Cross-Reactions of *Streptococcus mutans* Due to Cell Wall Teichoic Acid

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Antisera to the whole cells of *Streptococcus mutans* cross-reacted with antigen extracts from four other gram-positive species, as well as with those of three other oral streptococci. Similarly, antisera to these bacteria cross-reacted with extracts from *S. mutans* and with those from each other. Using a purified phenol extract of the walls of *S. mutans*, which was identified by chemical, immunochmical, and enzymatic analyses as glycerol teichoic acid, the cross-reactions were shown to be specific for a determinant of the teichoic acid backbone. Results were confirmed in immunodiffusion tests where clear bands of identity were shown. These observations point out the need for caution in serological research employing extracts of gram-positive bacteria and may be of interest in investigations of periodontal disease.

The prevalence of teichoic acids in gram-positive bacteria has been amply demonstrated (reviewed in reference 1) and their importance as antigens is well known (reviewed in reference 20). We have shown that humoral (7) and cell-mediated (3, 11) responses to the polyglycerolphosphate (PGP) backbone of teichoic acid occur frequently in normal individuals of several species. McCarty (21) first reported that cross-reactions between antisera to group A streptococci and hot acid extracts from a number of other gram-positive bacteria were of PGP specificity; however, he was unable to demonstrate cross-reactions with a number of gram-positive bacteria, including many streptococci. Stewart (31) confirmed the presence of a PGP determinant in certain gram-positive cocci. Recently, cross-reactions between antisera to group A streptococci and lactobacilli were shown to be partially inhibited by PGP (19) and by a phospholipid (cardiolipin) stripped of its fatty acids (33). Efforts to demonstrate identity in cross-reactions between gram-positive bacteria by immunodiffusion were reported by Sharpe et al. (27) and by Mukasa and Slade (26). In the former work, cross-reactions were demonstrated between an antiserum to *Lactobacillus acidophilus* and acid extracts of some gram-positive bacteria but were not observed with a number of others, including *Bacillus* species and 43 strains of streptococci. In the latter study, reactions of identity were shown between *L. fermenti* and examples of the four serotypes of *Streptococcus mutans* (*a, b, c, and d*).

Gram-positive bacteria are a major part of the oral flora, and streptococci have been implicated in dental caries and periodontal diseases (9, 28). Therefore, the demonstrated immunological impact of teichoic acids (3, 7, 11) and their cross-reactions have important implications in studies of these diseases. Yet, cross-reactions between many oral bacteria and with other gram-positive bacteria have not been established. We have investigated some of these organisms, using *S. mutans* as the reference organism because of its association with both diseases (12). In this connection, we had previously prepared purified cell walls of the latter organism and characterized it chemically (6).

**MATERIALS AND METHODS**

**Organisms.** *S. mutans* strain BHT was used for the preparation of purified cell walls, from which the purified teichoic acid was extracted. Other organisms used in this study were *S. mutans* strain AHT and *Streptococcus salivarius* strain HHT (38). In addition, a non-cariogenic unclassified *Streptococcus*, strain CHT (Zinner et al., 35), *Streptococcus pyogenes* (OSU 367), *Staphylococcus aureus* (FDA 209), *Bacillus cereus* (ATCC 9634), and a group 3 *Bacillus* sp. (OSU 372) were employed. The latter organism has been characterized (5). Cultures of the streptococci were grown at 37 C with stirring but no aeration for 24 h in Microferm fermenters (M-F-14, New Brunswick Scientific Co., New Brunswick, N.J.) containing 11 liters of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% dextrose. The bacilli were cultured in Trypticase soy broth (Baltimore Biologicals, Baltimore, Md.) for 48 h at 30 C with stirring and vigorous aeration. *S. aureus* was grown on Trypticase soy broth at 37 C.
with stirring and aeration for 24 h. Each fermenter was inoculated with 10 ml of a broth culture containing about 2.8 \times 10^{14} organisms.

Cells were harvested at 4 C by continuous-flow centrifugation (27,000 \times g) in a Sorvall RC-2-B centrifuge (Ivan Sorvall, Norwalk, Conn.). The harvested cells were washed three times with double-distilled demineralized (DDD) water and stored in an aqueous suspension (approximately 50\%, vol/vol) in the refrigerator for no more than 24 h before use.

**Preparation of cell walls.** Cell walls of *S. mutans* strain BHT were prepared as previously described (6).

A 5\% (wt/vol) aqueous suspension of bacterial cells was disrupted at 60,000 lb/in² by using a Ribi cell fractionator (Ivan Sorvall, Norwalk, Conn.). This suspension of disrupted cells was centrifuged (10,000 \times g, 20 min) and the cell lysate was discarded. The sediment was washed twice with 1 M NaCl and translucent cell wall fraction was recovered. This was layered over a linear sucrose gradient (40 to 70\%, vol/vol) in the refrigerator (approximately 50\%, vol/vol) in the refrigerator. The supernatant was discarded. The final product was then washed free of sucrose with DDD water and lyophilized.

**Antigen extraction and purification.** Phenol extraction of purified *S. mutans* strain BHT cell walls and of washed whole cells of all other organisms was performed by the method of Moskowitz (25). All extracts were dialyzed three times against DDD water and lyophilized. Lyophilized phenol extracts of *S. mutans* strain BHT cell walls and *Bacillus* sp. (OSU 372) were purified according to the procedure reported by Deck er et al. (7) for purifying bacillary teichoic acid.

**Synthetic PGP.** The synthetic polymer used in inhibition tests was prepared by the method of McCarty (21) and had been estimated to average four or five residues in length (10).

**Chemical analysis of the *S. mutans* antigen.** The purified antigen was analyzed for total hexose by the anthrone method of Steinecker and Rheins (30), for total hexosamine by the method of Boas (2), and for total pentose by the cysteine-sulfuric acid method of Dische (8). Glycerol and individual pentoses, hexoses, and hexosamines were identified by gas-liquid chromatography using a Varian Aerograph model 204-1 apparatus (Varian Instruments, Palo Alto, Calif.). Amino acids were identified and quantitated on a model 116 amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.). Total nitrogen content was determined by the Kjeldahl method (23). Glycerol was determined by the glycerol dehydrogenase assay detailed in the Worthington Biochemical Enzyme Manual. Samples were prepared for the glycerol assay as described by Deck er et al. (7). Total organic phosphorus was determined by the method of Chen et al. (4).

**Enzymatic assay of the purified antigen.** The purified antigen was treated with trypsin by mixing 0.1 mg of antigen with 1 mg of twice-crystallized enzyme (Sigma Chemical Co., St. Louis, Mo.) in 0.2 ml of 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.7, 0.05 M CaCl₂). After 24 h of incubation at 37 C, the mixture was dialyzed three times against DDD water to remove Ca²⁺ ions and diluted to 1 ml with DDD water. Separate 0.1-mg samples of antigen were similarly treated with 1 mg of twice-crystallized ribonuclease and deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.), except that CaCl₂ was not used. Other samples of antigen were treated with tetrachioracetic acid which was extracted from a soil bacterium (strain TAE) as described by Wise et al. (34). These authors kindly supplied the organism and extraction of the enzyme was carried out by their method. Assays were performed on 0.1-mg quantities of antigen with 0.1 mg of enzyme in 1 ml of maleate buffer (1 mM sodium maleate, 1 mM ethylenediaminetetraacetic acid; pH 6.5) with incubation at 37 C for 24 h. After incubation the mixture was dialyzed three times against DDD water and adjusted to a final volume of 1.0 ml. Periodate treatment of the purified antigen was carried out as described by Hofstad (13). After treatment, the mixture was dialyzed three times against DDD water and the final volume was adjusted so that the concentration of the antigen would be 100 \(\mu g/ml\) (this was based on the original weight of the extract).

**Antiserum.** Antisera were raised in 3- to 5-kg albino rabbits by injecting suspensions of whole heat-killed bacteria (1.2 \times 10⁸ organisms) in Freund complete adjuvant by both the footpad and subcutaneous routes. After 6 weeks, the animals were given an additional injection of 1.2 \times 10⁸ organisms by the intravenous route. Antisera were collected 5 days after this injection was given.

**Serologic assays.** For passive hemagglutination tests, suspensions of washed rabbit erythrocytes were standardized spectrophotometrically at 1.8 \times 10⁹ cells/ml (5% by volume) in phosphate-buffered saline and coated by incubating them with optimal concentrations of antigen (50 \(\mu g/ml\)) at 35 C for 25 min. Optimal antigen concentrations were determined by checkerboard titrations (5). After coating, erythrocytes were washed three times in cold phosphate-buffered saline and resuspended to a concentration of 1%. Coated cells were kept in the refrigerator or in an ice bath and discarded after 1 day of use. Testing was carried out in agglutination tubes (9 by 75 mm) using 0.05 ml of coated cell suspension and 0.05 ml of serum dilutions. Mixtures were incubated at 5 C for 30 min, centrifuged lightly for 15 s, and examined microscopically. Uncoated cells and a standard antiserum of specificity for PGP were included in all runs as controls.

Tests for inhibitors of the passive hemagglutination reaction were performed by mixing a selected concentration of the substance under test with varying arithmetic dilutions of antiserum (10\% intervals). Such tests were carried out for a series of inhibitor concentrations, so that the percentage of inhibition at a given concentration could be plotted on a 10-point scale. Antiserum and inhibitor were incubated at room temperature for 30 min, after which coated cells were added and the tests were reincubated at 5 C for 30 min. Immunofluorescence tests were performed in petri plates containing 5 ml of 1% lisonar no. 2 gel (Consolidated Laboratories, Inc., Chicago Heights.

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whole S. mutans were synthetic PGP reactants in test antiserum concentrations were essentially equivalent with the test antiserum by preliminary immunodiffusion tests.

RESULTS

The cell walls from S. mutans strain BHT were composed of 6.8% protein, 8.9% glycerol teichoic acid, 33.6% non-peptidoglycan polysaccharide, and 49.9% peptidoglycan, as previously reported (6). The purified phenol extract of these walls yielded the analysis shown in Table 1. It can be seen that glycerol and phosphorus were essentially equimolar and that 86% of the purified antigen appeared to be PGP. Results of inhibition tests (Fig. 1) confirm that PGP is the antigenic determinant responsible for passive hemagglutination reactions with the purified antigen, since complete inhibition was produced by both the synthetic polymer and by monomeric α, β-glycerophosphate. It is interesting that the purified antigen from whole baccillary cells also completely inhibited the reaction of anti-BHT antisera (Fig. 2). Furthermore, when the wall-associated sugars and amino acids (glucose, galactose, glucosamine, galactosamine, alanine, lysine, and glutamic acid) were tested, no inhibition was produced by amounts up to 2.5 mg/test, even though only 1 mg of monomeric glycerophosphate was required for complete inhibition. In gel precipitation tests, similar results were obtained (Fig. 3). Synthetic PGP, α,β-glycerophosphate and a purified antigen from Bacillus sp. OSU 372 inhibited the reaction between purified BHT antigen and antiserum to strain BHT. Further evidence as to the nature of the purified antigen from S. mutans strain BHT is furnished by the observation that activity of the antigen in immunodiffusion tests was not impaired by treatment with trypsin, ribonuclease, deoxyribonuclease, or periodate, but was abolished by treatment with teichoicase (Fig. 4).

Antisera to whole cells of S. mutans strain BHT cross-reacted in passive hemagglutination tests with extracted antigens of all other gram-positive organisms which were tested (Table 2). Likewise, antisera to the other organisms tested cross-reacted with the antigens of each organism. A particular antigen yielded the same titer regardless of the source of the antigen extract used to coat the test erythrocytes. These results were confirmed in immunodiffusion tests (Fig. 5), where clear bands of identity were

TABLE 1. Chemical analysis of purified phenol extract from cell walls of S. mutans

<table>
<thead>
<tr>
<th>Extract</th>
<th>μg/mg</th>
<th>μmol/mg</th>
</tr>
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<tbody>
<tr>
<td>Glycerol</td>
<td>451.0</td>
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<tr>
<td>Phosphorous</td>
<td>145.9</td>
<td>4.65</td>
</tr>
<tr>
<td>Hexose</td>
<td>62.0</td>
<td>0.34</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>23.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.0</td>
<td>0.06</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.0</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Fig. 1. Inhibition of passive hemagglutination by synthetic PGP and monomeric glycerophosphate. Erythrocytes were coated with purified teichoic acid from S. mutans walls and the antisera were raised to whole S. mutans cells in rabbits.

Fig. 2. Inhibition of passive hemagglutination by purified teichoic acid from Bacillus sp. (OSU 372), as compared to inhibition by purified teichoic acid from S. mutans strain BHT.
shown between the purified *S. mutans* antigen, purified bacillary antigen, and phenol extracts of the oral streptococci, *B. cereus*, *S. aureus*, and *S. pyogenes*.

**DISCUSSION**

On the basis of chemical analyses and enzymatic tests, the purified antigen employed in this study was shown to be a glycerol teichoic acid. These assays, coupled with the lack of any inhibition by wall-associated sugars or amino acids, establish the fact that the usual teichoic acid substituents were not involved in the reactions being studied. Complete inhibition of both passive hemagglutination and precipitation reactions by the synthetic polymer confirm the observation that the reactions were specific for the PGP backbone.

The presence of a glycerol teichoic acid in the walls of *S. mutans* strain BHT was reported by Vaught and Bleiweiss (32). These investigations confirm that finding and reveal that an important antigenic determinant of the *S. mutans* cell wall is associated with the PGP backbone of the teichoic acid, since injection of whole organisms produced considerable antibody of this specificity. The fact that this antibody cross-reacts with all other streptococci tested suggests caution when preparing monospecific antisera to other streptococcal antigens and is likely to

**TABLE 2. Cross-reactions (passive hemagglutination titers) between extracted antigens of streptococci, staphylococci, and bacilli**

<table>
<thead>
<tr>
<th>Antigens</th>
<th>AHT*</th>
<th>BHT</th>
<th>CHT</th>
<th>HHT</th>
<th>B-372</th>
<th><em>S. aureus</em></th>
<th><em>B. cereus</em></th>
<th><em>S. pyogenes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-AHT</td>
<td>512</td>
<td>521</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>Anti-BHT</td>
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<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>Anti-CHT</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>Anti-HHT</td>
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<td>512</td>
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<td>512</td>
<td>512</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>Anti-B-372</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
</tr>
</tbody>
</table>

*Antigens of AHT, CHT, HHT, *S. aureus*, *B. cereus*, and *S. pyogenes* were unpurified phenol extracts. Antigens of BHT and *Bacillus* sp. OSU 372 were purified phenol extracts. Test antigens were coated on rabbit cells. Antisera were prepared in rabbits by injection of whole bacterial cells.
Fig. 5. Cross-reactions in immunodiffusion tests between all eight gram-positive organisms included in this study: S. mutans strains AHT and BHT; noncariogenic streptococcus, CHT; S. salivarius strain HHT; Bacillus sp., OSU 372, (B-372); Bacillus cereus (B. cer); Staphylococcus aureus (S. aur); and S. pyogenes (S. pyog). Rabbit antisera were prepared against whole cells of AHT (a), BHT (b), CHT (c), HHT (d), and B-372 (e).

make preparation of potent antisera to substituent sugars of the teichoic acid difficult. It is significant that many methods for extraction of antigens (e.g., acid and heating) produce considerable levels of PGP in the extracts. In any case, employment of controls using purified PGP should be valuable in determining the specificity of such antisera.

Another area in which the demonstrated cross-reactions may be pertinent is in the etiology of periodontal disease. A number of studies (9, 28) have implicated different gram-positive bacteria in this disease, and it has been suggested that immunological injury may be an important factor (14–18, 22, 24). Therefore, since it would appear that multiple etiologic agents are involved, it could be assumed that different antigens may induce the single disease entity. The present observations demonstrate, however, that a single cross-reacting antigen, found in oral bacteria, could be involved. The present observations also expand on those of McCarty (21) relative to the occurrence of cross-reactions between most gram-positive bacteria due to the PGP backbone of the teichoic acids. We have demonstrated identity of precipitation bands between a variety of gram-positive bacteria and have also shown that these reactions are of PGP specificity. It is significant that antisera raised to each of the organisms produced such cross-reactions with each of the others.

LITERATURE CITED
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