular and cellular antigens of S. mutans in human serum, as competition experiments indicated (Fig. 4). If shared immunodeterminants were present and equally accessible, serum antibodies would be titrated out and unable to bind to the target antigens. This did not occur with either S. mutans LM7 or V403 when extracellular antigens were mixed with serum and then added to cellular target antigens or in the reverse situation, in which cellular antigens were mixed with serum and then added to extracellular target antigens.

The use of sera from rabbits which had been immunized with S. mutans LM7 extracellular proteins suggested the potential immunogenicity of many of these proteins. Figure 2 illustrates that there are at least 25 detectable immunoreactive polypeptides from LM7, ranging from approximately 24,000 to 320,000 daltons. S. mutans V403 had 13 detectable immunoreactive polypeptides when screened with antisera to LM7 extracellular antigens, which ranged from about 40,000 to 360,000 daltons. These presumably represent antigens cross-reactive with those of LM7. We believe that these data show that there are several proteins of S. mutans which can be potentially immunogenic and that at least a few seem to be strongly immunoreactive or cross S. mutans serotypes or both, at least in this rabbit system.

Figures 5 and 6 indicate that some of the antigens of S. mutans are similar immunologically to those of some other oral streptococci. S. salivarius seems to share the fewest common determinants, with S. sanguis sharing the most immunodeterminants. S. mitis seems to lie somewhere between these two. These results were not surprising, since cross-reactive antigens have been reported among the oral streptococci (22). Figure 6 indicates that the least cross-reactivity lies in the high-molecular-mass region above 100,000 daltons. These data do not prove that these antigens are unique to S. mutans, since there are many other microorganisms to consider, but the number of antigens which do share determinants are probably not protective and can be ruled out as prime vaccine candidates.

Since caries occurs far less frequently in adults, it is possible that caries immunity is eventually acquired over a long time. The sera of five young children 8 to 26 months old were examined for antibodies against S. mutans extracellular antigens. The results indicated some similarities in the immunoblot profiles below about 60,000 daltons as early 10 months old. However, as in adults, differences in reactivity to the high-molecular-mass antigens were seen, and one child appeared to have few, if any, antibodies to S. mutans extracellular proteins. It is interesting that antibodies reactive with S. mutans antigens are present as early as 8 months of age in some individuals, which is not long after initial tooth eruption and subsequent S. mutans colonization (13). Since almost all young children eventually acquire carious lesions, these early immunoreactive antigens are presumably not protective.

The human antibodies described in this paper are of the IgG class, since goat anti-human IgG antibody was used as the second antibody in the immunoblot assays. We have made no attempt to determine which antibody classes other than IgG are present in human serum against S. mutans antigens. We have also not looked at secretory IgA responses against specific S. mutans proteins. Some studies have demonstrated significant secretory IgA response against S. mutans (16, 17), and this may be a major immune response to this pathogen. However, other work has suggested that serum IgG antibodies can move into saliva via crevicular fluid and provide protection (1, 2, 14).

We believe that the work described here can have implications for development of vaccine against this organism in humans. First, it is evident that humans develop antibodies against many S. mutans proteins from an early age and that these antibodies do not seem to effect immunity, since more than 99% of the population eventually develop caries. Therefore, any of these proteins probably would not be vaccine candidates. Also, many of these S. mutans proteins cross-react immunologically with proteins of other oral streptococci. These proteins also would not make good vaccine candidates, since they are shared by other normal oral flora. We suggest using pools of adult human sera absorbed with a mixture of proteins from other oral streptococci when searching for immunogenic S. mutans proteins. Antigens detected by these serum pools then should be checked for reactivity with a pool of sera from young children. Antigens which react with the pooled adult sera but not with the pooled child sera should be further studied. Finally, there appeared to be several S. mutans extracellular proteins to which antibody was produced in the immunized rabbit but not in the human subjects. This may indicate that some S. mutans proteins are poor immunogens under normal circumstances but can be good immunogens if appropriate doses are used during immunization. Some of these are high-molecular-mass proteins over 100,000 in molecular mass.

We have begun to clone immunoreactive proteins of S.
mutans LM7 which are reactive to either human serum or hyperimmune rabbit serum. This is presently being done with a genomic library consisting of Escherichia coli cells containing the plasmid pOP203 (A\textsubscript{R}+) with \textit{S. mutans} chromosomal DNA inserts (20). We are also screening bacteriophage lambda libraries with each type of serum. The cloned immunoreactive proteins will be further examined individually as potential immunogens for vaccines against \textit{S. mutans}. Since \textit{S. mutans} antigen(s) appears to be cross-reactive with human heart tissue (4, 10), each of the cloned proteins can be screened for this property also. This approach may also aid in identifying proteins which have thus far not been cloned successfully from this organism (i.e., some glucosyltransferases). Such molecular genetic approaches should eventually allow the production of a safe and efficacious vaccine against dental caries.

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LITERATURE CITED


