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Cross-Reactivity of *Streptococcus mutans* Antigens and Human Heart Tissue

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Rocket and two-dimensional immunoelectrophoreses were used to demonstrate that antisera from rabbits immunized with *Streptococcus mutans* strain B13 cross-reacted with human heart tissue. Absorption of the anti-*S. mutans* serum with *S. mutans* whole cells removed all reactivity to heart tissue, but did not remove the reactivity of an added antibody marker to its corresponding antigen. The anti-*S. mutans* serum reacted most intensely with heart tissue antigen and to a lesser degree with skeletal muscle, but not with liver or kidney tissues. These results support the conclusion that antigens of *S. mutans* cross-react with mammalian heart tissue and, further, suggest that caution should be exercised in the formulation of a dental caries vaccine containing *S. mutans* antigens.

Streptococcus mutans is considered one of the primary etiological agents responsible for dental caries (6, 13), and there has been widespread interest and intensive research towards obtaining additional information about this organism. Among areas of particular interest are studies aimed at the development of an immunization procedure which would be effective in the prevention of dental caries, as well as the further identification and characterization of the antigens of this organism. A number of studies have shown that immunization of experimental animals with whole cells, broken cells, or various fractions of *S. mutans* results in a decreased incidence of caries when compared with control groups of nonimmunized animals (2, 3, 7, 15, 17, 20). The safety associated with a potential vaccine has come under question because of the possibility of immunological cross-reactions between streptococcal antigens and human tissues such as those described between group A, C, and G streptococci and human heart tissue (10–12, 18). van de Rijn et al. (22) have used immunofluorescence techniques to show that rabbit antibody to *S. mutans* is reactive with human myocardial tissue. More recently, reports by Hughes (8, 9) and a preliminary report of this study (5) have provided additional evidence which supports the existence of immunological cross-reactions between *S. mutans* and human heart tissue antigens.

In this communication, we present the results obtained from rocket and two-dimensional immunoelectrophoresis experiments which indicate that *S. mutans* possesses antigenic determinants similar to those found in human heart tissue.

MATERIALS AND METHODS

Bacteria. *S. mutans* strain B13, serotype *d*, was obtained from Lee R. Brown, The University of Texas Dental Branch, Houston, Tex., and was the primary strain used in all aspects of this study.

Medium. The chemically defined medium (FMC) reported by Terleckyj et al. (21) was used for the growth of *S. mutans*.

Antigen preparation and immunization. New Zealand white rabbits were injected with combinations of the three forms of *S. mutans* antigen preparations, i.e., live cells, ultraviolet-killed cells, and crude cell wall preparations obtained after breakage in a Braun cell homogenizer. Ultraviolet-killed cells, unlike formalinized cells used in other immunization procedures, had all antigenic components intact. Crude cell wall preparations presented the animal with increased antigen exposure to cell wall and membrane components. Live *S. mutans* cells have been reported to produce high titers of antibody according to an immunization procedure described by Pittman et al. (19); a modification of that immunization schedule was used in this study as follows. Briefly, eight injections of increasing volume (0.2 to 3.0 ml) containing equal volumes of a ultraviolet-killed cell suspension and crude cell wall preparation were administered over a period of 11 days by intravenous injection into the marginal ear vein. The rabbits were rested 1 week and were given four consecutive booster injections of increasing volume (0.38 to 2.25 ml) containing 2 parts live cell suspension to 1 part crude cell wall preparation. Subsequent booster injections were administered on 2 consecutive days at intervals of approximately 3 weeks. Sera were collected 7 days after each booster injection by ear artery bleeding. The serum samples were pooled, concentrated threefold by ultrafiltration (Amicon Centriflo, type CF 25), and stored at −70°C until used. Six rabbits were used in this study; two died during the first week of injections.

Mammalian tissue extracts. All tissue samples, except heart, were obtained at autopsy and were

stored at −70°C until used. None of the heart tissue samples was from subjects associated with rheumatic heart disease. The heart tissue sample was the papillary muscle of the mitral valve, which was obtained as a sterile surgical pathology specimen. Tissue samples were suspended in physiological sterile saline, and tissue extracts were prepared by homogenization in a Potter-Elvehjem homogenizer followed by 1 min of additional disruption by sonification and a final extraction of the particulate fraction with 4% Triton X-100. The soluble fractions were combined and used as the tissue extract antigens.

Rocket and two-dimensional immunoelectrophoreses. The techniques of rocket and two-dimensional immunoelectrophoreses used in these experiments were described in detail by Axelson and coworkers (1). Glass slides, 5 by 5 cm (Leitz cover glass plates, no. 19821), were covered with 5 ml of 1% agarose and 1% Triton X-100 in tris(hydroxymethyl)aminomethane-barbital-sodium barbital buffer, 0.03 ionic strength, pH 8.8. Trough buffer was 0.06 ionic strength for a discontinuous buffer system.

Absorption of antisera. Absorption of the antisera was performed according to the procedure described by Evans and Genco (4). Essentially, 1 ml of ultraviolet-killed packed cells and broken cell fragments was mixed with 1 ml of serum. The mixture was incubated at 37°C for 1 h with continuous gentle mixing, followed by an additional 1 to 2 h at 4°C. The cells were removed by centrifugation, and the above procedure was repeated, usually three to four more times, to remove all reactivity to *S. mutans* antigens.

RESULTS

Antisera to *S. mutans* B13 reacted with its corresponding antigens in two-dimensional immunoelectrophoresis experiments to form a large number of precipitin peaks. Utilizing this same reactive *S. mutans* antiserum, rocket immunoelectrophoresis experiments were performed with heart tissue antigen extracts. Antigen-antibody precipitation peaks were formed as shown in Fig. 1, and the quantitative nature of the reaction was illustrated by the differences in peak heights when increasing amounts of heart tissue extract were used. Similar results were obtained with extracts obtained from 10 other heart tissue samples. Precipitation peaks were occasionally observed with some preimmune rabbit sera and heart tissue extracts; however, these peaks were always smaller in size and diffuse in shape compared with those obtained with immune rabbit serum.

Two-dimensional immunoelectrophoresis of human heart tissue extract and *S. mutans* antibody resulted in two distinct precipitin peaks and possibly additional peaks (Fig. 2). It is not known whether these peaks represented reactions to different antigens or to similar antigens complexed to components which migrated differently. Precipitation peaks were never observed with preimmune rabbit serum and heart tissue extracts in two-dimensional immunoelectrophoresis experiments.

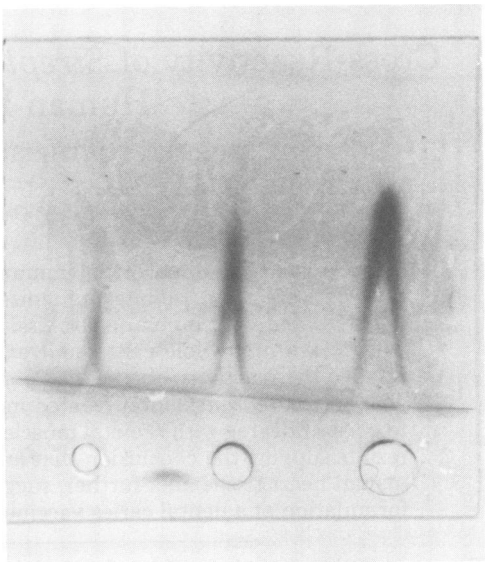

FIG. 1. *Rocket immunoelectrophoresis of increasing amounts of human heart tissue extract and S. mutans B13 antiserum. The concentrated antiserum was equivalent to 3.0 ml of immune serum, and the amounts of protein of the heart tissue extracts were, from left to right, 20, 100, and 300 μg.*

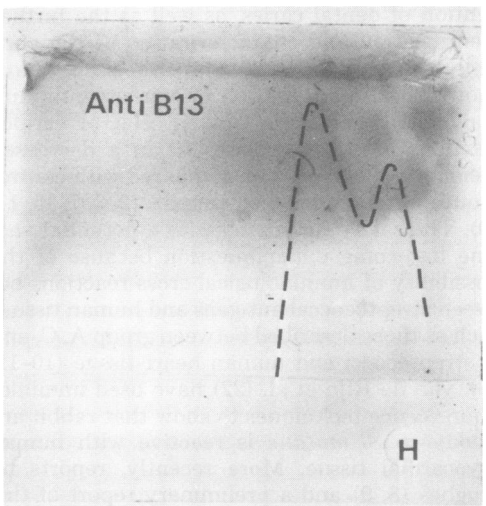

FIG. 2. *Two-dimensional immunoelectrophoresis of human heart tissue extract and S. mutans B13 antiserum. The concentrated antiserum was equivalent to 2.0 ml of immune serum, and the heart tissue extract (H) contained 150 μg of protein.*

To obtain information concerning the specificity of the *S. mutans* antibody for human heart tissue, an absorption experiment was performed.

An anti-*S. mutans* serum sample was absorbed by using a combination of *S. mutans* whole cells and broken cell fragments and allowed to incubate for 1 h at 37°C followed by 2 h at 4°C. The absorbing material was removed by centrifugation, and the procedure was repeated an additional four times with fresh absorbing material and the same serum sample. When the absorbed antiserum was used in rocket or two-dimensional immunoelectrophoresis experiments, no precipitin peaks were observed with human heart tissue extract.

The repeated absorptions of the *S. mutans* heart-reactive antibody with *S. mutans* cells raised the possibility that nonspecific absorption of antibody was taking place. To determine whether the absorption was of a specific or a nonspecific nature, an additional experiment was performed utilizing a known antigen and corresponding antibody as a marker. To the *S. mutans* B13 antibody was added a known amount of rabbit anti-bovine serum albumin (BSA), and rocket immunoelectrophoresis was performed with BSA in one well and heart tissue antigen in another well. In each case a precipitin peak was formed (Fig. 3a). An absorption experiment utilizing the combined anti-BSA and anti-*S. mutans* sera was performed, and the absorbed antiserum was subjected to rocket immunoelectrophoresis with BSA in one well and heart tissue extract in another well. A precipitin peak was observed with BSA; however, no precipitin peak occurred with heart tissue extract (Fig. 3b). The precipitin peak observed with BSA and the absorbed antiserum was taller than that seen with the nonabsorbed antiserum even though the same amount of antigen had been used in both experiments. The presence of a taller precipitin peak suggested that less antibody was present in the absorbed serum, or perhaps that the antibody present was diluted by the repeated exposure to *S. mutans* cells and fragments. The amount of antibody that was non-specifically removed was estimated from plots of peak heights of various BSA and anti-BSA concentrations. Approximately 30% of anti-BSA was removed as a result of nonspecific absorption or dilution. Removal of this amount of anti-*S. mutans* serum in subsequent control experiments did not eliminate the reactivity of the antiserum to heart tissue antigen.

To determine the specificity of the antibody obtained from rabbits immunized with *S. mutans*, rocket immunoelectrophoresis experiments were performed with heart, skeletal muscle, liver, and kidney tissue (Fig. 4). Precipitin peaks were observed with heart tissue extract, which gave the most intense reaction, and to a lesser extent with skeletal muscle. Essentially no reactions were observed with liver or kidney antigen preparations. The faint reactions seen with these two tissues were nonspecific in nature since they were also observed in the absence of immune serum.

DISCUSSION

The results of rocket and two-dimensional immunoelectrophoresis experiments indicate that *S. mutans* possesses antigenic determinants similar to those found in human heart tissue.

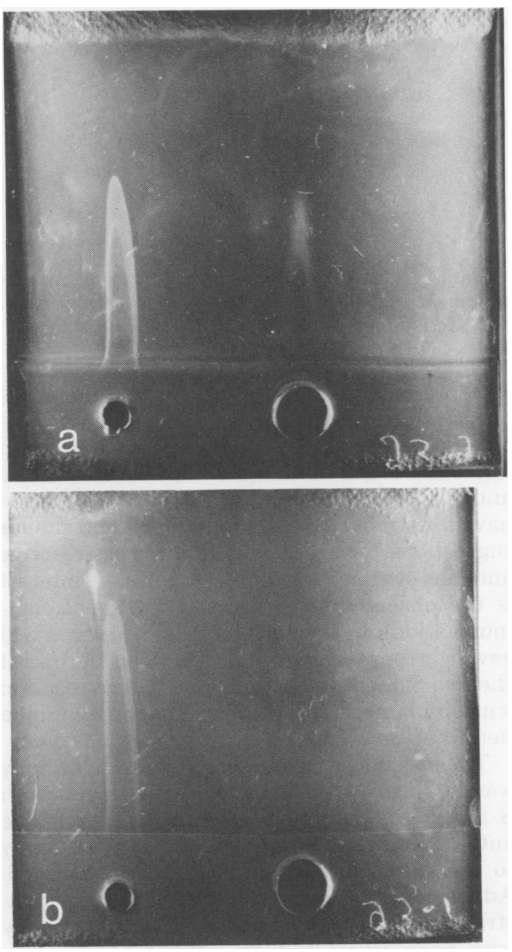

FIG. 3. *Absorption of human heart tissue cross-reactive S. mutans antiserum with S. mutans cells. The concentrated S. mutans antiserum was equivalent to 3.0 ml of immune serum to which was added 0.2 ml of rabbit anti-BSA. The heart tissue extract contained 215 μg of protein, and BSA contained 1 μg of protein. (a) Rocket immunoelectrophoresis of S. mutans and BSA antiserum to BSA (left well) and heart tissue extract (right well). (b) Rocket immunoelectrophoresis of S. mutans and BSA antiserum absorbed with S. mutans cells; BSA (left well) and heart tissue extract (right well).*

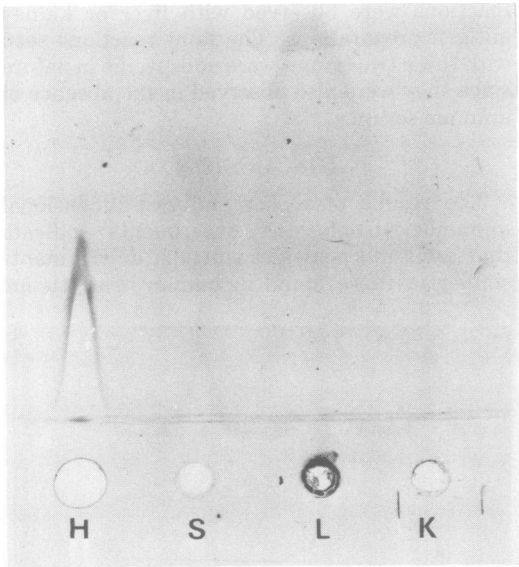

FIG. 4. *Rocket immunoelectrophoresis of human tissue extracts and S. mutans B13 antiserum: heart (H), skeletal muscle (S), liver (L), and kidney (K).*

The presence of two or more precipitin peaks observed with two-dimensional immunoelectrophoresis suggests the possibility of more than one cross-reactive antigen. Two cross-reactive antigens of *S. mutans* and human heart tissue have been reported by Hughes (8, 9) and similar suggestions have been made for cross-reactive antigens of group A streptococci (16). Of interest is the observation by Hughes (personal communication) that our antibody to *S. mutans* B13 gave a strong reaction to his antigen IF, one of the two *S. mutans* antigens that gave a reaction with immune antisera raised against human heart antigen.

The specificity of the heart-reactive antibody was ascertained by absorption experiments with *S. mutans* cells in which all of the heart-reactive antibody was removed but most of the antibody to a known antigen marker (BSA) remained. Additionally, tissue specificity was demonstrated by the reaction of *S. mutans* antibody with heart tissue and, to a lesser degree, with skeletal muscle, but not liver or kidney. These observations are in agreement with previous reports which have indicated that antibody to *S. mutans* or group A streptococci is cross-reactive with only heart or skeletal muscle (22).

The heart-reactive antibody appeared to possess low avidity since it was usually necessary to concentrate immune sera threefold to observe precipitin peaks with cross-reactive antigens. Additionally, repeated absorptions with *S. mutans* cells were required to remove the heart-reactive antibodies from serum samples. An explanation of low antibody avidity would be consistent with clinical observations that no adverse effects are observed in individuals known to possess circulating heart-reactive antibody.

We have taken several steps to eliminate the possibility of nonspecific interactions being responsible for the observed cross-reactions in this study. First, the organisms were grown in a chemically defined medium and the cells were thoroughly washed before immunization. These procedures eliminated the possibility that media components adhering to the organisms may have served as sources of contaminating cross-reactive material. Additionally, all heart tissue samples were obtained as sterile specimens from surgical pathology and were tested further for aerobic and anaerobic bacterial growth. Of 11 heart tissue samples tested, only 1 resulted in positive bacterial growth, and this sample was deleted from the study. Heart tissue samples obtained at autopsy, on the other hand, always had bacteria associated with the sample. Since autopsy heart tissue samples could possibly contain bacterial antigens reactive with antibody to *S. mutans*, this source of heart tissue was omitted from this study. Finally, in another communication we will present evidence that *S. mutans*, unlike group A, C, and G streptococci (14), does not react with nonimmune rabbit or human immunoglobulin G. These observations preclude explanations which suggest that apparent cross-reactions are a result of nonspecific interactions with Fc receptors on the surface of *S. mutans*.

Of some general interest and concern was the observation that a number of preimmune rabbit serum samples gave either faint or irregularly shaped precipitin peaks with heart tissue extract in rocket immunoelectrophoresis experiments. Whether these peaks represented the presence of heart-reactive antibody Fc reactivity of tissue components, or nonspecific aggregates of tissue and serum components is not clear. It is possible that a previous antigenic experience of the rabbits used to produce antibody may have been sufficient to allow the formation of a class of cross-reactive antibodies to a wide variety of antigens.

The results of this study, using rocket and two-dimensional immunoelectrophoreses, support the conclusion of van de Rijn et al. (22) that antigens of *S. mutans* cross-react with mammalian heart tissue. A similar conclusion was also reached by Hughes (8, 9), who used several different techniques to demonstrate immunological cross-reactions between *S. mutans* and mammalian tissues. Although the nature, mechanism, and significance of these apparent immunological cross-reactions remain to be eluci-

dated, there appears to be sufficient reason to exercise caution in the formulation of a dental caries vaccine containing *S. mutans* antigens.

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