INFECTION AND IMMUNITY, Apr. 1991, p. 1544–1551
0019-9567/91/041544-08$02.00/0

Vol. 59, No. 4

Evidence that the Serotype b Antigenic Determinant of *Actinobacillus actinomycetemcomitans* Y4 Resides in the Polysaccharide Moiety of Lipopolysaccharide

MARK E. WILSON* AND ROBERT E. SCHIFFERLE

Department of Oral Biology, Periodontal Disease Clinical Research Center, and Dental Research Institute, School of Dental Medicine, State University of New York at Buffalo, Buffalo, New York 14214*

Received 27 August 1990/Accepted 10 January 1991

A high-molecular-weight polysaccharide-containing antigen was isolated from a phenol-water extract of *Actinobacillus actinomycetemcomitans* ATCC 43718 (formerly Y4) by gel permeation chromatography in lipopolysaccharide (LPS)-disaggregating buffer. The polysaccharide antigen formed a precipitin band with rabbit serotype b-specific antiserum but not with rabbit antisera to serotype a or c. Electroblotted serotype b antigen was probed with serum from a patient with localized juvenile periodontitis (LJP), resulting in a diffuse "smear" in the upper region of the lane. By utilizing an enzyme-linked immunosorbent assay, it was demonstrated that the geometric mean immunoglobulin G antibody titer to the serotype b polysaccharide was significantly higher in sera from LJP patients than in sera from periodontally healthy individuals. Moreover, LJP antibody titers to the serotype b polysaccharide exhibited age-dependent variation. Double immunodiffusion analysis revealed that the serotype b antigen formed a line of identity with low-molecular-weight LPS following reaction with serotype b-specific antiserum. Incubation of LJP serum in the presence of a lipid-free polysaccharide moiety obtained by mild acid hydrolysis of LPS from *A. actinomycetemcomitans* Y4 markedly reduced immunoglobulin G titer to the serotype b antigen. In contrast, solubilized lipid A was only weakly inhibitory. The results of this study indicate that the serotype b-specific determinant of *A. actinomycetemcomitans* resides in the polysaccharide moiety of LPS and represents a major target for immunoglobulin G antibody in serum of LJP subjects colonized by this organism.

Actinobacillus actinomycetemcomitans is a nonmotile, gram-negative, capnophilic, fermentative coccobacillus which has been implicated in the etiology and pathogenesis of localized juvenile periodontitis (LJP) (27, 28, 32). This organism has also been associated with some cases of rapidly progressing and refractory periodontitis (27), periodontitis of the primary dentition (8), and serious extraoral infection (32). Three distinct serotypes (designated a, b, and c) have been identified among oral *A. actinomycetemcomitans* strains (33). Among LJP subjects, however, serotype b isolates are recovered more frequently than serotype a or c (33). This has fostered speculation that serotype b strains may exhibit a greater periodontopathic potential than other *A. actinomycetemcomitans* serotypes (32).

LJP subjects often exhibit high titers of serum antibody to *A. actinomycetemcomitans* antigens (9, 19). The humoral response of LJP subjects to *A. actinomycetemcomitans* includes production of antibodies which recognize leukotoxin, lipopolysaccharide (LPS), and outer membrane proteins (3, 10, 11). Recently, Califano et al. (6) reported that the immunodominant antigen of *A. actinomycetemcomitans* Y4 appears to be a papain-resistant, heat-stable molecule which is distinct from LPS. This antigen, which was obtained by cold ethanol precipitation of a phenol-water extract of whole bacteria, formed a line of identity with rabbit serotype b-specific antiserum and LJP serum. Although the characteristics of this antigen were indicative of a carbohydrate (possibly capsular polysaccharide), no chemical analysis of the immunodominant antigen of strain Y4 was performed.

Amano et al. (1) described the purification of a serotype b-specific polysaccharide antigen from *A. actinomycetemcomitans* Y4. This material, which was obtained by ion-exchange and subsequent gel permeation chromatography of an autoclaved extract of whole cells, appeared to be a polymer consisting of a repeating disaccharide unit containing rhamnose and fucose. Antibody titers to this serotype b antigen in sera of periodontally healthy and LJP subjects were not determined. Hence, the relationship between this antigen and the immunodominant antigen described previously has not been defined.

Serum immunoglobulin G (IgG) antibody titers to *A. actinomycetemcomitans* LPS from a serotype b strain were found to be significantly elevated in LJP patients compared with other types of periodontal disease and periodontally healthy individuals (11). Immunization of human subjects or experimental animals with purified LPS leads to the production of specific antibody directed principally to the repeating oligosaccharide subunits (21) known to account for the serologic specificity of enteric gram-negative bacteria (20). In the present study, we examined the relationship between the polysaccharide moiety of LPS and the serotype b-specific carbohydrate antigen of *A. actinomycetemcomitans*. We describe the isolation and partial characterization of a serotype b-specific polysaccharide antigen from a phenol-water extract of *A. actinomycetemcomitans* Y4. Low-molecular-weight LPS from this strain, prepared by the phenol-chloroform-petroleum ether (PCP) method, formed a line of identity with the serotype b antigen following the reaction with rabbit serotype b-specific antiserum. We provide evidence that the serotype b antigen of *A. actinomycetemcomitans* resides in the polysaccharide side chain of LPS.

* Corresponding author.

MATERIALS AND METHODS

Bacteria. *A. actinomycetemcomitans* ATCC 43718 (previously designated strain Y4) was used in the isolation and characterization of the serotype b-specific antigen. Midlogarithmic-phase cultures of this strain were prepared as described elsewhere (2).

Human sera. Sera were obtained from 35 patients with LJP (mean age, 18.5 years; range, 10 to 31 years) receiving treatment through the Periodontal Disease Clinical Research Center, State University of New York at Buffalo. Diagnosis of LJP was made on the basis of alveolar bone loss and periodontal destruction limited to the first molars and incisors and not more than two additional teeth. Sera were also obtained from 35 periodontally healthy subjects (mean age, 30.6 years; range, 19 to 45 years) with no loss of attachment >2 mm. The sera were collected by standard venipuncture technique and stored in aliquots at −70°C until needed. Informed consent was obtained from all subjects.

Isolation of the serotype b antigen. The serotype-defining polysaccharide antigen was obtained from *A. actinomycetemcomitans* ATCC 43718 by extraction in hot aqueous phenol, followed by gel filtration chromatography in LPS-disaggregating buffer, nuclease digestion, and rechromatography. Briefly, thioglycolate-grown bacteria were extracted with 45% phenol at 65 to 70°C as described by Westphal and Jann (30). A 100-mg amount of phenol-extracted material was suspended in 10 to 15 ml of LPS-disaggregating (NAD) buffer consisting of 1.5% sodium deoxycholate, 0.05 M glycine, and 0.001 M EDTA, pH 10. The extract was applied to a column (2.5 by 90 cm) of Sephacryl S-400 equilibrated with NAD buffer, pH 9.0, and chromatographed at a flow rate of 16 ml/h. Fractions were collected and monitored for the presence of high-molecular-weight polysaccharide and LPS on silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels (see below). Polysaccharide-containing fractions exhibited a yellow to light brown zone in the top 1 to 2 cm of the gel, while low-molecular-weight LPS migrating to the bottom of the gels exhibited a characteristic dark brown to black staining pattern. Serotype polysaccharide-containing fractions were pooled, exhaustively dialyzed against distilled H_2O, and lyophilized. This yielded 9.8 mg of crude serotype polysaccharide. Residual RNA contamination was eliminated by dissolving 9 mg of polysaccharide in 9 ml of RNase digestion buffer consisting of 10 mM Tris, 300 mM NaCl, and 5 mM EDTA, pH 7.5, after which 50 μl each of RNase A and RNase T_1 (each at 10 mg/ml) were added. The mixture was incubated at ambient temperature for 1 h, followed by overnight incubation at 4°C. This material was rechromatographed on Sephacryl S-400 in NAD buffer, dialyzed against H_2O, and lyophilized. Serotype-defining polysaccharide thus prepared (final yield, 7.6 mg) contained <1% protein and nucleic acid.

Extraction of LPS. LPS was extracted from *A. actinomycetemcomitans* by the PCP method described by Galanos and co-workers (12). Residual nucleic acid and protein contamination was removed by sequential nuclease and proteinase K digestion. The LPS was pelleted by ultracentrifugation for 1 h at 100,000 × *g*, rinsed with distilled H_2O, and centrifuged again. The pellet thus obtained was dispersed in a small volume of distilled H_2O and lyophilized. Lipid-free oligosaccharide from PCP-extracted LPS was obtained by incubating a 1% (wt/vol) suspension of LPS in 1% (vol/vol) acetic acid for 3 h at 100°C (15). The white precipitate (containing lipid A) was recovered by centrifugation at 2,000 × *g* for 10 min. The supernatant fraction (containing lipid-free oligosaccharide) was extracted with chloroform to eliminate residual lipid, desalted on Sephadex G-10, and lyophilized. The lipid A-containing precipitate was washed three times in distilled H_2O, resuspended in chloroform-methanol (2:1), and evaporated to dryness under vacuum.

Chemical analysis. Protein content was determined by automated amino acid analysis on a Beckman 6300 system analyzer following hydrolysis in 6 N HCl at 105°C for 28 h under a nitrogen atmosphere, using α-amino-β-guanidinopropionic acid (Sigma Chemical Co., St. Louis, Mo.) as an internal standard (23). Nucleic acid content was determined by A_{260}. Neutral sugar composition was determined by gas chromatographic analysis of alditol acetate derivatives, using arabinose as an internal standard (23). Samples were hydrolyzed in 2 N HCl for 6 h at 100°C, followed by acid removal by passage through Dowex-1. The samples were reduced with sodium borohydride, acidified with acetic acid, and dried by evaporation multiple times after being mixed with methanol. The samples were subsequently acetylated with acetic anhydride at 100°C for 30 min and dried by evaporation. The alditol acetate derivatives were dissolved in chloroform and analyzed with a Varian model 3400 gas chromatograph (Varian, Walnut Creek, Calif.) with flame ionization detection. The samples were analyzed on chromatography columns packed with 3% OV-225 on 80/100 Supelcoport (Supelco, Bellefonte, Pa.) at an initial temperature of 190°C, which was increased to 210°C at a rate of 1°C/min and then increased to 240°C at 5°C/min and held at 240°C for 30 min. Sugars were identified by comparison to alditol acetates prepared from neutral sugars (rhamnose, arabinose, mannose, galactose, and glucose purchased from Sigma; mannoheptose and glucoheptose purchased from Supelco). Amino sugar composition was determined by analysis with the Beckman 6300 system analyzer following hydrolysis in 4 N HCl for 5 h at 100°C, drying to remove acid, and using α-amino-β-guanidinopropionic acid as an internal standard. Lipid content was determined following transesterification with methanolic-HCl (3 M) at 80°C for 18 h. The fatty acid methyl ester derivatives were analyzed on the Varian 3400 gas chromatograph utilizing a column of 3% SP-2100-DOH on 100/120 Supelcoport at an initial column temperature of 140°C, which was increased to 240°C at 5°C/min and held at 140°C for 5 min. Fatty acids were identified by comparison to a bacterial fatty acid methyl ester standard mixture (Supelco).

ELISA. Serum IgG antibody titers to the *A. actinomycetemcomitans* serotype b polysaccharide were determined by an enzyme-linked immunorbent assay (ELISA) described elsewhere (31). Briefly, enzyme immunoassay plates were coated with an optimal concentration (10 μg/ml in 0.015 M carbonate, pH 9.6) of serotype b polysaccharide. The plates were washed and then incubated with blocking buffer. The plates were subsequently incubated for 1 h at 37°C with human sera diluted in blocking buffer. Unbound antibodies were removed by washing, after which the plates were incubated for 1 h at 37°C with alkaline phosphatase-conjugated goat anti-human IgG diluted 1:1,000 in blocking buffer. The plates were washed and incubated for 30 min at ambient temperature with *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine substrate buffer. Reactions were terminated via addition of 1 N NaOH, after which the A_{405} nm was measured with a microplate reader. Each experiment was performed in duplicate and included a positive control serum. Negative controls consisted of (i) antigen-coated wells reacted with alkaline phosphatase conjugate alone and

(ii) mock-coated wells reacted with human serum and conjugate. Optical density readings of the negative controls were always <0.1. Serum IgG antibody titers to the serotype-defining polysaccharide were expressed as a reciprocal of the serum dilution yielding an optical density of 0.6, which is roughly the midpoint of the linear range of the assay (26). For the purpose of calculating geometric mean antibody titer, sera exhibiting titers of <125 were assigned a value of 100.

SDS-PAGE and immunoblot analysis. LPS and serotype b antigen preparations were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), using the discontinuous buffer system of Laemmli (18). Electrophoresis was performed with a vertical slab electrophoresis cell 11 by 8 cm; Hoefer Scientific Instruments, San Francisco, Calif.). Samples were solubilized by treatment at 100°C for 10 min in 0.063 M Tris hydrochloride, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.003% bromophenol blue. The samples were applied to slots of a Laemmli gel containing a 3% stacking gel and a 14% separating gel and electrophoresed at a constant power of 6 W until the tracking dye was approximately 1 cm from the bottom of the gel. High-molecular-weight polysaccharide and LPS bands were subsequently visualized by silver staining by the method of Tsai and Frasch (29). Electrophoretic transfer of the serotype b polysaccharide antigen (10 μg) onto nitrocellulose membranes (0.2 μm) was performed with a semidry electroblotter (Janssen Life Sciences Products, Piscataway, N.J.) according to the manufacturer's instructions. Following transfer, the nitrocellulose membranes were blocked for 45 min in 1% bovine serum albumin (fatty acid-free) in 20 mM Tris, pH 7.5, containing 0.5 M NaCl (TBS), washed for 5 min in TBS with 0.2% Tween 20 and then with TBS. The membranes were subsequently incubated overnight at 4°C with LJP serum diluted 1:250 in TBS with 1% bovine serum albumin. The membranes were rinsed and incubated for 1 h at ambient temperature with a 1:1,000 dilution of biotinylated goat anti-human IgG (γ-chain specific; Calbiochem-Behring, La Jolla, Calif.) in TBS with 1% bovine serum albumin. The blots were rinsed and incubated for 1 h in the dark at ambient temperature with a 1:1,000 dilution of avidin-conjugated horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.). The membranes were then rinsed and developed with horseradish peroxidase color development reagent (Bio-Rad). Biotinylated low-molecular-weight standards (Bio-Rad) were included in each gel.

Statistics. Reciprocal antibody titers to the serotype-defining polysaccharide in sera from periodontally healthy and LJP subjects were compared by using a *t* test for unpaired samples after log transformation of the data.

RESULTS

Logarithmic-phase cultures of *A. actinomycetemcomitans* Y4 were prepared in NIH thioglycolate broth supplemented with 5 mg of yeast extract and 1 mg of sodium bicarbonate per ml. The bacteria were extracted in hot aqueous phenol and the aqueous phase was lyophilized. The phenol-water extract was subsequently chromatographed on Sephacryl S-400 in LPS-disaggregating buffer containing 1.5% sodium deoxycholate (Fig. 1). A single large peak, eluting at the void volume, consisted largely of nucleic acid (as determined by 260/280 UV absorbance ratio). Fractions eluting after this peak (indicated by arrow) were subjected to SDS-PAGE, followed by silver staining to visualize polysaccharides and LPS (Fig. 2). A high-molecular-weight material eluted first,

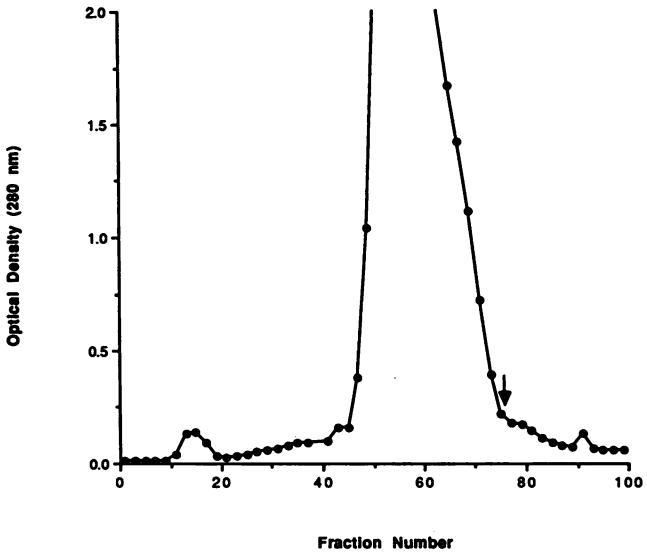

FIG. 1. Elution profile of a phenol-water extract of *A. actinomycetemcomitans* Y4 chromatographed on Sephacryl S-400 in LPS-disaggregating buffer containing 1.5% sodium deoxycholate. The large peak eluting at the void volume contained large amounts of nucleic acid. Material eluting after this peak was retained and subsequently analyzed on silver-stained SDS-polyacrylamide gels. Fraction 75 (indicated by arrow) corresponds to the first lane in Fig. 2.

followed by low-molecular-weight, disaggregated LPS. The high-molecular-weight material exhibited a pale yellow staining with ammoniacal silver, while LPS stained dark brown to black. Both the high-molecular-weight material and LPS stained weakly with periodic acid-Schiff reagent (not shown). Fractions containing high-molecular-weight material were pooled, digested with RNase to remove residual nucleic acid, and rechromatographed on Sephacryl S-400 in LPS-disaggregating buffer. This material, which was essentially free of contaminating protein or nucleic acid, was used in subsequent chemical and immunochemical studies.

LPS was isolated from *A. actinomycetemcomitans* Y4 by the PCP extraction method. This technique permits extraction of lipophilic LPS molecules into a monophasic mixture of phenol-chloroform-petroleum ether while excluding water-soluble nucleic acids, proteins, and polysaccharides. PCP-extracted LPS showed no evidence of high-molecular-weight polysaccharide on silver-stained SDS-polyacrylamide gels (not shown), exhibiting an electrophoretic profile identical to that of low-molecular-weight LPS resolved by deoxycholate chromatography (Fig. 2, fraction 93). This preparation was utilized in studies defining the chemical and immunochemical relationship, if any, between the high-molecular-weight fraction and LPS.

Double immunodiffusion analysis. The high-molecular-weight substance obtained by chromatography of the phenol-water extract in LPS-disaggregating buffer was tested for reactivity with rabbit antisera to the three known serotypes of *A. actinomycetemcomitans*. This material formed a precipitin band with rabbit serotype b-specific antiserum, but exhibited no reactivity with rabbit antisera specific for serotype a or c (Fig. 3). Moreover, rabbit serotype b-specific antiserum and LJP serum formed a line of identity with respect to this polysaccharide antigen. Hence, this substance was subsequently referred to as the serotype b

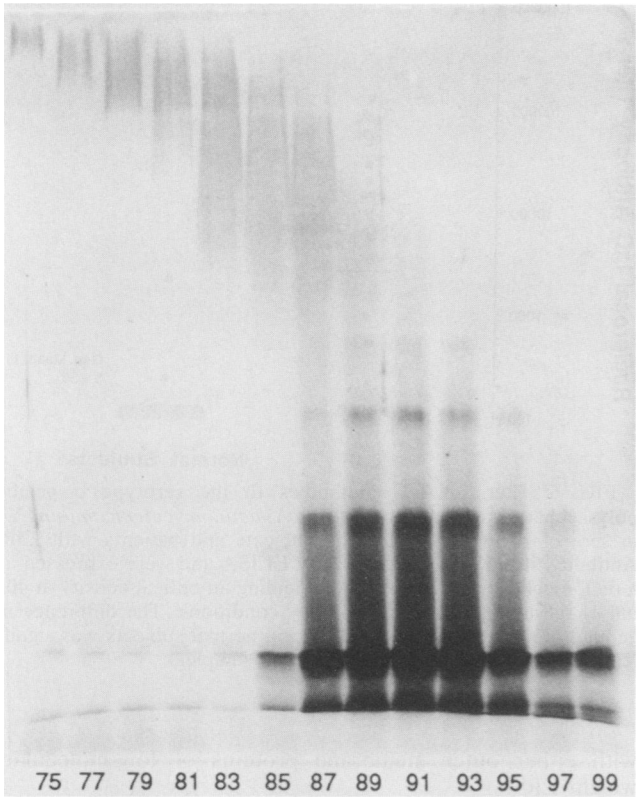

FIG. 2. Electrophoretic analysis of a phenol-water extract of strain Y4 chromatographed on Sephacryl S-400 in deoxycholate-containing buffer. Samples of each fraction were applied to slots of a 14% polyacrylamide gel containing 0.1% SDS. Following electrophoresis, the gels were stained with ammoniacal silver to visualize polysaccharides and LPS. A high-molecular-weight material eluted first, followed by disaggregated LPS. The high-molecular-weight material in fractions 75 to 83 was retained, treated with RNase to remove residual nucleic acid, and rechromatographed on Sephacryl S-400 as described previously. This material was used in all subsequent chemical and immunologic analyses of the serotype antigen from *A. actinomycetemcomitans* Y4.

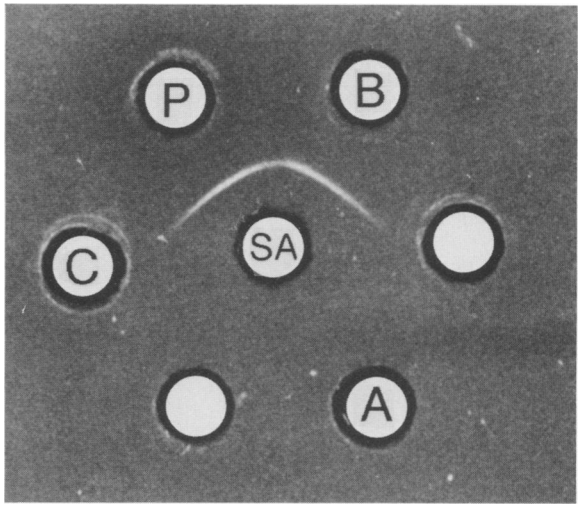

FIG. 3. Double immunodiffusion analysis of the high-molecular-weight polysaccharide obtained from a phenol-water extract of *A. actinomycetemcomitans* Y4. Peripheral wells contained serum from an LJP patient (P) or rabbit antisera specific for *A. actinomycetemcomitans* serotypes a, b, and c. The center well contained the serotype-specific polysaccharide (SA; 1 mg/ml). Note that LJP serum and rabbit serotype b-specific antiserum recognize the same antigen.

antigen. The relationship between the serotype b antigen and low-molecular-weight LPS was also assessed. Rabbit serotype b-specific antiserum recognized an antigenic determinant common to both LPS and the serotype b antigen (Fig. 4). The reaction of LJP serum with these two preparations also produced a line of identity (not shown).

Chemical analyses. The serotype b antigen and PCP-extracted LPS were further analyzed with respect to carbohydrate and fatty acid composition (Table 1). The results of this analysis revealed distinct compositional differences both qualitatively and quantitatively between the two antigens. The carbohydrate composition was determined by gas chromatography of alditol acetates for neutral sugar and by analysis on an automated amino acid analyzer for hexosamine. The methylpentoses rhamnose and fucose were represented in both preparations, but constituted a greater proportion of the sugar content of the serotype b antigen. Glucose was also present in both preparations, while galactose was detected only in LPS. Heptoses were detected in the serotype b antigen and LPS; however, mannoheptose and glucoheptose were present as major components of LPS, whereas the serotype antigen contained a reduced proportion of mannoheptose and no glucoheptose. Glucosamine constituted the principal amino sugar present in LPS, while the serotype b antigen contained only galactosamine.

The fatty acid composition, determined by gas chromatography of fatty acid methyl ester derivatives, revealed that fatty acids constituted 2% of the weight of the serotype b antigen and 9.8% of that of the LPS. LPS contained 3-hydroxytetradecanoic acid and tetradecanoic acid in a ratio of approximately 2.6:1. Five different fatty acids were identified in the serotype b antigen preparation including 3-hydroxytetradecanoic acid, a unique component of LPS. The $C_{16:0}$, $aC_{17:0}$, and $C_{18:0}$ acids may represent noncovalently associated cellular lipids (5).

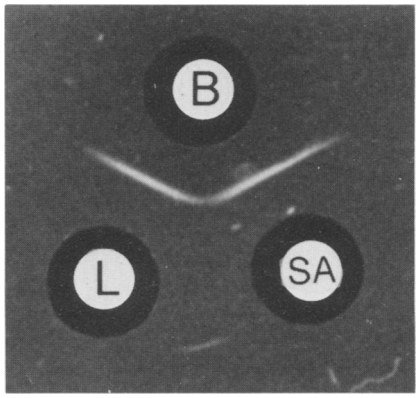

FIG. 4. Double immunodiffusion analysis showing identity between the serotype b antigen and LPS extracted from strain Y4 by the phenol-chloroform-petroleum ether method. The serotype b antigen (SA) and LPS (L) were added to the wells (at 1 mg/ml; LPS solubilization was enhanced via addition of 0.25% deoxycholate) and allowed to react with rabbit serotype b-specific antiserum (B).

TABLE 1. Carbohydrate and fatty acid composition of the serotype-specific antigen and PCP-extracted LPS from *A. actinomycetemcomitans* Y4 (ATCC 43718)

Component[a]	%[b]	
	Serotype-specific antigen	PCP-extracted LPS
$C_{14:0}$	5	28
3-OH-$C_{14:0}$	24	72
$C_{16:0}$	15	ND
$aC_{17:0}$	10	ND
$C_{18:0}$	47	ND
Rhamnose	17	5
Fucose	16	3
Galactose	ND	12
Glucose	39	23
Mannoheptose	8	21
Glucoheptose	ND	26
Glucosamine	ND	10
Galactosamine	20	ND

[a] $C_{14:0}$, Tetradecanoic acid; 3-OH-$C_{14:0}$, 3-hydroxytetradecanoic acid; $C_{16:0}$, hexadecanoic acid; $aC_{17:0}$, 14-methylhexadecanoic acid; $C_{18:0}$, octadecanoic acid.

[b] The relative amount of each substance (corrected for molar response factor) is expressed as a weight percentage of the sum of all sugars or fatty acids identified (considered to be 100% for each group). ND, Not detected.

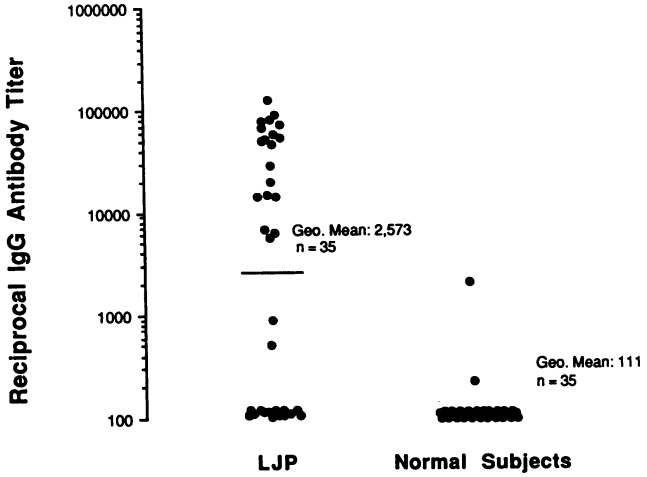

FIG. 6. Titers of IgG antibodies to the serotype b-specific polysaccharide antigen derived from *A. actinomycetemcomitans* Y4 in sera of periodontally healthy subjects and patients with LJP. Antibody titers were determined by ELISA and were expressed as a reciprocal of the serum dilution yielding an optical density at 405 nm of 0.6 under standardized assay conditions. The difference in geometric mean titer between LJP and normal subjects was significant ($P < 0.0001$ by t test for unpaired samples).

Immunoblot analysis. The serotype b antigen was resolved by SDS-PAGE, transferred to nitrocellulose and reacted with LJP serum. This reaction yielded a diffuse band (or "smear") in the upper region of the lane (Fig. 5, lane 1), particularly in the area corresponding to an apparent molecular mass of >100 kDa. Immunoblot analysis did not reveal the presence of any contamination of the serotype b antigen with either outer membrane proteins or low-molecular-weight LPS.

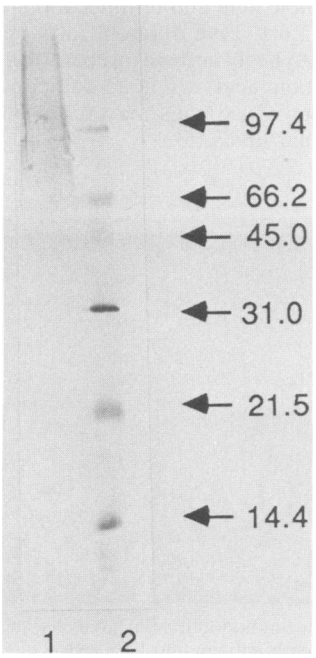

FIG. 5. Immunoblot of the *A. actinomycetemcomitans* serotype b polysaccharide developed with serum (1:250 dilution) from an LJP patient. Lane 1, Serotype b polysaccharide; lane 2, biotinylated molecular weight standards (10^3).

Antibody titer to the serotype b antigen in sera of periodontally healthy and LJP subjects. IgG antibody titers to the serotype b antigen were determined in 35 LJP serum samples and an equal number of sera from periodontally healthy individuals. Of 35 serum samples from periodontally healthy subjects, 33 had IgG antibody titers of <1:125 (Fig. 6). The other serum samples from these subjects had titers of 1:223 and 1:2,097. In contrast, 21 of 35 (60%) LJP sera tested had IgG titers to the serotype b antigen which were >2 standard deviations from the geometric mean titer of the periodontally healthy group. The geometric mean titer of sera from the LJP group was >20-fold higher than that of the periodontally healthy group ($P < 0.0001$), although antibody titers varied widely among LJP subjects. A number of LJP sera exhibited low antibody titers to the serotype b antigen.

Relationship between LJP subject age and antibody titer to the serotype b antigen. It has been suggested that periodontal destruction involving the incisor-molar teeth may occur prior to the development of a protective antibody response (13, 18). This hypothesis may help explain the dichotomous pattern of antibody response to the serotype b antigen seen among LJP patients. Accordingly, we examined the relationship between IgG titer to the serotype b antigen and chronologic age of the LJP patients. Antibody titers to the serotype b antigen exhibited age-dependent variation (Fig. 7). Patients < 18 years of age ($n = 16$) exhibited a modest antibody response (geometric mean titer, 1,391), while LJP subjects ≥ 18 years of age ($n = 19$) had a mean antibody titer of 4,318. This difference in antibody titer between the two LJP subgroups was significant ($P < 0.05$). Mean IgG titers to the serotype b antigen rose sharply in the 18- to 21-year age group (mean titer, 6,487).

Relationship between LPS and the serotype b antigen. As discussed previously, the serotype b antigen and PCP-extracted LPS formed a line of identity following the reaction with either rabbit antiserum or LJP serum. Moreover,

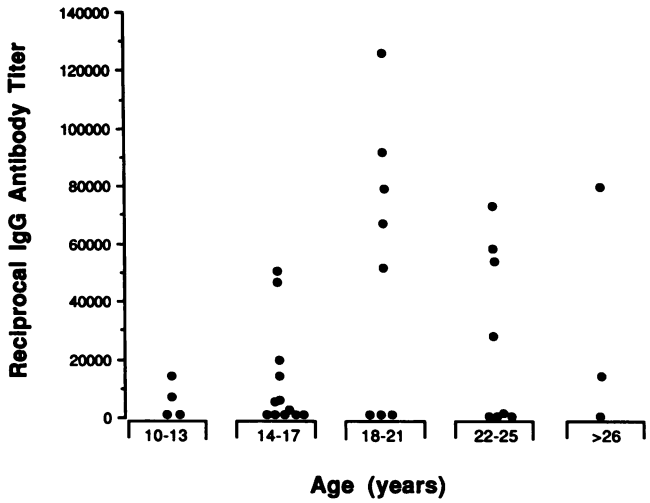

FIG. 7. Relationship between IgG antibody titer to the serotype b polysaccharide antigen from *A. actinomycetemcomitans* Y4 and LJP subject age. Geometric mean values for each LJP subgroup were as follows: 10 to 13 years, 996; 14 to 17 years, 1,555; 18 to 21 years, 6,487; 22 to 25 years, 2,247; >26 years, 8,325.

the serotype b antigen contained significant amounts of mannoheptose and 3-hydroxytetradecanoic acid, components unique to LPS. As specific antibody to LPS is principally directed to the polysaccharide moiety, we examined the relationship between the polysaccharide component of LPS and the serotype b antigen. Lipid-free oligosaccharide was obtained by mild acid hydrolysis of PCP-extracted LPS. The lipid-free oligo-saccharide fraction contained trace glucosamine and <1% fatty acid (and undetectable amounts of 3-hydroxytetradecanoic acid or tetradecanoic acid) but a significant amount of mannoheptose (18.8%), glucoheptose (16.7%), glucose (15.8%), galactose (9.2%), and rhamnose (2.3%). Incubation of LJP serum with lipid-free oligosaccharide markedly reduced IgG antibody titer to the serotype b antigen (Table 2). In contrast, lipid A (which contained glucosamine and 3-hydroxytetradecanoic acid as the major components, but trace amounts of neutral sugar) was poorly inhibitory. These results indicated that IgG antibody which recognizes the serotype b antigen of *A. actinomycetemcomitans* cross-reacts with the polysaccharide moiety of LPS.

TABLE 2. Inhibition of ELISA-reactive IgG antibody to the *A. actinomycetemcomitans* serotype b antigen by lipid-free oligosaccharide (LFO) and free lipid A

Inhibitor[a]	Concn (μg/ml)	% Reduction in IgG titer to serotype b antigen
Lipid A[b]	500	27.2
	250	13.0
	100	4.3
LFO	500	87.1
	100	13.8
	50	9.5

[a] LJP serum (10%) was incubated with the indicated concentration of lipid A or LFO for 1 h at 0°C. Residual IgG antibody titer to the serotype b antigen was determined by ELISA, as described in Materials and Methods.

[b] Free lipid A was solubilized via addition of 2 μl of triethylamine per ml of solution and briefly sonicated prior to use.

DISCUSSION

Analysis of the serotype distribution of *A. actinomycetemcomitans* among LJP patients indicates that serotype b strains are more frequently represented in the oral cavity of such patients than serotype a or c strains (33). This observation suggests that serotype b strains possess a virulence factor(s) which may enhance the periodontopathic character of these strains relative to other serotypes of *A. actinomycetemcomitans*. Efforts to evaluate the importance of the serotype b-specific antigen as either a virulence factor or a potential target for host defense have been hampered by a paucity of information regarding the precise nature of the molecule.

The phenol-water extraction technique is commonly used in the preparation of LPS from various species of gram-negative bacteria. However, the aqueous phase obtained through this method often consists of a complex mixture of both hydrophilic LPS and polysaccharides (7, 13, 17, 23). Separation of polysaccharides and LPS present in phenol-water extracts can be achieved by either isopycnic density gradient centrifugation (13) or gel permeation chromatography in LPS-disaggregating buffer containing sodium deoxycholate (7, 16, 23, 25). In the present study, we used chromatography in deoxycholate-containing buffer to isolate a high-molecular-weight polysaccharide-containing antigen from the aqueous phase of a phenol-water extract of *A. actinomycetemcomitans* Y4. This antigen reacted with rabbit serotype b-specific antiserum but not with antisera to other *A. actinomycetemcomitans* serotypes. IgG antibody in sera of LJP subjects reacted with the same antigen recognized by rabbit antiserum.

Serum IgG antibody titers to the serotype b polysaccharide antigen were found to be significantly higher among LJP subjects than among a group of periodontally healthy subjects. Sixty percent (21 of 35) of the LJP patients tested had antibody titers >2 standard deviations from the geometric mean titer of the periodontally healthy group. Within the group of LJP subjects exhibiting elevated antibody titers to the serotype b antigen, 10 patients had titers exceeding 1:50,000. In contrast, only 2 of 35 periodontally healthy subjects (6%) had IgG titers >1:125, the sera from these two subjects showing only modest titers. The latter result is consistent with previous studies indicating that approximately 10% of periodontally healthy subjects have elevated serum antibody titers to *A. actinomycetemcomitans* Y4 (32).

Despite the fact that the geometric mean IgG titer to the serotype b polysaccharide was more than 20-fold greater than that of the periodontally healthy group, it was evident that not all LJP patients exhibited a significant antibody response to this antigen. Previous studies have shown that antibody directed toward *A. actinomycetemcomitans* antigens may be protective (14, 22). It has been suggested that such a protective antibody response may emerge after initial periodontal injury to the incisor-molar teeth has occurred. Were this the case, it might be anticipated that antibody titers to *A. actinomycetemcomitans* antigens would exhibit age-dependent variation. Accordingly, we examined the relationship between LJP subject age and IgG antibody titer to the serotype b polysaccharide. High levels of antibody to the serotype b polysaccharide were observed predominantly in LJP subjects ≥18 years of age. The geometric mean antibody titer among LJP patients in this subgroup was threefold higher than the mean titer of LJP patients <18 years of age, a difference which was significant ($P < 0.05$). A similar pattern of age dependency has been noted with respect to the

IgG response of LJP patients to the heat-modifiable outer membrane protein of *A. actinomycetemcomitans* (31). These findings suggest that LJP subjects of circumpubertal age may be particularly vulnerable to *A. actinomycetemcomitans*-initiated periodontal destruction as a consequence of inadequate antibody-mediated protection during this period.

Chemical analysis of the serotype b polysaccharide and LPS revealed that both were essentially free of protein and nucleic acid contamination. PCP-extracted LPS from *A. actinomycetemcomitans* Y4 contained 3-hydroxytetradecanoic acid as the most abundant fatty acid, followed by tetradecanoic acid. Similar results have been reported by Brondz and Olsen (4, 5). The LPS contained small amounts of rhamnose and fucose. The serotype b antigen also contained 3-hydroxytetradecanoic acid and mannoheptose, which are considered to be unique constituents of LPS. The serotype b antigen contained a proportionately lesser amount of mannoheptose, and greater amounts of rhamnose and fucose, than PCP-extracted LPS. The chemical profile of the serotype b antigen was consistent with that of a high-molecular-weight LPS containing a lesser amount, by weight, of core region determinants relative to the more hydrophobic PCP-extracted LPS.

A relationship between the serotype b antigen and LPS was further suggested by immunodiffusion studies which revealed that these two antigens yielded a line of identity following their reaction with either rabbit serotype b-specific antiserum or LJP serum. Moreover, IgG antibody activity in LJP serum reactive toward the serotype b antigen could be markedly reduced by a lipid-free oligosaccharide from LPS, but not by lipid A. These results indicate that the polysaccharide moiety of LPS and the serotype b antigen are immunochemically identical. It appears likely that these two species contain the same repeating polysaccharide subunit structure, but differ in number of subunits per molecule.

The results of this study indicate that the serotype b-specific antigen of *A. actinomycetemcomitans* is contained within the polysaccharide moiety of LPS. The importance of the serotype b determinant as a potential target for opsonic and/or bactericidal antibody against *A. actinomycetemcomitans* remains unclear. We have attempted to address this question by preparing antisera against the purified serotype b antigen. However, this antigen has proven to be poorly immunogenic in rabbits. Other bacterial polysaccharides and LPS have also been found to exhibit poor immunogenicity, although high levels of serum antibodies have been obtained following immunization with polysaccharide-protein conjugates (24). Accordingly, current efforts are directed toward the preparation of serotype b polysaccharide-protein conjugates which may facilitate studies designed to evaluate the role of antibody to the serotype b determinant in host defense against *A. actinomycetemcomitans*.

ACKNOWLEDGMENTS

We thank Paul Bronson and Marcy Salvo for technical assistance. We also thank R. Genco and the staff of the Periodontal Disease Clinical Research Center at Buffalo for providing the normal and LJP sera employed in these studies and for conducting dental examinations of these patients. We also thank J. Zambon for providing rabbit serotype-specific antisera.

This work was supported by U.S. Public Health Service grants DE08240 and 04898 and by Physician Scientist Award DE00137 (to R.E.S.) from the National Institute of Dental Research.

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