Purification and Immunochemical Studies of Type b Carbohydrate Antigen of Oral Streptococcus milleri

TSUYOSHI YAKUSHIJI, MASAKAZU INOUE, AND TOSHIHIKO KOGA

Department of Preventive Dentistry, Kagoshima University Dental School, 1208-1 Usuki-cho, Kagoshima 890, and Department of Dental Research, National Institute of Health, 2-10-25 Kamiyosaki, Shinagawa-ku, Tokyo 141, Japan

Received 25 February 1988/Accepted 24 May 1988

The type-specific antigen of serotype b Streptococcus milleri was extracted with trichloroacetic acid from a purified cell wall preparation of the strain NCTC 10708 and then purified on a DEAE-Sephadex A-25 column. The antigen was composed of rhamnose and glucose in a molar ratio of 1.7:1.0, with a trace of galactosamine (0.1). The quantitative precipitin inhibition test with various haptenic sugars showed that rhamnose gave the greatest inhibition, whereas glucose and its related carbohydrates were less effective. The major carbohydrate components of the Rantz-Randall extracts from cells of all four serotype b strains tested were also rhamnose and glucose. These results suggest that rhamnose is structurally involved in the immunodeterminant of the serotype b-specific antigen of oral S. milleri.

Since Streptococcus milleri was originally isolated from human dental abscesses by Guthof (13), the etiologic role of the streptococcal species in systemic infections has been increasingly appreciated (1, 15, 24, 28, 30). The streptococcal species is found among the normal human flora (2, 26), and one of its habitats is the dentate oral cavity, especially dental plaque (3, 10, 19, 25).

S. milleri is serologically unique among streptococcal species. Some strains of the streptococcus, even if they are nonhemolytic, have been shown to carry one of the Lancefield group antigens, A, C, F, or G, and other strains, even if hemolytic, do not possess any group antigens (4, 5, 18, 28). Furthermore, as Ottens and Winkler (23) first demonstrated, significant numbers of the group F, C, and G streptococcal strains, which can now be considered S. milleri species morphologically and physiologically, carry the carbohydrate antigens, designated I to V, apart from the Lancefield group antigens. Michel and co-workers (20, 31–34) have extended the studies of Ottens and Winkler to immunochemically characterize the type antigens. Meanwhile, many ungroupable and untypeable strains have been isolated from various clinical specimens (4, 18).

We have previously classified S. milleri strains isolated from human dental plaque into 10 serotypes, a to j, based on the serological specificities of the cellular antigens (T. Yakushiji, R. Konagawa, M. Oda, and M. Inoue, J. Med. Microbiol., in press). As a first attempt to establish the serotyping of oral S. milleri, serotype b-specific antigen was purified and its immunochemical properties were examined.

MATERIALS AND METHODS

Strains. S. milleri NCTC 10708 (biotype Ia [T. Yakushiji, M. Katsuki, A. Yoshimitsu, J. Mizuno, and M. Inoue, Microbiol., in press]) and three serotype b strains (Yakushiji et al., J. Med. Microbiol., in press) isolated from the mouth were used. They were grown in BH1 broth (Difco Laboratories, Detroit, Mich.) anaerobically at 37°C for 18 h, washed three times with distilled water, and lyophilized as described previously (Yakushiji et al., J. Med. Microbiol., in press).

Preparation of purified cell walls. Purified cell walls of NCTC 10708 were prepared as described by Okahashi et al. (22). Briefly, whole cells were disrupted in a Braun cell homogenizer (model MSK; B. Braun Apparatebau, Münsingen, Federal Republic of Germany) with glass beads (0.17 to 0.18 mm in diameter). The broken-cell suspension was separated from glass beads by decantation, and the cell walls were precipitated by centrifugation (26,000 × g for 60 min at 4°C). The crude cell wall preparation was treated with 40 mg of trypsin (1:250, Difco) in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0, at 37°C for 2 h. The cell walls collected by centrifugation were extensively washed with distilled water and lyophilized (designated purified cell wall preparation). The yield of the purified cell walls was 1.6 g from 47.7 g (wet weight) of whole cells.

Extraction of carbohydrate antigens. Preliminary experiments showed that extraction with hot trichloroacetic acid (TCA) at 90°C for 15 min destroyed immunological activity. Then, the purified cell walls (1.4 g, dry weight) were extracted in 50 ml of 5% TCA at 4°C for 6 h with continuous stirring. The reaction mixture was centrifuged (15,000 × g for 20 min at 4°C) to collect the supernatant, and the extraction procedure was repeated three times on the residual cell walls. The supernatants obtained by four successive extractions were combined, neutralized with 1 N NaOH, and dialyzed against distilled water. The nondialyzable portion of extracts was concentrated to about one-fourth of its original volume with a rotary evaporator at 50°C. Five volumes of acetone were added to one volume of the concentrated solution and stored at −20°C for 18 h. The precipitated cell wall carbohydrates were collected by centrifugation (25,000 × g for 30 min at 4°C) and dissolved in 10 ml of distilled water.

Serotype antigens were also extracted from whole cells by the method of Rantz and Randall (27). Whole cells (40 mg, dry weight) were heated at 121°C for 20 min in 2 ml of saline and then removed by centrifugation. The supernatant was dialyzed against distilled water and lyophilized.

Purification of type antigen. The crude carbohydrate preparation (10 ml) was applied to a DEAE-Sephadex A-25...
FIG. 1. DEAE-Sephadex A-25 ion-exchange chromatography of the TCA extract of NCTC 10708 cell walls. The column (1.5 by 45.5 cm) was eluted first with 0.05 M (NH₄)₂CO₃, followed by a linear gradient of 0 to 1 M NaCl in the same buffer. Pi, Phosphorus.

(Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.5 by 45.5 cm). The column was eluted first with 0.05 M (NH₄)₂CO₃ (100 ml) followed by a linear gradient of 0 to 1 M NaCl (150 plus 150 ml) in the starting solution. The fractions (11 ml each) that gave a positive reaction with the type b-specific antiserum were combined, dialyzed against distilled water, and finally concentrated in a rotary evaporator. The immunologically active concentrate (5 ml) was applied to a Sephadex G-100 (Pharmacia) column (1.5 by 45 cm) and eluted with distilled water. The fractions (2.8 ml each) reactive with the antiserum were combined and lyophilized. The yield of purified carbohydrate antigen was 44 mg from 1.4 g of cell walls.

Chemical analyses. Neutral sugars and amino sugars were quantitatively analyzed by gas-liquid chromatography by the method of Griggs et al. (12) with some modifications. Briefly, the purified type b antigen (1 mg), the purified cell wall preparation (1 mg), or the Rantz-Randall antigen (5 mg), together with inositol (0.5 mg) as an internal standard, were hydrolyzed in 2 ml of 3 N HCl at 100°C for 4 h. After the hydrolysates were neutralized with Ag₂CO₃, alditol acetate derivatives were obtained by treatment first with sodium borohydride and then with acetic anhydride. The alditol acetates were analyzed on a glass column (3 mm by 2 m) of 0.2% ethylene glycol succinate (EGS) plus 0.2% ethylene glycol adipate (EGA) plus 1.4% XE-60 on Gas chrom P (100/120 mesh; Gasukuro Kogyo Inc., Tokyo) mounted in a gas-liquid chromatograph (GC-4CPF; Shimadzu Works, Kyoto) fitted with dual hydrogen-flame ionization detectors. The initial temperature of 150°C was increased at 1°C/min to 205°C.

The amounts of phosphorus and protein were determined by the methods of Lowry et al. (16) and Lowry et al. (17), respectively. Total hexoses and N-acetylhexosamines were estimated by the anthron method (28) and by the method described by Ghuyzen et al. (11), respectively.

Immunological methods. The type b-specific antiserum was prepared by immunizing rabbits with whole cells of S. milleri NCTC 10708 as described previously (Yakushiji et al., J. Med. Microbiol., in press). Reactivities of the carbohydrate antigen preparations with the type-specific antiserum were determined in capillary tubes and by immunodiffusion in an agar gel. Immunodiffusion was performed in a 1% Noble agar (Difco) gel in 0.01 M phosphate buffer (pH 7.2) as described previously (Yakushiji et al., J. Med. Microbiol., in press).

The quantitative precipitin reaction and its inhibition by haptenic sugars were carried out as described by Hamada and Slade (14). To obtain the quantitative precipitin curve of the reaction between the purified type b antigen and the type-specific serum, 5-μl portions of serum and 0 to 30 μg of antigen in the reaction mixture adjusted to 50 μl with saline were incubated at 37°C for 1 h and then stored at 4°C for 24 h. For the hapten inhibition test, the serum was preincubated with 20 μmol (unless otherwise described) of hapten in a total volume of 30 μl at 37°C for 1 h, and then the antigen was added.

RESULTS

Purification of type b-specific antigen. Cold TCA extracts of purified NCTC 10708 cell walls were fractionated on a DEAE-Sephadex A-25 column (Fig. 1). A single carbohydrate peak was obtained, which was adsorbed on the column and eluted at 0.38 M NaCl. Only the peak fractions reacted with type b-specific antiserum. The antigenic component was primarily composed of hexose moieties, and small amounts of phosphorus were detected in the peak fractions. No protein was detected in any fraction.

Fractions (no. 18 to 22) reactive with the antiserum were combined, concentrated, and then applied to a Sephadex G-100 column (Fig. 2). The elution profile indicated a single asymmetrical peak composed of hexose and phosphorus (trace). All the fractions were reactive with the type-specific antiserum. These fractions were combined and lyophilized and are hereafter designated type b antigen.

Immunochromatographic characterization of type b antigen. The immunodiffusion test (Fig. 3) revealed that precipitin lines of the Rantz-Randall antigen, the TCA extract, and the type b antigen with the type-specific antiserum were completely fused, indicating that all the preparations contained an identical determinant.
Chemical analyses revealed that the purified cell walls of strain NCTC 10708 contained significant amounts of various sugars, amino sugars, and peptide (Table 1). However, the purified type b antigen was composed of only rhamnose, glucose, and galactosamine in a molar ratio of 1.7:1.0:0.1. Neither glycerol nor ribitol was detected. The amounts of protein and phosphorus were also negligible.

The maximum amount of antibody protein was precipitated when 2 µg of the type b antigen was reacted with 5 µl of the antisera in the reaction mixture (Fig. 4). As shown in Table 2, the quantitative precipitin inhibition test with various haptenic sugars showed that among the sugar components of the purified antigen, rhamnose was the most effective inhibitor, whereas glucose and its related disaccharides were less effective. The precipitin inhibition test with various amounts of the purified antigen components, rhamnose, glucose, and galactosamine confirmed that rhamnose was the most effective inhibitor at all concentrations tested, although glucose also showed significant inhibition at higher concentrations (Fig. 5).

The sugar and amino sugar compositions of the Rantz-Randall extracts from whole cells of the four serotype b strains, including NCTC 10708, are summarized in Table 3. The extracts from all strains tested contained rhamnose, glucose, and galactosamine in an average molar ratio of 1.9:1.0:0.2. Significant amounts of ribitol and phosphorus were also detected in the extracts of all strains.

**DISCUSSION**

Wide serological varieties of oral S. milleri strains have been demonstrated in our previous studies (Yakushiji et al., J. Med. Microbiol., in press). Strain NCTC 10708, chosen first in the present study, is one of the representative S. milleri strains (5, 9, 18, 19) from which serotype b antigen was extracted with cold TCA, purified through column chromatography, and characterized immunochemically.

Type b antigen was composed mainly of rhamnose and glucose, with galactosamine as a minor component (Table 1). Among the major sugars constituting the type antigen,

**TABLE 1. Chemical composition of purified NCTC 10708 cell walls and purified type b antigen**

<table>
<thead>
<tr>
<th>Component</th>
<th>Content (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified cell walls</td>
</tr>
<tr>
<td>Glucose</td>
<td>49 (1.0)</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>425 (9.5)</td>
</tr>
<tr>
<td>Galactose</td>
<td>79 (1.6)</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>98 (2.0)</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>140 (2.9)</td>
</tr>
<tr>
<td>Peptide</td>
<td>210 (&lt;0.05)</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1 (&lt;0.05)</td>
</tr>
</tbody>
</table>

* Glycerol and ribitol were not detected.
* Numbers in parentheses indicate molar ratio to glucose.
* As glycine.
rhamnose exhibited the highest inhibition at all concentrations tested in the quantitative precipitin reaction (Table 2 and Fig. 5), suggesting that the immunodeterminant of the type b antigen of \textit{S. milleri} is rhamnose. All the Rantz-Randall antigens extracted from the four serotype b strains, including strain NCTC 10708, had a similar carbohydrate composition (Tables 1 and 3), indicating the validity of the proposed chemical background of the immunodeterminant of serotype b \textit{S. milleri}.

It is well known that \textit{S. milleri} strains often carry the Lancefield group antigens and/or the Ottens type antigens (23). It has been demonstrated that rhamnose is structurally involved in the immunodeterminant of the group B and G antigens (6, 7). Curtis and Kraus (7) have demonstrated that the group B antigen is composed of rhamnose, galactose, and glucosamine (molar ratio, 5:1:1) and the group G antigen consists of rhamnose, galactose, and \textit{N}-acetylgalactosamine (2:1:1) and that both antigens possess a rhamnose residue as a terminal immunodeterminant, although they are not serologically identical. It is apparent that the sugar compositions of the serotype b antigen of \textit{S. milleri} (Table 1) are not completely identical to those of the group antigens. We have previously shown that the type b antigen is serologically distinguishable from the group antigens A to G (Yakushiji et al., J. Med. Microbiol., in press).

Furthermore, the immunochemical difference of the serotype b antigen from any one of the Ottens type antigens is also indicated by their chemical composition and reaction to the haptenic sugars in the precipitin inhibition test. Immunodeterminants of the Ottens type antigens have been suggested to be \textit{N}-acetylgalactosamine for the type I and II antigens (20, 33), glucose for the type III and V antigens (32, 33), and galactose for the type IV antigen (31, 33). Consequently, the type b antigen of \textit{S. milleri} is considered a unique antigen, different from any group or type antigen previously detected in \textit{S. milleri} strains.

Significant amounts of ribitol and phosphorus were detected in the Rantz-Randall extracts of the four serotype b strains tested (Table 3). Although chemical and immunological confirmations have not yet been done, the findings suggest that serotype b \textit{S. milleri} carries ribitol teichoic acid in the cell wall as a common structure. However, the chemical composition of the purified serotype b antigen (Table 1) indicates that the teichoic acid is not involved in an important part of the serotype b-specific antigen of \textit{S. milleri}.

Drucker and Green (8) once reported that the NCTC 10708 strain carried the group F antigen; however, they later retracted this statement (9), and it was not supported by the results of other investigators, including us (5; Yakushiji et al., J. Med. Microbiol., in press). It has been suggested that the group antigen possesses the glucose-\textit{N}-acetylgalactosamine sequence as a serologically specific moiety (21).

\[ \text{TABLE 2. Hapten inhibition of the quantitative precipitin reaction between the purified serotype b antigen and the anti-type b serum}^a \]

<table>
<thead>
<tr>
<th>Hapten</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>13.9</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>38.4</td>
</tr>
<tr>
<td>(\alpha)-Methylglucoside</td>
<td>4.0</td>
</tr>
<tr>
<td>(\alpha)-Methylgalactoside</td>
<td>13.3</td>
</tr>
<tr>
<td>(\beta)-Methylgalactoside</td>
<td>19.9</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>21.7</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0</td>
</tr>
<tr>
<td>\textit{N}-Acetylglucosamine</td>
<td>8.8</td>
</tr>
<tr>
<td>\textit{N}-Acetylgalactosamine</td>
<td>5.3</td>
</tr>
<tr>
<td>Maltose</td>
<td>3.3</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>2.2</td>
</tr>
<tr>
<td>Melibiose</td>
<td>12.1</td>
</tr>
<tr>
<td>Lactose</td>
<td>7.7</td>
</tr>
</tbody>
</table>

\[ a \text{ The anti-b serum (5 \(\mu\)l) was incubated with 20 \(\mu\)mol of hapten for 1 h at 37°C, and then the purified serotype b carbohydrate antigen (2 \(\mu\)g) was added.} \]
We have previously suggested that the NCTC 10708 strain carries two antigenic determinants (Yakushiji et al., J. Med. Microbiol., in press): one is serotype b specific, as immunologically characterized in the present study, and the other may be in common with the one carried by the so far untypeable K214-2K strain, which was used to absorb non-type-b-specific antibodies present in the crude anti-NCTC 10708 serum (Yakushiji et al., J. Med. Microbiol., in press). The column chromatographies (Fig. 1 and 2) did not result in clear separation of the two antigenic moieties from the TCA extracts of the purified cell walls, as suggested by the appearance of only one carbohydrate peak in either elution profile, although the one obtained by the Sephadex G-100 gel filtration was asymmetrical (Fig. 2). Our recent preliminary study has also revealed that the serotype b antigen preparation reacted with the anti-K214-2K serum, yielding a precipitin line to form a spur with the line produced between the serum and the Rantz-Randall extracts of the K214-2K cells (unpublished). Clarification of the non-type b antigen determinant of the 10708 strain, which is in common with the K214-2K cells, should await further studies. It should be noted here that the chemical composition of the Rantz-Randall extract of the K214-2K whole cells, which is composed of glucose, rhamnose, and galactosamine in a molar ratio of 1.0:3.0:0.2 (unpublished), seems not to differ significantly from that for the NCTC 10708 strain (Table 3).

ACKNOWLEDGMENT

We thank Shigeuyki Hamada for his encouragement and advice.

LITERATURE CITED


