

## Binding of *Streptococcus mutans* Antigens to Heart and Kidney Basement Membranes

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Using indirect immunofluorescence, alkali-extracted components of *Streptococcus mutans* were found to bind in vitro to capillary walls and sarcolemmal sheaths of monkey cardiac muscle and to glomerular and tubular basement membranes of monkey kidney. Adsorption of *S. mutans* components to tissue fragments was also detected by indirect radioimmunoassay and immunoblotting on nitrocellulose paper. Antibodies did not bind to untreated, control tissues in these experiments, proving that antigens shared by *S. mutans* and tissue components were not involved. Rabbit and monkey heart and kidney components bound *S. mutans* antigens of 24,000, 35,000, and 65,000 *M<sub>r</sub>*. Monkey heart also bound molecules of 90,000 and 120,000 *M<sub>r</sub>*. Rabbits immunized by intravenous injection of disrupted *S. mutans* cells developed severe nephritis that was characterized by the deposition of immunoglobulins, complement component C3, and *S. mutans* antigens in the glomeruli. Immunoglobulin G eluted from nephritic kidneys reacted in immunoblots with the 24,000, 35,000, and 65,000 *M<sub>r</sub>* components of *S. mutans* extract, indicating that the antigens that bound to tissue in vitro also bound in vivo and reacted with antibodies in situ. Antibodies to other *S. mutans* antigens were not detected in the kidney eluate, although they were present in the serum of the same rabbit.

*Streptococcus mutans* has been identified as the most important etiological agent of dental caries (reviewed in references 9 and 16). Immunizations of rats and monkeys that result in increased salivary immunoglobulin A (IgA) specific for *S. mutans* can inhibit the colonization of teeth by these bacteria and reduce the incidence of dental caries (5, 13, 17, 28). Therefore, it has been suggested that development of an *S. mutans* vaccine may be of value in controlling dental caries in humans. Yet, because dental caries is not a life-threatening disease, *S. mutans* antigens that exert adverse side effects are unacceptable vaccine components (16).

Surface components of *S. mutans* have been shown to bind selectively glycoproteins of human saliva (8, 15, 20, 24) and cardiac antigens of Todd-Hewitt broth (25). Crude extracts of a number of *S. mutans* serotypes contain antigens that bind in vitro to components of cardiac and skeletal muscle (26). Recently, it has been shown that rabbits immunized with *S. mutans* strains KIR and MT703 develop kidney disease that exhibits many characteristics of streptococcus-associated nephritides (SAN) in humans (27). This study was undertaken to identify the components of *S. mutans* that bind to heart and kidney tissues in vitro and to kidney tissue in vivo using an indirect radioimmunoassay and immunoblotting (Western blotting).

### MATERIALS AND METHODS

**Bacteria and cultural conditions.** *S. mutans* strains AHT (serotype *a*), BHT (serotype *b*), 10449 (serotype *c*), KIR (serotype *d/g*), MT703 (serotype *e*), and OMZ175 (serotype *f*) were grown on a dialysate of tryptic soy broth supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.) and on chemically defined medium (31). All cultures (1 liter of medium in a 2-liter Erlenmeyer flask) were incubated at 37°C and mixed at 25 rpm on a gyratory shaker

(New Brunswick Scientific Co., New Brunswick, N.J.). Growth was followed turbidimetrically at 600 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. Cultures with optical densities of 0.8 to 1.2 (late-logarithmic to early-stationary phases of growth) were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. The cells were then washed twice with phosphate-buffered saline (PBS; 0.01 M NaH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, containing 0.15 M NaCl).

**Immunization.** Bacteria grown in the complex culture medium were disrupted by shaking with glass beads in a Braun cell homogenizer (Bronwill Scientific Inc., Rochester, N.Y.) at 4°C as described by Bleiweis et al. (2). The vaccine of disrupted bacteria was heated at 70°C for 30 min, adjusted to 10 mg of protein per ml in PBS and stored at -20°C. New Zealand white rabbits were immunized with the sterile preparations of disrupted streptococci by intravenous injection three times a week as previously described (27). The antisera used in this study were obtained 8 to 24 weeks after the start of the immunization regimen, a period in which many rabbits developed symptoms of nephritis (27). The rabbits were test-bled every 4 weeks. Antibody titers to intact *S. mutans* were determined by indirect immunofluorescence assay to be between 640 and 2,560.

**Extraction of bacteria.** Intact cells were extracted sequentially with acetone, chloroform-methanol (2:1), and ammonium hydroxide at pH 10 as previously described (23). Lyophilized extracts were dissolved at 5 mg (dry weight) per ml of PBS for tissue-binding experiments.

**Immunofluorescence.** Antigens in *S. mutans* extracts were tested for their abilities to bind to 4-μm-thick cryostat-cut tissue sections of cynomolgus monkey (*Macaca fascicularis*) heart and kidney (23, 26). Briefly, tissue sections were incubated with the bacterial extract (5 mg [dry weight]/ml) for 30 min and washed with PBS for 15 min. Tissue-bound antigens were detected by indirect immunofluorescence (IIF) assay with rabbit antiserum elicited by immunization with the homologous bacterium and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG as previously de-

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scribed (26). Normal rabbit serum obtained before immunization and anti-*S. mutans* serum were incubated with both untreated, native tissue and tissue that was pretreated with bacterial antigens.

The components present in immune deposits within the tissues of immunized rabbits were identified by direct immunofluorescence as previously described (27), using FITC-labeled goat anti-rabbit IgG, IgM, IgA, and C3 (Cappel Laboratories Inc., West Chester, Pa.). Sections of tissues obtained from immunized rabbits were also examined by direct immunofluorescence for the presence of *S. mutans* antigens by using chicken anti-*S. mutans* antibodies that were conjugated with FITC by the procedures of Nairn (19). This latter antiserum was elicited in chickens by injection of disrupted *S. mutans* cells mixed with an equal volume of complete Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.). The chickens received 1 mg of protein in each footpad at 2-week intervals. The animals were exsanguinated after 8 weeks.

**Radioiodination of protein A.** Protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) was radioiodinated by the chloramine-T procedure of Hunter (12). The specific radioactivity of the  $^{125}\text{I}$ -protein A preparations ranged from 10,000 to 16,000 cpm/ng.

**Tissue homogenization.** Five grams each of monkey and rabbit heart and kidney tissues were cut into roughly 2-mm<sup>2</sup> sections with razor blades and washed five times with 50 ml of ice-cold PBS to remove blood components. The fragments were suspended in 20 ml of PBS containing 2 mM phenylmethylsulfonyl fluoride and 0.05% sodium azide and homogenized at 4°C. After removing clumps of connective tissue by filtration through cotton gauze, each suspension was adjusted with PBS to 10 mg of protein per ml and stored at -70°C.

**Indirect radioimmunoassay.** Homogenized monkey and rabbit tissue was fixed to enzyme-linked immunosorbent assay plates (Immulon; Dynatech Laboratories, Alexandria, Va.) and washed with PBS-1% bovine serum albumin (BSA) as previously described (27). The tissue components were incubated with 50  $\mu\text{l}$  of bacterial extract for 45 min at room temperature. Empty and tissue-containing wells were incubated with the PBS-BSA solution as negative controls. All wells were washed five times with PBS to remove unbound reactants followed by incubation with 50  $\mu\text{l}$  of diluted rabbit antiserum or normal rabbit serum for 45 min at 22°C. After five more washings with PBS, the wells were incubated with 15 ng of  $^{125}\text{I}$ -protein A for 45 min. After a final series of washings, the contents of the wells were extracted with 150  $\mu\text{l}$  of 2% sodium dodecyl sulfate (SDS) solution at 50°C for 10 min, and a 100- $\mu\text{l}$  portion was removed and counted in a gamma counter (Beckman Instruments Inc., Berkeley, Calif.). The data were corrected for the  $^{125}\text{I}$ -protein A bound to control wells coated with BSA. Although these values varied with the preparation of radioiodinated protein A, the one used in the experiment described here gave 18,685 cpm with a standard deviation of 1,438 cpm.

**Binding of bacterial antigens to tissue.** One milligram (dry weight) of *S. mutans* extract was dissolved in 200  $\mu\text{l}$  of PBS and mixed with 200  $\mu\text{l}$  of homogenized tissue and stirred for 2 h at 4°C. The suspension was centrifuged at 15,000  $\times g$  for 30 min. The pellet of tissue particles was suspended in 1 ml of ice-cold PBS and collected by centrifugation. This washing procedure was repeated three times to remove unbound reactants. Before the final centrifugation, the suspension was transferred to a clean centrifuge tube so that any antigen adsorbed to the walls of the original vessel would be eliminated. The final pellet was suspended in 1 ml of SDS-

polyacrylamide gel electrophoresis (PAGE) buffer and boiled for 15 min. After removing insoluble material by centrifugation, 40  $\mu\text{l}$  of the supernatant fluid was separated by SDS-PAGE. As a control, tissue was incubated with saline instead of bacterial extract and then washed and extracted in the same manner.

**SDS-PAGE.** Extracted bacterial and tissue components were separated by SDS-PAGE in the absence of reducing agents (33). The separating gel contained 7.5% acrylamide and 6 M urea. The molecular weight markers (Sigma Chemical Co., St. Louis, Mo.) and the streptococcal and tissue components were solubilized at 100°C in SDS-PAGE sample buffer. The molecular weight markers were: bovine serum albumin, 66,000; ovalbumin, 45,000; trypsinogen, 24,000;  $\beta$ -lactoglobulin, 18,400; and lysozyme, 14,300. Proteins were detected by staining with Coomassie brilliant blue.

**Immunoblotting.** Proteins were transferred electrophoretically from SDS-polyacrylamide gels to nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) and reacted with rabbit antiserum and  $^{125}\text{I}$ -protein A according to the procedure of Burnette (3) but with the buffer systems of Smith and Summers (22). XAR-5 X-ray film (Eastman Kodak, Rochester, N.Y.) was exposed to the antigen-antibody- $^{125}\text{I}$ -protein A complex for 0.5 to 4 h at -70°C with enhancement by a Dupont Cronex intensifying screen. Molecular weight markers were transferred to nitrocellulose and detected directly with amido black stain. In all blotting experiments, duplicate antigen blots were incubated with normal rabbit serum, obtained before immunization, to evaluate the specificity of antibody binding to antigen bands.

**Antibody elution.** For the elution of immunoglobulins deposited in kidney tissue, a kidney (20 g [wet weight]) of a rabbit immunized with *S. mutans* MT703 for 22 weeks was homogenized as described above and centrifuged at 15,000  $\times g$  for 30 min. This kidney was selected because it contained immune deposits of IgG in the glomeruli as determined by direct immunofluorescence assay. The pellet was suspended in 80 ml of 20 mM citrate buffer (pH 3.2) and stirred for 4 h at room temperature (34). After centrifugation at 87,000  $\times g$  for 1 h, the supernatant fluid was collected, adjusted to pH 7.0, and concentrated to 10 ml by ultrafiltration (UM 10 membrane; Amicon Corp. Danvers, Mass.) at 4°C. Similar extracts were prepared from kidneys of healthy, nonimmunized rabbits and compared with that from the diseased kidney in all serological assays. This technique was not useful for eluting tissue-bound bacterial antigens because most of the proteins in the *S. mutans* extract were insoluble at pH values below 4.5.

**Serum absorption.** Dilutions of rabbit anti-*S. mutans* sera were mixed with preparations of bacterial or tissue antigens and incubated at 37°C for 1 h and then at 4°C for 18 h. The immune complexes were removed by centrifugation at 105,000  $\times g$  for 1 h.

**Protein determination.** Protein concentrations were determined by the Hartree modification (10) of the Lowry method, using bovine serum albumin as a standard.

## RESULTS

**Binding of bacterial antigens to tissue sections.** *S. mutans* extracts were incubated with cryostat-cut sections of monkey heart and kidney which were stained subsequently by rabbit anti-*S. mutans* serum in the IIF assay. Figure 1 shows immunofluorescence staining along the sarcolemmal sheath and in perivascular areas of cardiac muscle. This indicated the presence of streptococcal antigens on these structures

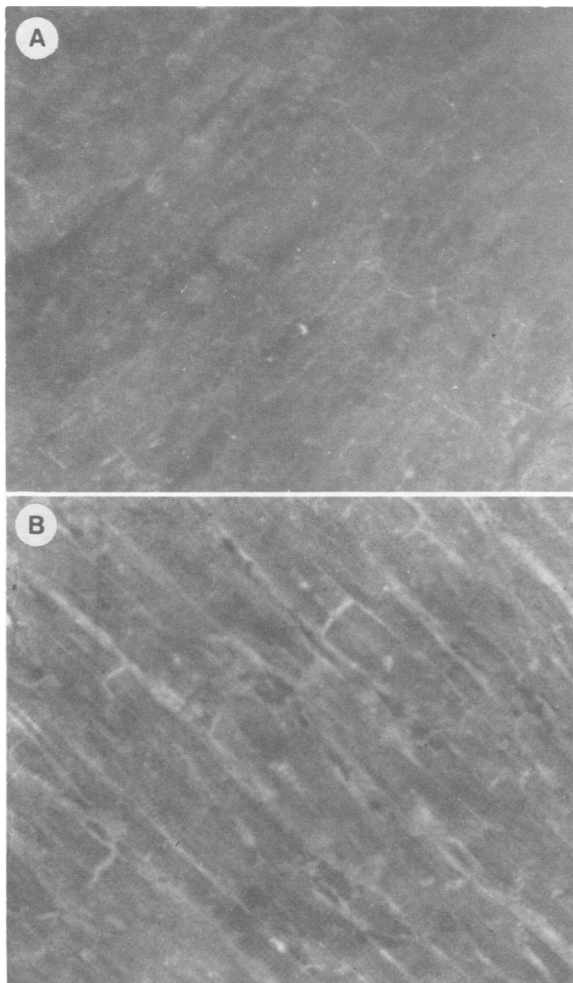


FIG. 1. IIF staining of *S. mutans* MT703 antigens bound to monkey cardiac muscle. (A) Tissue section treated with a 1:40 dilution of rabbit anti-*S. mutans* MT703 serum. Striations and intercalated disks are stained weakly. (B) Tissue section treated with alkali-extracted *S. mutans* antigens (5 mg/ml) followed by the antiserum. The sarcolemmal sheaths and perivascular regions are stained in addition to striations and intercalated disks. The antiserum used in this experiment and the one shown in Fig. 2 was obtained 8 weeks after the first immunization. Bacteria were grown in tryptic soy-yeast extract broth.

since the antiserum did not react with tissue sections that were not pretreated with the extract (Fig. 1A). Binding of *S. mutans* antigens to kidney sections is shown in Fig. 2. Immunofluorescence can be seen on both tubular and glomerular basement membranes of *S. mutans*-treated tissue but not on untreated tissue. Serum obtained from this rabbit before immunization with *S. mutans* did not react with either tissue nor with tissue treated with bacterial extract. Extracts prepared from *S. mutans* strains AHT, BHT, 10449, MT703, KIR, and OMZ175 produced similar staining patterns in IIF assays with homologous antisera on monkey heart and kidney tissues. It has been previously reported (26) that antigens extracted from *Lactobacillus casei*, *Staphylococcus aureus*, *Streptococcus salivarius* and *Streptococcus sanguis* did not bind to basement membranes of these tissues. To evaluate the effect of nutritional conditions on the production of basement membrane-binding factors,

streptococci were grown on either tryptic soy-yeast extract or chemically defined media. Extracts of both cell populations contained activity (Fig. 1 and 2).

**Identification of tissue-bound streptococcal antigens.** *S. mutans* MT703 was selected for these experiments because its tissue-binding antigens reacted strongly with antiserum in IIF assays, and administration of disrupted cell vaccines induced severe glomerulonephritis in rabbits (27). Binding of streptococcal antigens to monkey tissue in vitro was determined by radioimmunoassay (Fig. 3). The quantity of bound  $^{125}\text{I}$ -protein A was proportional to the concentrations of *S. mutans* extract and antiserum used to treat the tissue. Control experiments showed that the *S. mutans* antigens were indeed bound to tissue components and not to the BSA-coated plastic. Incubation of *S. mutans* extract in BSA-coated wells, followed by incubation with antiserum and  $^{125}\text{I}$ -protein A, produced radioactivity within the background range; that is, the amount of radioactivity obtained in BSA-coated wells treated with  $^{125}\text{I}$ -protein A. Assays with an extract of *Lactobacillus casei* and a homologous antiserum did not result in increased binding of radioactivity, indicating that this bacterium did not possess tissue-binding proteins.

Rabbit anti-*S. mutans* MT703 sera, collected 8 weeks after the first immunization, had antibody titers of 320 to 1,280 on

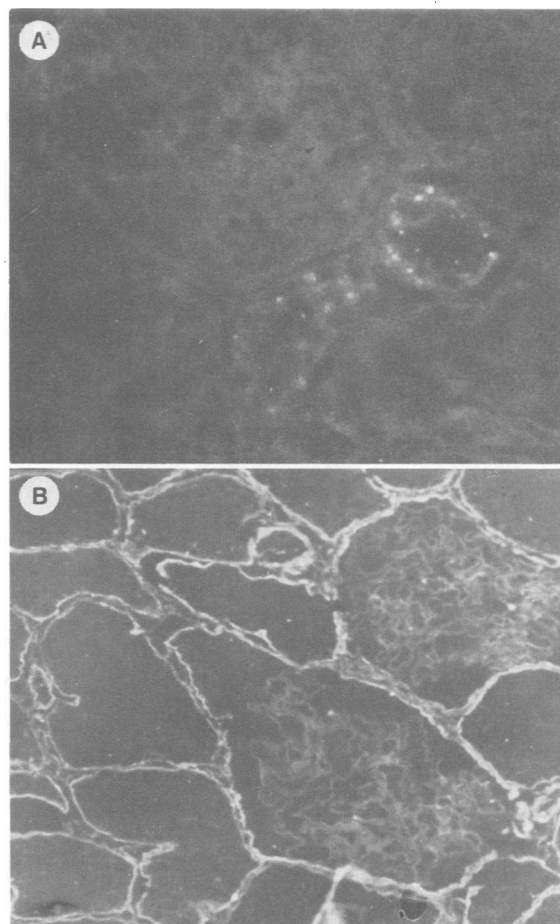


FIG. 2. IIF staining of *S. mutans* MT703 antigens bound to monkey kidney. Tissue sections A and B were treated as described in the legend to Fig. 1. Bacteria were grown in chemically defined medium.

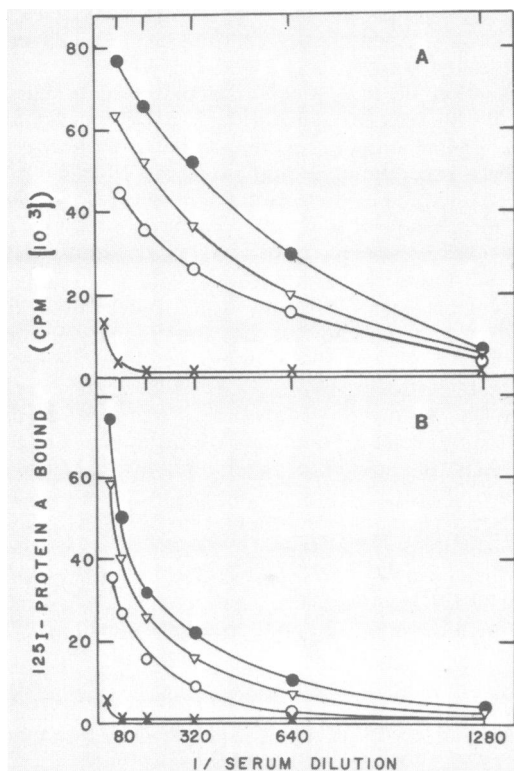


FIG. 3. Indirect radioimmunoassay showing antibody reactions with *S. mutans* antigens bound to immobilized components of monkey (A) cardiac muscle and (B) kidney. The anti-*S. mutans* MT703 serum was collected 8 weeks after the first immunization. Symbols: ●, tissue treated with 100 µg of bacterial antigens; ▽, tissue treated with 50 µg of bacterial antigens; ○, tissue treated with 25 µg of bacterial antigens; × untreated tissue. Bacteria were grown in tryptic soy-yeast extract broth.

tissue pretreated with *S. mutans* extract and only 10 to 40 on untreated, normal tissue. Interestingly, antisera obtained 12 weeks or more after the first immunization showed increased titers, ranging from 160 to 1,280, of antibodies reactive with normal tissues. The binding of these antibodies to tissue often obscured the antibody reactions with tissue-bound *S. mutans* bacterial antigens in this assay system. Therefore, an antiserum with a titer of 10,240 to bacterial antigens and of 40 or less to animal tissue was selected for all subsequent serological assays for the detection of tissue-bound bacterial antigens (Fig. 3 included). It should be noted that this antiserum did not react with plastic wells coated with components of tryptic soy-yeast extract broth. Absorption of the antiserum with *S. mutans* MT703 grown in chemically defined medium reduced the antibacterial titer from 10,240 to 20, proving that the antibodies were not elicited to constituents of the complex medium that may have been in the vaccine preparation.

To identify the streptococcal components bound *in vitro* to fragments of monkey kidney and heart, antigen-treated tissues were extracted with 2% SDS, and the solubilized components were resolved by PAGE and immunoblotting. The resulting patterns were compared with those obtained separately with crude extracts of *S. mutans* and with extracts of untreated tissue. Of the 25 *S. mutans* MT703 components stained by Coomassie blue in polyacrylamide

gels, 11 reacted with antiserum to *S. mutans* after transfer to nitrocellulose paper (Fig. 4); none of the bands bound antibodies from a similarly diluted serum obtained from the rabbit before immunization. Extracts of monkey and rabbit heart and kidney tissue that had been pretreated with *S. mutans* extract showed two common antigenic bands (65,000 and 35,000  $M_r$ ). Monkey heart contained additional bacterial bands with apparent molecular weights of 90,000 and 120,000. The rabbit and monkey kidneys also bound smaller amounts of a bacterial antigen(s) of ca. 24,000  $M_r$ . The antiserum to *S. mutans* did not react with SDS extracts of saline-treated control tissues under these conditions, proving that the antibodies were binding to bacterial antigens and not to tissue components.

**Binding of streptococcal antigens *in vivo*.** Rabbits injected intravenously with disrupted *S. mutans* vaccines were reported previously to develop extensive histopathological changes in the kidneys (27). Microscopic examination of direct immunofluorescence-stained kidney sections obtained from rabbits during periods of proteinuria revealed immune deposits of C3, IgG, IgM, or IgA in the glomeruli. The IgG-specific strains showed smooth ribbon-like patterns along the GBM and granular deposits in the mesangium and on some sections of the GBM (Fig. 5). Seven of 10 rabbits also contained streptococcal antigens in some glomeruli (Fig. 6); the intensity of the direct immunofluorescence with FITC-chicken antibodies to *S. mutans* was greatest on kidneys

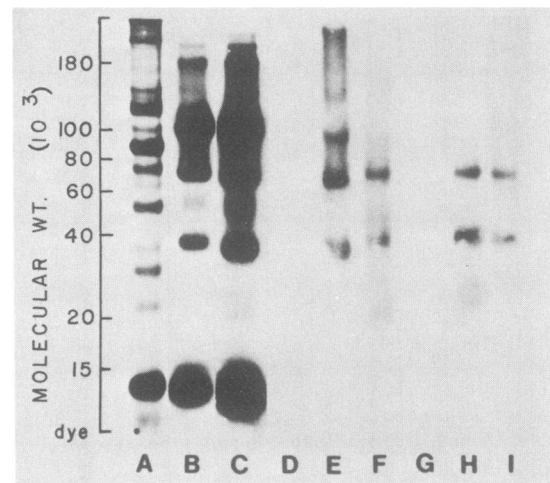


FIG. 4. SDS-PAGE and autoradiograms of immunoblots of free and tissue-bound *S. mutans* MT703 antigens. Lane A, 200 µg of extracted *S. mutans* components stained with Coomassie blue; lane B, 75 µg of bacterial protein immunoblotted with a 1:100 dilution of the rabbit anti-*S. mutans* MT703 serum used in the experiment shown in Fig. 3 (30-min development); lane C, same as lane B but with a 2-h development; lane D, 300 µg of monkey heart proteins immunoblotted against the antiserum (2-h development); lane E, 300 µg of protein extracted from complexes of monkey cardiac muscle-*S. mutans* components immunoblotted against the antiserum (1-h development); lane F, 300 µg of protein extracted from complexes of rabbit cardiac muscle-*S. mutans* components immunoblotted against the antiserum (1-h development); lane G, 300 µg of monkey kidney proteins immunoblotted with the antiserum (2-h development); lane H, 300 µg of protein extracted from complexes of monkey kidney-*S. mutans* components immunoblotted with antiserum (1-h development); lane I, 300 µg of protein extracted from complexes of rabbit kidney-*S. mutans* components immunoblotted with antiserum (1-h development). In all instances, bacteria were grown in tryptic soy-yeast extract broth.

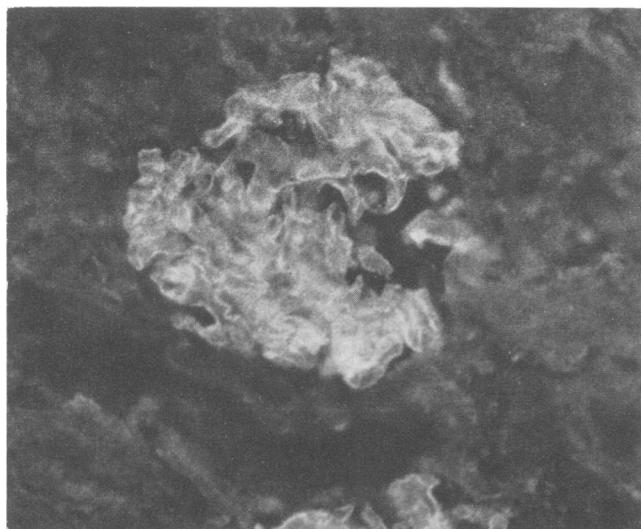


FIG. 5. Direct immunofluorescence staining of kidney from a rabbit immunized with *S. mutans* MT703. The FITC-goat antibodies to rabbit IgG show a granular pattern on GBM and in the mesangium. The kidney was obtained 16 weeks after the first injection of vaccine.

obtained during early disease, 8 to 12 weeks after the first injection of vaccine. The intensity and diffuse distribution of the IIF stain made identification of the sites of antigen deposition within the glomerulus difficult to determine (Fig. 6). To identify the accumulated bacterial components, nephritic kidneys were extracted with SDS and analyzed by immunoblotting with rabbit anti-*S. mutans* MT703 serum; however, bacterial antigens were obscured by a variety of protein A-binding substances, presumably IgG and its degradation products, that were smeared throughout the polyacrylamide gel. Alternatively, the IgG contained in the immune complexes within the kidney tissue was dissociated and extracted by citrate buffer (pH 3.2). The eluted rabbit antibodies were reacted with nitrocellulose blots of SDS-PAGE-separated *S. mutans* antigens (Fig. 7). Four bands, with apparent molecular weights of 65,000, 50,000, 35,000, and 24,000, were detected. Most of these corresponded to the bacterial antigens that bound to kidney and heart tissue in vitro (Fig. 4). Comparison of this immunoblot with that of *S. mutans* extract and serum obtained from the animal at sacrifice showed that a number of major serum antibodies were absent in the kidney extract. This indicates that the eluted antibodies were bound to kidney tissue via tissue-bound *S. mutans* antigens and were not free serum antibodies.

#### DISCUSSION

The immunofluorescence staining of tissues treated with extracted components of *S. mutans* shows that this oral bacterium contains antigens that can bind directly to capillary walls and sarcolemmal sheaths of cardiac muscle and to basement membranes of kidney. Absorption of extracts of *S. mutans* MT703 with homogenized monkey and rabbit heart and kidney removed a number of bacterial antigens that were subsequently recovered and identified by immunoblotting techniques. Rabbit and monkey heart and kidney bound *S. mutans* antigens of 35,000 and 65,000  $M_r$ . Rabbit and monkey kidneys also bound an antigen of approximately 24,000  $M_r$  and monkey heart bound antigens with 90,000 and

120,000  $M_r$ . Moreover, the presence of streptococcal antigen deposits in nephritic kidneys of rabbits immunized with disrupted *S. mutans* MT703 indicates that these bacterial components may bind in vivo and may be involved in the pathogenesis of kidney disease. However, attempts to extract the *S. mutans* antigens from the diseased kidneys with citrate buffer (pH 3.2) or 1% SDS and to determine whether they correspond to the antigens that were absorbed by the kidney in vitro were unsuccessful. The detection of these bacterial components was severely hindered by the large quantities of IgG and partially degraded IgG fragments present in the kidney extract; their intense reactions with radioiodinated protein A obscured any bacterial band that may have been present in the immunoblot. As an alternative means to show the pathogenic potential of these bacterial components, antibodies were eluted from diseased kidneys and tested for reactivity with *S. mutans* antigens. The eluted antibodies reacted with *S. mutans* antigens with apparent molecular weights of 65,000, 50,000, 35,000, and 24,000. Three of these corresponded to *S. mutans* antigens that bound to rabbit kidney in vitro, indicating that the components that bind in vitro also bind in vivo and may react in situ with immunoglobulins.

The *S. mutans*-induced nephritis reported by us (27) has many histopathological and immunopathological similarities with streptococcus-associated nephritides in humans. In both diseases there is diffuse involvement of glomeruli marked by endotheliomesangial proliferation, infiltration by polymorphonuclear cells, and deposition of electron-dense "humps" on the basement membrane (18, 27). These deposits contain predominantly complement component C3 and IgG; streptococcal antigens are also deposited early in the disease process (27). Although SAN is well documented, the precise mechanism of tissue injury is unknown. Three mechanisms have been proposed to explain the pathogenesis of SAN: (i) direct tissue toxicity of the bound streptococcal components; (ii) immune reactions with antigens in situ, including tissue-bound streptococcal antigens, cross-reacting renal antigens, and altered renal antigens; and (iii) deposition of circulating immune complexes.

The best characterized heart- and kidney-binding component of streptococci is lipoteichoic acid (LTA) (4, 14, 21, 29,



FIG. 6. Direct immunofluorescence staining of *S. mutans* antigens in a kidney of an immunized rabbit. The FITC-chicken antibodies to *S. mutans* were deposited on the GBM and throughout the mesangium. This kidney was obtained 16 weeks after the first injection of vaccine.



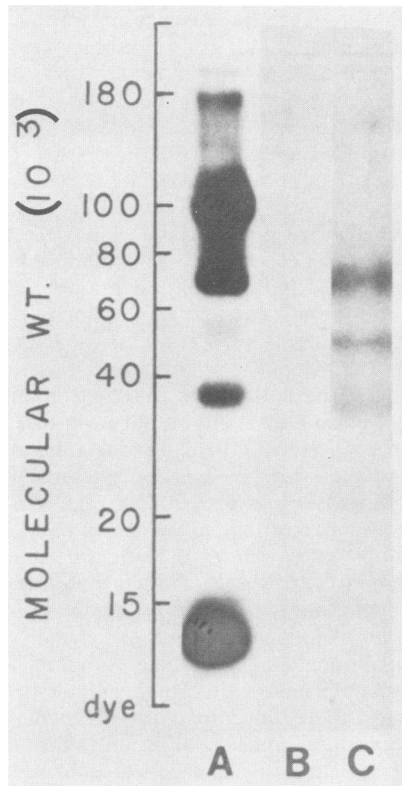


FIG. 7. SDS-PAGE and autoradiograms of immunoblots of *S. mutans* MT703 antigens with rabbit anti-*S. mutans* serum and immunoglobulins eluted from a nephritic kidney of a rabbit immunized with *S. mutans* MT703 for 22 weeks. Lane A, 75  $\mu$ g of bacterial proteins immunoblotted with a 1:100 dilution of antiserum obtained from the rabbit just before death (30-min development); lane B, 75  $\mu$ g of bacterial protein immunoblotted with a citric acid extract of a control, normal rabbit kidney (1-h development); lane C, same as lane B except that a citric acid extract of a nephritic kidney was used as a source of antibodies (1-h development).

32). However, the *S. mutans* extracts used in the studies reported here did not contain biologically active LTA as indicated by passive hemagglutination assays using an antiserum to *L. casei* LTA. Moreover, the *S. mutans* components bound to kidney or heart tissue fragments did not react with this antiserum, indicating that the polyglycerol phosphate moiety of LTA was absent (23, 26). The tissue-binding factors of *S. mutans* do not exhibit the same tissue-binding properties as LTA. The tissue-binding factors bind only to basement membrane-containing structures of mammalian tissue, whereas LTA binds predominantly to cytoplasmic membranes (unpublished data). Serum albumin and nonionic detergents inhibit LTA binding but not binding of tissue-binding factors to animal tissues (23, 26). The tissue-binding activity of LTA but not that of tissue-binding factor is destroyed by incubation at room temperature in KOH (pH 10) for 16 h (23).

Sera of rabbits immunized with *S. mutans* have been reported to contain antibodies that bind to normal heart and kidney tissue components in vitro (6, 11, 27, 30). Some of these antibodies appear to cross-react with *S. mutans* antigens as evidenced by the ability of streptococcal components to remove the tissue-reactive antibodies from serum in absorption experiments. Yet, it is not known to what extent

or when these cross-reactive antibodies bind to tissue components in vivo. The deposition of IgG and IgM observed in nephritic rabbit kidneys often appears in a linear pattern along the glomerular basement membrane. This could reflect direct antibody binding to kidney components or to *S. mutans* antigens previously deposited on this structure (see Fig. 2). Most frequently, IgG is accumulated in nephritic kidneys in a granular pattern on the GBM (Fig. 5), which may reflect the deposition of circulating immune complexes or in situ immune complex formation (27). Direct immunofluorescence staining of *S. mutans* antigens in nephritic glomeruli showed both granular and linear patterns, which substantiates results published by Andres et al. (1) and Fillit et al. (7) showing streptococcal antigens in diseased glomeruli of human kidney. The antibodies specific for *S. mutans* antigens appear to contribute more to the formation of immune complexes in the rabbit kidney than the cross-reactive antibodies, because antibodies eluted from nephritic kidney bound readily to *S. mutans* components in immunoblots (Fig. 7) but only weakly to extracted components of rabbit kidneys (unpublished data). The manner and sequence in which the reactants accumulate in tissues may be best resolved by organ perfusions with preparations of *S. mutans* antigens followed by antiserum or with mixtures of serum and antigens.

Because of the tissue-binding properties of the 65,000 and 35,000  $M_r$  *S. mutans* components, it is tempting to propose that in situ immune complex formation is most important in initiating glomerular injury. However, a prominent role for circulating immune complexes in this disease cannot be excluded. Immune complexes have been detected in the serum of some rabbits, although they appeared only several hours after intravenous injections of the *S. mutans* preparation (27). These immune complexes may ultimately localize in vessels and glomeruli, causing vasculitis and glomerulonephritis. The attachment of immune complexes to tissue may be mediated by the tissue-binding components of *S. mutans* or by a physical entrapment mechanism. The pathogenetic potential of circulating immune complexes is exemplified by acute serum sickness in rabbits, an experimental animal model that has some resemblance to SAN of humans (18). However, some aspects of acute serum sickness differ from those of SAN. The prominent polymorphonuclear leukocyte infiltration of the glomerulus in SAN is generally absent in acute serum sickness.

The nature of the immunopathology, serology and microbiology of *S. mutans*-induced nephritis in rabbits indicates that this is an appropriate and reproducible animal model for studying streptococcus-associated kidney diseases. Attempts to develop a reproducible animal model with group A streptococci have, up to now, been unsuccessful (18). With respect to prospective dental caries vaccines, we recommend that *S. mutans* antigens be purified to homogeneity and tested for tissue-binding activities as well as immunological cross-reactivities with heart and kidney components before use in humans. The desired vaccine antigen should not exert any deleterious effects on the host but must stimulate production of salivary immunoglobulins that will bind to the surface of *S. mutans* cells and inhibit the formation of dental caries.

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