Immunological and Structural Characterization of a Serotype-Specific Polysaccharide Antigen from *Actinobacillus actinomycetemcomitans* Y4 (Serotype b)

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A serotype-specific polysaccharide antigen of *Actinobacillus actinomycetemcomitans* Y4 (serotype b) was extracted from whole cells by autoclaving. The extract was purified by chromatography on DEAE-Sephadex A-25 and Sephacryl S-300 columns. The purified polysaccharide antigen formed a single precipitin line with anti-type b serum but not with anti-type a serum and anti-type c serum. The antigen was composed of 43.9% L-rhamnose, 49.1% D-fucose, and a trace amount of fatty acid. Methylation analysis, Smith degradation, and optical rotation data showed that the antigen was a polymer consisting of a disaccharide repeating unit, --3)-α-D-fucopyranosyl-(1→2)-β-L-rhamnopyranosyl-(1→. In quantitative precipitin inhibition tests, D-fucose and L-rhamnose showed very low inhibition, but the partial hydrolysate of the purified antigen was an effective inhibitor, suggesting that the serotype b specific antiserum recognizes the larger oligosaccharide units.

*Actinobacillus actinomycetemcomitans* is a nonmotile, gram-negative, capnophilic, fermentative rod. This organism is believed to be associated with localized juvenile periodontitis (25, 26, 28), endocarditis (7, 9), pericarditis (11), thyroid abscess (2), and vertebral osteomyelitis (18).

Oral *A. actinomycetemcomitans* strains are serologically classified into three distinct serotypes, a, b, and c, on the basis of immunodiffusion and indirect immunofluorescence data. Serotypes a and b occur in the oral cavity more frequently than does serotype c (29). Serotype b is the most frequently isolated serotype in localized juvenile periodontitis patients, suggesting a particularly high periodontopathic potential for serotype b strains (28).

Serotype antigens of *A. actinomycetemcomitans* are heatable polysaccharides (27). The antigens of serotypes a, b, and c have been purified and shown to be predominantly mannan-containing carbohydrates suggestive of mannan (30, 31). We report here the isolation and purification of a mannan-free, serotype-specific antigen from whole cells of *A. actinomycetemcomitans* serotype b. The chemical structure and immunological properties of the serotype b antigen are described.

**MATERIALS AND METHODS**

**Microorganisms.** *A. actinomycetemcomitans* ATCC 29523 (serotype a), SUNYaB 75 (serotype a), Y4 (serotype b), ATCC 29522 (serotype b), NCTC 9710 (serotype c), and SUNYaB 67 (serotype c) were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) yeast extract at 37°C for 3 days in a 5% CO₂ atmosphere (21). *Haemophilus aphrophilus* NCTC 5906 and *Haemophilus paraphrophilus* NCTC 10557 were grown in brain heart infusion broth (Difco) supplemented with 10 ml of IsoVitalex enrichment (BBL Microbiology Systems, Cockeysville, Md.) per liter at 37°C for 3 days in a 5% CO₂ atmosphere. The organisms of these strains were harvested by centrifugation, washed three times with distilled water, and lyophilized.

**Extraction of antigens.** (i) **Autoclaved extract.** Lyophilized cell suspension (300 mg/ml) in saline was autoclaved at 120°C for 15 min (22). After being autoclaved, the suspension was cooled and centrifuged at 10,000 × g for 20 min, and the supernatant was collected. The extraction was repeated on residual whole cells. The supernatants were combined, dialyzed extensively against distilled water, and lyophilized.

(ii) **Sonic extract.** Lyophilized cells (100 mg) were suspended in 5 ml of saline and disrupted by sonication (Ultrasonic Disrupter, model UR-200P; Tomy Seiko Co., Tokyo, Japan) at 60-s intervals for a total sonication time of 30 min at maximum output in ice. After the disrupted cell suspensions were centrifuged, the supernatants were dialyzed against distilled water and lyophilized.

**Purification of serotype antigen.** The autoclaved extract of *A. actinomycetemcomitans* Y4 was solubilized with 0.01 M Tris hydrochloride, pH 8.2, to give a final concentration of 100 mg (dry weight) of bacterial extract per ml and dialyzed against the buffer at 4°C for 2 days. A 5-ml portion of the antigen suspension was applied to a column of DEAE-Sephadex A-25 (2 by 30 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with the buffer and eluted with 200 ml of the buffer followed by a linear gradient of 0 to 1 M NaCl in the buffer at 4°C. Fractions (10 ml each) were monitored for total sugar, protein, and phosphorus. Fractions which showed a positive reaction with anti-Y4 serum (see below) by immunodiffusion were combined and concentrated in a rotary evaporator. This preparation was dialyzed against distilled water at 4°C for 3 days, applied to a column of Sephacryl S-300 (1.5 by 100 cm; Pharmacia), and eluted with distilled water. Fractions containing the serotype-specific antigen were pooled and lyophilized. The molecular weight of the antigen was estimated by using dextran T2000, T250, and T20 (Pharmacia) and glucose as molecular weight markers.

**Colorimetric analyses.** Amounts of total sugar, hexosa-
mine, RNA, and phosphorus were determined by the colorimetric methods of Dubois et al. (4), Dische and Shettes (3), Mejbaum (17), and Lowry et al. (15), respectively. Protein content was determined by using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.).

**Sugar composition.** The serotype b antigen was hydrolyzed with 2 M trifluoroacetic acid at 121°C for 2 h. Neutral sugars in the hydrolysate were converted into alditol acetates (24) and analyzed by gas-liquid chromatography (GLC) (model GC-9A; Shimadzu Works, Tokyo, Japan) with a fused-silica capillary column (CP Sil-88 [0.22 mm by 2 or 10 m]; Chrompack Inc., Bridgewater, N.J.) at 180°C. GLC-mass spectrometry was carried out with a mass spectrometer (model M-80; Hitachi Co., Tokyo, Japan) fitted with an electron impact ionization detector. Sugar composition was also analyzed by a Shimadzu LC-4A high-performance liquid chromatograph equipped with a postcolumn labeling system for reducing sugars and an anion exchange column (Shim-pack ISA-07; Shimadzu). Absolute configuration of the component sugars was determined by the method of Leontin et al. (14). One drop of trifluoroacetic acid and 0.2 ml of D(-)-2-octanol were added to the hydrolysate of the serotype b antigen and heated overnight at 130°C in an oil bath. The solution was then concentrated, and the resulting mixture of glycosides was acetylated. The acetylated octyl glycosides were analyzed by GLC on the same column used for the analysis of alditol acetates. Optical rotation was determined by an optical rotatory analyzer (model DIP-140; Japan Spectroscopic Co., Tokyo, Japan).

**Methylation analysis.** Methylation of the purified antigen was performed by the method of Hakomori (10). The antigen (1 mg) was dissolved in dimethyl sulfoxide (0.5 ml) under a nitrogen atmosphere and then methylated with methylsulfon- nyl carbonate (0.1 ml) and methyl iodide (0.1 ml). After the methylation was completed, the reaction mixture was subjected to chromatography on Sephadex LH-20 (Pharmacia) equilibrated with chloroform-methanol (1:1, vol/vol). The resulting sugar-containing fractions were combined and concentrated under reduced pressure. The fully methylated polysaccharide was hydrolyzed with 90% (vol/vol) formic acid at 100°C for 1 h and then with 1 M trifluoroacetic acid at 121°C for 1 h. The hydrolysates were reduced and acetylated. The partially methylated alditol acetates were analyzed by GLC and GLC-mass spectrometry as described above.

**Smith degradation.** The purified antigen (10 mg) was oxidized with 10 ml of 0.05 M sodium periodate for 7 days in the dark at 4°C (8). After the addition of a drop of ethylene glycol to destroy the excess periodate, the product was reduced overnight with 50 mg of sodium borohydride at room temperature. Acetic acid was carefully added to destroy the excess borohydride, and the solution was applied to a Bio-Gel P-2 (Bio-Rad) column (1.5 by 60 cm) equilibrated with distilled water. Sugar-containing fractions were combined and lyophilized. The lyophilized material (0.1 mg) was hydrolyzed with 2 M trifluoroacetic acid at 121°C for 2 h. Sugars in the hydrolysate were converted to alditol acetates and analyzed by GLC.

**Preparation of partial hydrolysate of the purified antigen.** The purified antigen (0.1 mg) was partially hydrolyzed for 1 h at 80°C with 0.5 ml of 0.1 M trifluoroacetic acid. The hydrolysate was washed twice with distilled water and then suspended in 0.1 ml of distilled water.

**Immunological methods.** Antisera against *A. actinomyctemcomitans* Y4, ATCC 29523, and NCTC 9710 whole cells were prepared in rabbits by intravenous injections of the

**RESULTS**

The results of DEAE-Sepahex chromatography of the autoclaved extract of *A. actinomyctemcomitans* Y4 whole cells are shown in Fig. 1. A single carbohydrate peak reacting with rabbit anti-Y4 serum was eluted at the void volume. The antigenic peak fractions were contaminated with protein. The fractions were combined, concentrated, and applied to a Sephadryl S-300 column. The antigen was eluted as a single symmetrical peak (Fig. 2). Most of the contaminating protein was adsorbed on the gels at the top of the column, and a trace amount of protein was eluted close to the total bed volume. All fractions which reacted with anti-Y4 serum were combined, lyophilized, and designated serotype b antigen. The molecular weight of the purified antigen was estimated by gel filtration to be 150,000.

The purified serotype antigen was composed of 93% neutral sugar and a trace amount of fatty acid (Table 1). Protein, hexosamine, RNA, and phosphorus were not detected. GLC and high-performance liquid chromatography analyses showed that the antigen was composed of almost equal amount of rhamnose and fucose. The absolute configurations of these sugars were determined to be L and D for rhamnose and fucose, respectively, by GLC analysis of the corresponding D(-)-2-octyl glycoside acetate (Fig. 3). Optical rotatory analysis showed that [α]_D values of the purified antigen, the hydrolysate of the antigen, and a mixture of L-rhamnose and D-fucose (1:1, wt/wt) were +105°, +32°, and +40°, respectively. The high positive rotation of the purified antigen suggests the existence of β-linked L-rhamnose and α-linked D-fucose residues. Methylation analysis of the purified antigen showed that the hydrolysate of the fully
methylated antigen gave almost equal amounts of 3,4-di-O-methyl rhamnose and 2,4-di-O-methyl fucose, suggesting that the antigen consists of 1,2-linked l-rhamnose and 1,3-linked d-fucose residues at a 1:1 ratio (47.2 and 52.8%, respectively, for the derivatives 1,2,5-tri-O-acetyl-3,4-di-O-methyl rhamnitol →2 Rha →1 and 1,3,5-tri-O-acetyl-2,4-di-O-methyl fucitol →3 Fuc →1, where Rha is a rhamnopyranosyl residue and Fuc is a fucopyranosyl residue). The purified antigen was also subjected to Smith degradation (8), including oxidation of the polysaccharide, reduction with sodium borohydride, and hydrolysis with acid. Complete acid hydrolysis of the resulting polyalcohol gave only fucose, supporting the conclusion derived from the methylation analysis. These results indicate that the serotype b antigen is a polymer consisting of a disaccharide repeating unit, →3) α-D-Fucopyranosyl-(1→2)-β-L-rhamnopyranosyl-(1→5) (Fig. 4).

Immunodiffusion tests revealed that anti-Y4 serum formed a fused precipitin line with autoclaved extracts from Y4 and ATCC 29522 but not with autoclaved extracts from other A. actinomycetemcomitans and Haemophilus strains (Fig. 5). The serum formed a fused precipitin line with the serotype b antigen and with autoclaved and sonicated extracts of Y4. Anti-29523 serum and anti-9710 serum formed no precipitin line with the purified antigen (data not shown).

**TABLE 1. Chemical composition of purified serotype b antigen of A. actinomycetemcomitans Y4**

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition (μg/mg)</th>
</tr>
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<tbody>
<tr>
<td>Rhamnose</td>
<td>439</td>
</tr>
<tr>
<td>Fucose</td>
<td>491</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Peptide</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>26</td>
</tr>
</tbody>
</table>

*Quantitative analyses were carried out by colorimetric methods after qualitative determination of component sugars by GLC and high-performance liquid chromatography.*

The quantitative precipitin curve of the reaction between the purified serotype b antigen and the anti-Y4 serum is shown in Fig. 6. The maximal precipitation of antibody protein occurred with 0.5 μg of the antigen when 10 μl of the antiserum was used in a total volume of 35 μl. The partial hydrolysate tests showed that the component sugars, l-rhamnose and d-fucose, were very poor inhibitors (Table 2). On the other hand, the partial hydrolysate (0.06%, wt/vol) of the purified antigen produced 85.6% inhibition.

**DISCUSSION**

Zambon et al. (30, 31) purified serotype a, b, and c antigens of A. actinomycetemcomitans with fractional ethanol precipitation of the culture supernatants, sequential
ion-exchange chromatography, and gel filtration. GLC analyses of the purified antigens showed 84% mannose and 16% glucose in the serotype a antigen from strain SUNYaB 75 (31); 82% mannose, 7% galactose, and 12% glucose in the serotype b antigen from strain Y4 (31); and 84 to 90% mannose and 5 to 16% glucose in the serotype c antigen from strain SUNYaB 67 (30). These results suggest that the serotype antigens of *A. actinomycetemcomitans* are basically mannose polymers such as mannan. In this study, we have isolated a serotype b-specific antigen from whole cells of *A. actinomycetemcomitans* Y4 by the autoclaving procedure and purified it with ion-exchange chromatography and gel filtration. Analysis of sugar composition, including deter-

mation of absolute configuration, methylation analysis, Smith degradation, and optical rotation data, showed that the serotype b antigen purified in this study is a polymer consisting of the disaccharide repeating unit $\beta-1\rightarrow 3$-$\alpha$-D-fucopyranosyl-(1$\rightarrow$2)-$\beta$-L-rhamnopyranosyl-(1$\rightarrow$). We were unable to detect mannosyl residues in the antigen by GLC, GLC-mass spectrometry, or high-performance liquid chromatography. Serotype b *A. actinomycetemcomitans* strains may produce multiple serotype-specific polysaccharides that differ from one another in chemical structure. Hammond et al. (B. F. Hammond, M. Darkes, and C. C. Tsai, J. Dent. Res. 59[Special Issue A, Am. Assoc. Dent. Res. Abstr.],512, 1980) isolated a carbohydrate antigen (similar in chemical composition to the antigen purified in this study) from whole cells of strain Y4 by the phenol-water extraction procedure. The antigen purified by Hammond et al. (J. Dent. Res., 1980) is composed of methyl pentose, rhamnose, and fucose (total of 50%) and glucosamine (8%), heptose (5%), ribose (4%), and galactose (2%).

Quantitative precipitin inhibition tests showed that the degrees of inhibition of the serotype b antigen by the component sugars L-rhamnose and D-fucose were very low (Table 2). On the other hand, the partial hydrolysate of the serotype b antigen was an effective inhibitor of the precipitin reaction. Serotype b-specific antiserum may recognize larger oligosaccharide units rather than any monosaccharide units of the polysaccharide antigen. We have recently isolated several monoclonal antibodies to the antigen. Studies are under way to determine the recognition sites of these monoclonal antibodies.

Several investigators (1, 12, 19, 20) have indicated that lipopolysaccharide (LPS) from *A. actinomycetemcomitans* Y4 contains rhamnose, fucose, galactose, glucose, l-glycero-D-mannoheptose, d-glycero-D-mannoheptose, glucosamine, and galactosamine. It is possible that the serotype b antigen purified in this study is the O-specific chain of LPS. Our preliminary experiments demonstrated that monoclonal antibodies to the serotype b antigen did not react with the O-specific polysaccharide obtained from purified Y4 LPS (unpublished data). No monoclonal antibodies to purified Y4 LPS reacted with the serotype b antigen. These findings suggest that the serotype b antigen differs from the O-polysaccharide of LPS.

It has been reported that patients with localized juvenile periodontitis exhibit elevated levels of antibody to *A. actinomycetemcomitans* Y4 in serum and saliva (5, 6, 16, 23). In these studies, whole cells (5, 16), sonicated extracts from whole cells (6, 23), leukotoxin (6), LPS (6), and group carbohydrate (6) were used as antigens for the detection of antibodies to *A. actinomycetemcomitans*. Antibigen prepara-

**TABLE 2.** Haemoglobin of the quantitative precipitin reaction between serotype b antigen and anti-Y4 serum

<table>
<thead>
<tr>
<th>Haemoglobin (conc or amount)</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>L-Rhamnose (200 mM)</td>
<td>8.3</td>
</tr>
<tr>
<td>2-Fucose (200 mM)</td>
<td>7.5</td>
</tr>
<tr>
<td>L-Fucose (200 mM)</td>
<td>5.9</td>
</tr>
<tr>
<td>L-Rhamnose (100 mM) + 2-fucose (100 mM)</td>
<td>8.5</td>
</tr>
<tr>
<td>L-Rhamnose (100 mM) + L-fucose (100 mM)</td>
<td>6.4</td>
</tr>
<tr>
<td>Partial hydrolysate of serotype b antigen (5 µg)</td>
<td>60.5</td>
</tr>
<tr>
<td>Partial hydrolysate of serotype b antigen (20 µg)</td>
<td>85.6</td>
</tr>
</tbody>
</table>

a Anti-Y4 serum (10 µl) was incubated with various amounts of haptons for 1 h at 37°C, and then the purified serotype b antigen (0.5 µg) was added. The final volume of the reaction mixture was adjusted to 35 µl. L-glucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine showed less than 5% inhibition.

**FIG. 5.** Immunodiffusion reaction of anti-Y4 serum with autoclaved and sonicated extracts of whole cells of *A. actinomycetemcomitans* and *Haemophilus* species and with the purified serotype b antigen. Well 1, Autoclaved extract of ATCC 29523 (serotype a); well 2, autoclaved extract of SUNYaB 75 (serotype a); well 3, autoclaved extract of Y4 (serotype b); well 4, autoclaved extract of ATCC 29522 (serotype b); well 5, autoclaved extract of NCTC 9710 (serotype c); well 6, autoclaved extract of SUNYaB 67 (serotype c); well 7, purified serotype b antigen; well 8, sonicated extract of Y4; well 9, autoclaved extract of NCTC 5906; well 10, autoclaved extract of NCTC 10557; well A, anti-Y4 serum.

**FIG. 6.** Quantitative precipitin curve for serotype b antigen of *A. actinomycetemcomitans* Y4. Anti-Y4 serum (10 µl) was mixed with various amounts of the antigen. The final volume was adjusted to 35 µl. The reaction mixture was incubated for 1 h at 37°C and then stored for 18 h at 4°C. The amounts of protein precipitated were measured by using the Bio-Rad protein assay reagent.
tions such as whole cells and sonicated extracts may contain common antigens that complicate clinical tests for the diagnosis and treatment of juvenile periodontitis. In fact, Zambon et al. (29) have shown that the sonicated extracts from whole cells of A. actinomycetemcomitans contain several common antigens among the A. actinomycetemcomitans serotypes and between A. actinomycetemcomitans and various other Actinobacillus and Haemophilus species. In this study, the crude autoclaved extract of strain Y4 formed a strong precipitin line with anti-serotype b whole-cell serum in immunodiffusion analyses but not with anti-serotype a and anti-serotype c whole-cell sera. In addition, our preliminary immunodiffusion tests showed that the autoclaved extracts of serotype a or c A. actinomycetemcomitans strains formed strong precipitin lines only with the corresponding rabbit antisera (unpublished data). The extraction of serotype antigens by autoclaving may be useful for the serotyping of A. actinomycetemcomitans strains and for the purification and characterization of the antigens.

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