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The Immunodominant Outer Membrane Antigen of *Actinobacillus actinomycetemcomitans* Is Located in the Serotype-Specific High-Molecular-Mass Carbohydrate Moiety of Lipopolysaccharide

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Most patients with juvenile periodontitis manifest serum antibodies, sometimes at very high titers, to antigens of *Actinobacillus actinomycetemcomitans*, but the antigens inducing the immune response have been only partly characterized. We separated *A. actinomycetemcomitans* serotype b cells into protein, lipopolysaccharide (LPS), and soluble polysaccharide fractions and characterized them. Coomassie blue- and silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels were used to detect protein and LPS components, and gas-liquid chromatography was used to determine their carbohydrate and fatty acid composition. Western blots, dot blots, and enzyme-linked immunosorbent assay inhibition with high-titer sera from juvenile periodontitis patients revealed which components were highest in antibody binding activity. These results showed that the major portion of the immunoglobulin G binding activity resides in the purified mannan-free LPS, with lesser amounts in the total protein fraction. Using Sephacryl S-300 chromatography, we separated LPS into high-molecular-mass components with high carbohydrate contents by gas-liquid chromatography and a low-molecular-mass component consisting mainly of lipid A and the inner core sugar heptulose. The results of quantitative dot blot assays and enzyme-linked immunosorbent assay inhibition show that the serotype-specific antibody binding activity is highly concentrated in the high-molecular-mass carbohydrate-rich LPS fraction and is almost completely absent in the low-molecular-weight lipid-rich fraction. Our observations contrast with previous reports that the predominant serotype antigen of *A. actinomycetemcomitans* resides in a mannan-rich polysaccharide isolated from spent culture medium. These observations support the conclusion that the immunodominant antigen of the outer membrane is the O antigen of the LPS.

Actinobacillus actinomycetemcomitans has been strongly implicated as the causative agent in juvenile periodontitis (JP) (11, 18, 23, 24, 34, 35, 37), and many patients manifest serum antibodies that are reactive with antigens of *A. actinomycetemcomitans* (1, 3, 5, 7–10, 15, 17, 21, 22, 26, 27, 29, 37). The role these antibodies play in the progress of the disease is unknown, in part because the antigens responsible for inducing them have not been identified and characterized.

Three serotypes of *A. actinomycetemcomitans* designated as a, b, and c have been identified (35). Serotype b (Y4) has been most frequently associated with oral infections (7, 37). The serotype b-specific antigens have been of particular interest because they are the quantitatively major antigen class as measured by antibody binding from high-titer sera of patients with JP (3, 7, 22) and because of their possible role in pathogenicity and induction of potentially protective immune responses (37). We recently showed that the serotype-specific antigens of *A. actinomycetemcomitans* serotype b account for 72 to 90% of the total antibody from high-titer sera that binds to enzyme-linked immunosorbent assay (ELISA) plates coated with sonic extracts of whole serotype b cells and that the antigens are found in the purified lipopolysaccharide (LPS) fraction obtained from *A. actinomycetemcomitans* (22). These observations, however, contrast with other studies reporting that the serotype-specific antigenic activity resides in polymers of capsular polysaccharide that contain large amounts of mannose (36, 37) or in a carbohydrate polymer consisting of repeating units of rhamnose and fucose (1).

In the present report we focus on the biochemical identity of the serotype-specific, quantitatively dominant class of antigens of *A. actinomycetemcomitans* serotype b as recognized by high-titer sera from patients with JP. Protein, LPS, and polysaccharide fractions were prepared from *A. actinomycetemcomitans* serotype b and biochemically and immunochemically characterized. Our present data and those of a previous report (22) show that the major serotype-specific antigens of the outer membrane reside in the carbohydrate O-antigen side chains of LPS.

MATERIALS AND METHODS

Serum samples. Sera were obtained from patients ranging in age from 10 to 38 years and diagnosed as having JP by using standard criteria, including radiographic evidence of alveolar bone loss and pocket depths of 5 mm or greater around the first permanent molars and/or incisors with onset in the circumpubertal period. A pool of patient sera was used in some experiments. The pool was made by combining equal volumes of sera from 10 patients previously shown to have high immunoglobulin G (IgG) titers against antigens of *A. actinomycetemcomitans* serotype b. Serum samples were also obtained from subjects judged to be periodontally normal by radiographic and clinical examination and ranging in age from 5 to 38 years; one of these periodontally normal subjects was a sibling of one patient. Serum from clotted

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venous blood or, in a few cases, plasma containing 1.0 IU of heparin per ml was harvested by centrifugation and stored at −70°C until tested. Rabbit serum that was specific for *A. actinomycetemcomitans* serotype b was a gift from J. J. Zambon (State University of New York, Buffalo).

Bacteria and culture conditions. *A. actinomycetemcomitans* serotype b strain ATCC 43718 was obtained from the American Type Culture Collection, and strain Y4 was provided by J. J. Zambon. The two strains yielded comparable results. Organisms were cultured to the midlogarithmic stage of growth in enriched Trypticase soy broth (25) in an anaerobic glove box. Cells were harvested by centrifugation at 10,000 × *g* for 10 min, washed twice in phosphate-buffered saline and once in distilled water, and then immediately stored at −70°C until used. The protein contents of cells and fractions were determined by the method of Lowry et al. (16).

Antigen preparations. Lyophilized bacteria were suspended in distilled water at 10 mg (dry weight) per ml, heated in a boiling water bath for 30 min to inactivate heat-labile proteases, disrupted with a Cole-Parmer 4710 ultrasonic homogenizer (5 min, 50% pulse mode, 40% power), and diluted in buffer appropriate for polyacrylamide gel electrophoresis (PAGE) or an immunoblot assay as described below.

For the preparation of total protein fractions, we used a modification of the method of Sims et al. (22). Briefly, cell homogenates were sonicated in carbonate buffer containing 1% sodium dodecyl sulfate (SDS) at pH 8.0, boiled for 30 min, and centrifuged at 100,000 × *g* for 1 h to remove the peptidoglycan and insoluble debris. The supernatant was diluted 1:1 with cold 20% trichloroacetic acid to precipitate proteins, and centrifuged at 12,000 × *g* at 4°C for 30 min. The trichloroacetic acid-precipitated pellet was washed once with 10% trichloroacetic acid, resuspended in water, dissolved by titration to neutral pH with 0.01 N NaOH, and dialyzed against phosphate-buffered saline and then water. To remove residual LPS from the protein solution, it was centrifuged at 200,000 × *g* for 2 h and the resulting supernatant was lyophilized.

LPS was prepared by a modification of the hot phenol-water method of Westphal and Jann (30). Briefly, after dialysis the aqueous phase was centrifuged at 200,000 × *g* for 2 h in the presence of 25 mM $MgCl_2$. The pellet was suspended in 10 mM Tris (pH 8) and treated with 200 μg of DNase per ml and 50 μg of RNase per ml for 2 h at 37°C. Pronase E was added to a concentration of 200 μg/ml and allowed to react overnight. The preparation was dialyzed, centrifuged twice in the presence of 25 mM $MgCl_2$ at 200,000 × *g* as described above, suspended in deionized water, and lyophilized. LPS from *E. coli* (O217:B8), prepared by the Westphal and Jann (30) method, was purchased from Sigma, St. Louis, Mo. LPS was also purified by the method of Darveau and Hancock (4). Purified LPS was fractionated on a Sephacryl S-300 (2.5 by 90 cm) column eluted with buffer containing 10 mM Tris, 0.25% deoxycholate, 0.2 M NaCl, 1 mM EDTA, and 0.02% sodium azide at pH 8.0 (20). The column was eluted at the ambient temperature at 10 ml/h, and 5-ml fractions were collected. Fractions were monitored with the sugar assay of Dubois et al. (6). Fractions were pooled (see Fig. 4) and dialyzed. To check the specificity and purity, the fatty acid and sugar composition of all antigen fractions was assessed by gas-liquid chromatography (GLC) with a Hewlett-Packard 5890 system equipped with an HP-1 capillary column by the method of Bryn and Jantzen (2) as described previously (22).

Soluble polysaccharide was prepared from spent culture medium by the method of Zambon et al. (36). Polysaccharide fractions were also prepared from washed cells and from spent culture medium as follows. A phenol-water extract was made as described by Westphal and Jann (30). The aqueous phase was made 50 mM in $MgCl_2$ and centrifuged at 200,000 × *g* for 1 h to sediment aggregated LPS. The supernatant of this fraction was dialyzed extensively against water and lyophilized and is designated as the crude phenol polysaccharide. Crude polysaccharides were then further purified by affinity chromatography on polymyxin B-agarose as described by Issekutz (13) to remove residual LPS. Five milligrams of crude phenol polysaccharide dissolved in 10 ml water was passed five times through a 5-ml column bed at a flow rate of 10 ml/h. The passage was repeated twice with fresh column beds, and the final effluent was lyophilized. The total protein content of each bacterial homogenate and fraction was measured (16). Total sugar and heptulose were measured by the phenol-sulfuric acid method (6) and the cysteine-sulfuric acid procedure (33), respectively. The ratio of nucleic acid and protein in the fractions was determined by UV A_{260} and A_{280}. The fractions were also analyzed by SDS-PAGE (14). Protein bands were stained with Coomassie blue, and LPS migration patterns were made visible with ammoniated silver nitrate stain (12).

Nitrocellulose immunoblots. Western immunoblots of the whole-cell sonic extract and purified fractions were prepared as described by Towbin et al. (28). Samples were suspended in buffer (100 mM Tris, 10% sucrose, 1% SDS, 1% 2-mercaptoethanol [pH 6.8]) at a protein concentration of 1 mg/ml and heated in a boiling water bath for 10 min, and samples of 25 μl were separated in 11 or 15% polyacrylamide gels. The gels were placed in contact with nitrocellulose sheets, immersed in blot buffer (25 mM Tris, 0.1 M glycine, 20% methanol [pH 8.8]) between two wire grid electrodes, and subjected to an electrical field of 7 V/cm for 1 h. The nitrocellulose sheets were washed for 5 min with five changes of blocking buffer (10 mM *N*-Tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid [TES], 1% gelatin, 0.1% Tween 20 [pH 7.5]), incubated in serum diluted in blocking buffer for 2 h on a rocking platform, washed again, incubated for 2 h in goat anti-human IgG (γ chain specific)-alkaline phosphatase conjugate (Sigma) diluted 1:500, washed again, and incubated in 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium phosphatase substrate solution (Kirkegaard and Perry, Gaithersburg, Md.) until antibody-bearing bands were visible.

For the dot blot assay, whole sonic extract, LPS, carbohydrate, and protein (see Fig. 3) samples were suspended at a concentration of 1 mg/ml in sodium carbonate buffer containing 20 mM $MgCl_2$ and diluted serially 1:1 in microtest plate wells. A 4-μl sample from each well was then spotted onto a sheet of nitrocellulose with a 12-channel micropipetter. Sheets bearing rows of spots representing dilutions of different antigen preparations were then incubated with blocking buffer, washed, incubated with pooled patient serum, and processed to reveal bound IgG as described above for electrophoretic immunoblots. The dot blots shown in Fig. 11 were generated in the same manner, except they were incubated in serotype b-specific serum generated in rabbits (kindly provided by J. J. Zambon) and developed with goat anti-rabbit IgG. The dot blot assay of column fractions (see Fig. 7) was developed with pooled patient sera. The first dot contained 720 ng of sugar, as determined by the phenol-sulfuric acid assay (6), and samples for each

dot were serially diluted 1:1 such that dot 12 contained 0.35 ng of sugar.

ELISA inhibition. Polystyrene microtest plate wells were coated with LPS, subfractions of LPS, or ultrasonically disrupted cells at 1 μg/ml in 50 mM carbonate buffer (pH 9.6) containing 20 mM $MgCl_2$ as described previously (22). Inhibitors (LPS, column fractions, or disrupted cells) were then added to coated wells in 50-μl aliquots at a concentration of 200 μg/ml (dry weight) and diluted serially 1:1 in blocking buffer (0.1% Tween 20 in 10 mM N Tris [pH 7.5]), such that 12 concentrations of each were established. Serum previously shown to have a high IgG titer against Y4 cells was then diluted 1:2,000 in blocking buffer and added to each well containing an inhibitor and to controls without an inhibitor, resulting in a further 1:1 dilution of both serum (1:4,000 final dilution) and the inhibitors (100 μg/ml, final maximum concentration). The plates were then incubated for 1 h and further processed to measure specific IgG binding in the presence of an inhibitor, in accordance with the standard ELISA protocol described previously (22). The percent inhibition (PI) due to each inhibitor at each concentration was calculated as follows: PI = [1 − (ELISA absorbance with inhibitor/ELISA absorbance without inhibitor)] × 100.

Linear regression analysis was then performed on the series of percent inhibition values for each inhibitor versus the natural log of the inhibitor concentration. The linear regions of these dilution curves (Pearson r value of >0.95) were used to calculate the units of inhibitor activity for each sample relative to that of a control inhibitor, for which the dilution curve was arbitrarily assigned a value of 100 activity units. For a given plate, the control curve used was one in which the inhibitor and the plate-coating antigen were identical. Activity units calculations were done by using a BASIC computer program written in our laboratory and based on the calculation algorithms described by Peterman and Butler (19).

RESULTS

Protein, LPS, and polysaccharide were prepared from whole-cell sonic extracts, and LPS, and polysaccharides from spent culture medium of *A. actinomycetemcomitans* serotype b. To assess purity, these fractions, whole-cell sonic extracts, and virgin culture medium were analyzed by GLC and other biochemical and immunologic procedures described in Materials and Methods.

Biochemical composition of the major fractions. The GLC data for the fractions prepared from the spent culture medium are shown in Fig. 1A. The polysaccharide prepared from the medium by the method of Zambon et al. (36) contained 33.0% mannose and 1.8% glucose, with amounts of galactose, fucose, rhamnose, and heptulose ranging from 0.1 to 1.9%. The growth medium polysaccharide purified by our phenol-water method resembled that prepared by the Zambon et al. (36) method in that it contained 38% mannose, but values for glucose, galactose, fucose, and rhamnose were considerably higher. Both polysaccharide fractions contained traces of heptulose and 16:0 fatty acid. After further purification of the phenol-water polysaccharide by passage over a column of polymyxin B-agarose, only traces of markers for LPS were observed, and these in only some patterns. Virgin culture medium was also analyzed and found to contain 16.8% mannose and 6.3% glucose. Galactose, fucose, heptulose, and rhamnose were present at 3.3, 1.1, 0.6, and 0.5%, respectively.

The GLC data for the whole-cell sonic extracts and cell fractions are shown in Fig. 1B. Whole-cell sonic extracts contained the markers for the core carbohydrates, lipid A of LPS including 3-hydroxy 14:0 and 14:0 fatty acids, and heptulose in amounts of 2.1, 1.9, and 1.1%, respectively. Notably, mannose was not detected in any analysis of whole cells. The polysaccharide fraction obtained directly from the whole cells differed greatly from the medium-derived fractions. Notably, as with the whole-cell sonic extracts, mannose was not detectable. Rhamnose and fucose were enriched relative to the medium fraction with values of 11.0 and 9.0%, respectively; glucose and galactose accounted for 5.0 and 2.8%, respectively, and the markers for LPS were present, demonstrating contamination by LPS. After the polymyxin B-agarose purification step, however, only trace amounts of the LPS markers were observed.

The LPS extracted from the whole cells (Fig. 1B) and that obtained from the medium (Fig. 1A) with the phenol-water method (30) had remarkably similar compositions (Table 1). Mannose was not detected, and glucose accounted for more than 25% of the total dry weight. Glucosamine was present, as were the markers for the core carbohydrates and lipid A, including 3-hydroxy 14:0, heptulose, and 14:0 fatty acids. Notably, ketodeoxyoctonic acid (KDO) was generally not detected in the analyses of whole cells culture medium, or purified fractions. The composition of the LPS obtained from the cells with the method of Darveau and Hancock (4) differed from that obtained with the phenol-water method in that it contained a much lower proportion of glucose and two- to threefold more hydroxy fatty acids (Table 1; Fig. 1).

The protein fraction contained barely detectable amounts of glucose, undetectable amounts of rhamnose, fucose, galactose, mannose, and heptulose, and barely detectable amounts of the markers for LPS including 3-hydroxy 14:0 fatty acids (Table 1).

The various fractions were evaluated for contamination and composition by SDS-PAGE. The LPSs from *Escherichia coli* and from *A. actinomycetemcomitans* (Fig. 2, gel 1, lanes A and B, respectively) manifest no staining, indicating that these preparations are free of amounts of contaminating protein detectable by this procedure. The pattern for extracted protein (Fig. 2, gel 1, lane E) demonstrates that most if not all of the protein bands present in the starting material can be accounted for in the protein fraction (Fig. 2, gel 3, lanes C and E). A pattern for fractions stained with silver to demonstrate components of LPS is shown in Fig. 2, gel 2. LPS from *E. coli* (lane A) and that from *A. actinomycetemcomitans* extracted by the phenol-water method (lane B) stain intensely in both the high- and low-molecular-mass regions of the gels and show typical ladders. The pattern for the whole-cell sonic extract (lane C) shows intense staining in the low-molecular-mass region and several faintly staining components throughout the other regions of the lane. The Zambon polysaccharide material (lane D) manifests staining only at the origin, whereas the pattern for the extracted protein fraction (lane E) is free of staining except for the very low-molecular-mass zone near the advancing front. A comparable pattern stained with silver for LPS and counterstained with Coomassie blue for protein is shown in Fig. 2, gel 3. Coomassie blue staining did not alter the character of the patterns for lanes containing LPS but did permit visualization of the protein bands in the whole-cell sonic extract, again supporting the conclusion that the extracted protein fraction contains all of the protein component bands observed in the whole-cell sonic extract. Staining

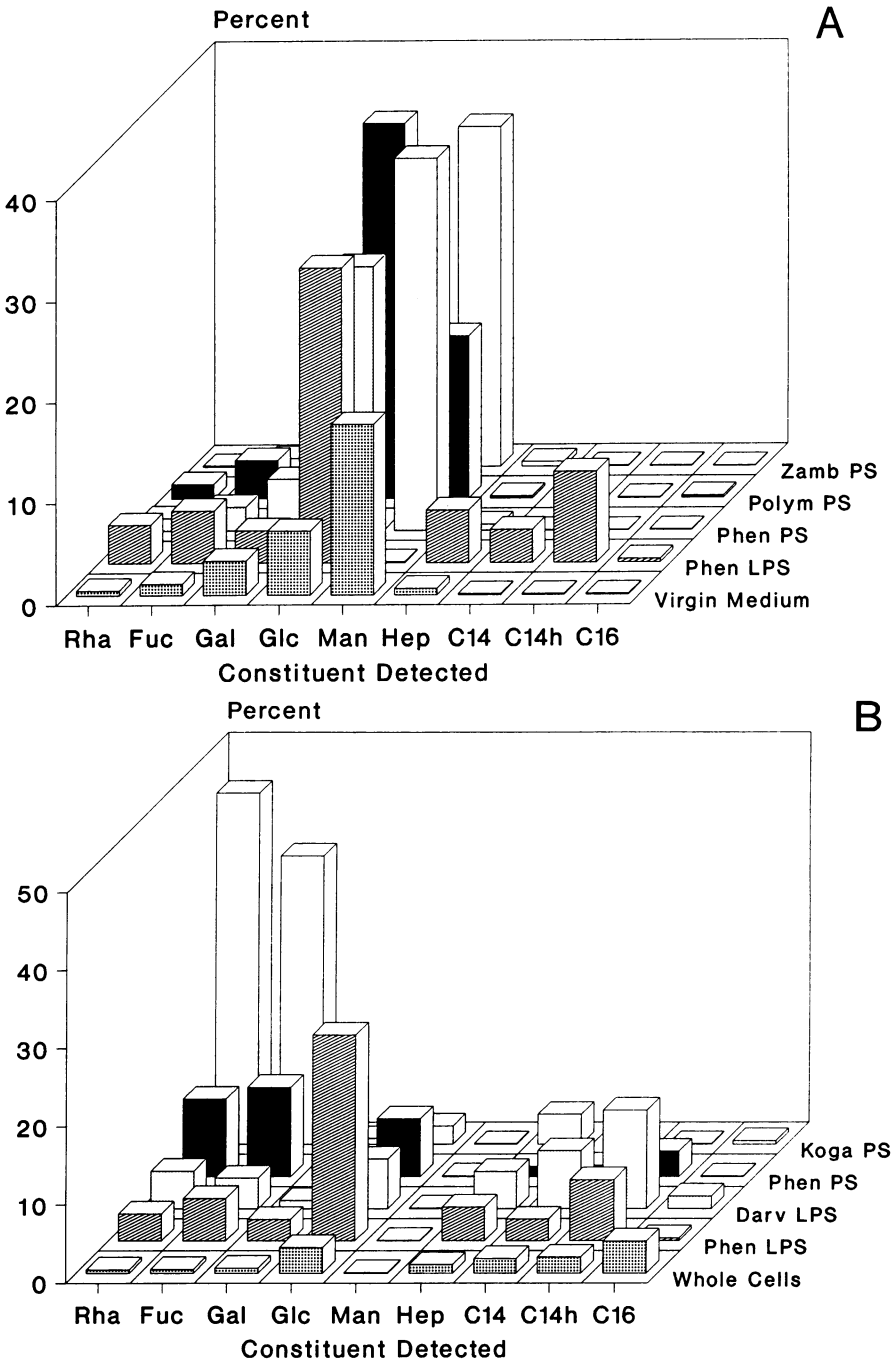

FIG. 1. (A) Composition of virgin culture medium and fractions purified from the spent culture medium by the method of Zambon et al. (36) (Zamb PS) or by our phenol-water method (Phen PS) and further purified by chromatography on a polymyxin B-Agarose column (13) (Polym PS) and LPS purified by the method of Westphal and Jann (30) (Phen LPS), as determined by GLC. Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Hep, heptose; C14, 14:0 fatty acid; C14h, 3-OH,14:0 fatty acid; C16, 16:0 fatty acid. (B) Composition of whole *A. actinomycetemcomitans* cells and fractions purified from whole cells. Polysaccharide was prepared by the phenol-water method (Phen PS), and LPS was prepared by the method of Darveau and Hancock (4) (Darv LPS) or by the method of Westphal and Jann (30) (Phen LPS). The Koga antigen (Koga PS) was prepared as described by Amano et al. (1) and was provided by T. Koga. Designations on the horizontal axis are as in panel A.

was still not observed except at the origin for the Zambon-prepared polysaccharide (lane D).

Immunoreactivity of major fractions. SDS-PAGE patterns in which the lanes contain samples identical to those in gels 2 and 3 were used to prepare Western blots developed with pooled high-titer JP patient sera (Fig. 2, blot). No staining was observed in lane A containing *E. coli* LPS, but intense staining was observed, especially in the high-molecular-mass material containing *A. actinomycetemcomitans* LPS extracted by the phenol-water procedure (lane B). Intense

TABLE 1. Sugar and fatty acid content of LPS and protein isolated from cells or culture medium of *A. actinomycetemcomitans*

Component	% (dry wt) of:			
	Medium-derived LPS (Westphal-Jann[a])	Cell-derived LPS		Cell-derived protein
		Westphal-Jann	Darveau-Hancock[b]	
Rhamnose	3.74	3.47	4.81	ND[c]
Fucose	5.13	5.45	3.94	ND
Galactose	3.16	2.76	1.09	ND
Mannose	ND	ND	ND	ND
Glucose	29.20	26.40	6.37	0.30
Heptulose	5.15	4.35	4.79	ND
14:0 fatty acid	3.23	2.80	7.44	0.05
14:0,3:OH fatty acid	8.96	7.82	12.61	0.01
16:0 fatty acid	0.36	0.40	1.63	0.36

[a] Derived by the method of Westphal and Jann (30).
[b] Derived by the method of Darveau and Hancock (4).
[c] ND, not detected.

staining was also noted in protein bands (lanes C and E) and in the smear material located in the region where high-molecular-mass LPS is known to migrate (lane C). Immunostaining in lane D, containing the polysaccharide fraction from serotype b prepared by the method of Zambon et al. (36), was negative except for a very faint band at the midpoint of the gel.

A quantitative assessment of binding of antibodies from pooled high-titer sera from JP patients was performed by using the dot blot assay. As shown in Fig. 3, row A, intense immunostaining was still apparent in the whole-cell sonic extract, even at dilution step 12, where the dot contained only 1.95 ng (dry weight) of the sample. Staining was also intense in phenol-water-extracted LPS (30) through dilution step 7 and faintly positive at step 12 (row B). In contrast, the phenol-water-prepared polysaccharide from spent culture medium and not further purified was immunopositive, but staining was not visible past step 7, where the dot represented 63 ng of the sample (row C). The polysaccharide fraction, after further purification with polymyxin B-agarose affinity chromatography to remove contaminating LPS, was only faintly immunopositive even at step 1 (row D). The polysaccharide fraction obtained from the spent medium by the method of Zambon et al. (36) was immunopositive at the highest concentrations, but no staining was visible past dot 4 (250 ng) (row E). The rhamnose-fucose serotype antigen (Koga antigen) (1) was intensely immunopositive at step 1, but staining could not be seen past step 7 (63 ng) where staining was comparable to that of LPS at step 12 (row F). The purified protein was intensely positive at step 1, but staining was not visible beyond step 8 (32 ng of antigen) (row G). Patterns obtained with an individual high-titer JP serum did not differ significantly from those developed with pooled high-titer JP patient sera (data not shown).

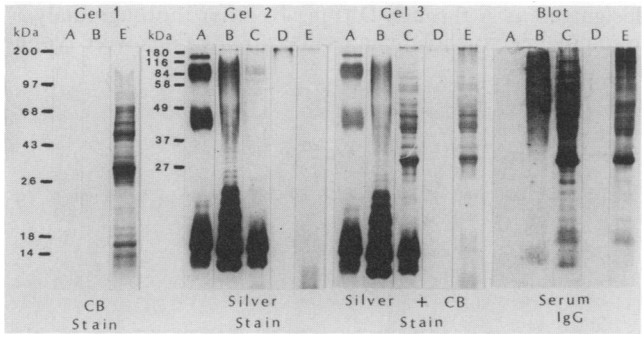

FIG. 2. SDS-PAGE patterns or gels stained with Coomassie blue for protein and with silver for LPS components and blotted with pooled high-titer patient sera. Gel 1 was 11% polyacrylamide, and gels 2 and 3 and the blot were 15% polyacrylamide; markers for the molecular mass standards are shown on the left. Gel 1, lanes A and B, contain LPS from *E. coli* (Sigma) and LPS from *A. actinomycetemcomitans* serotype b (30), respectively, each at a total load of 40 μg, and lane E contains the purified protein fraction from *A. actinomycetemcomitans* at 100 μg. The gels labeled 2, 3, and Blot contained *E. coli* LPS (40 μg) (lanes A), *A. actinomycetemcomitans* LPS (40 μg) (lanes B), whole *A. actinomycetemcomitans* sonic extract (100 μg) (lanes C), 100 μg of medium-derived polysaccharide prepared by the method of Zambon et al. (36) (lanes D), and 100 μg of purified protein (lanes E). Gel 2 is stained with silver to display components of LPS, and gel 3 is stained with silver and Coomassie blue to display LPS and protein components. The Western blot was developed with pooled high-titer patient sera.

Subfractionation of LPS. Because most of the antibody binding activity in the present and previous studies (22) appeared to reside in the purified LPS fraction from *A. actinomycetemcomitans*, we chose to fractionate phenol-water-extracted LPS further by using a Sephacryl S-300 column as described in Materials and Methods. The column effluent was monitored for sugar content by the method of Dubois et al. (6), and a typical chromatogram is shown in Fig. 4. A small peak was observed at the void volume of the column, followed by a plateau and then a large peak. Starting at the void volume, every second fraction was subjected to SDS-PAGE and stained with silver (data not shown for fractions 2 to 34). As shown in Fig. 5, a faint relatively wide band of silver-staining material can be observed beginning with fraction 42 and extending with increasing molecular mass through fraction 56. Intensely staining low-molecular-mass material was observed from fractions 64 through 76; the high-molecular-mass material was not visible in these fractions. Western blots were prepared with fractions 35 to 74 and developed with pooled high-titer JP sera. As shown in Fig. 6, the high-molecular-mass material in fractions 42 to 63 stained rather intensely. Faintly staining material of intermediate molecular mass was observed in fractions 64 to 68, and faintly staining low-molecular-mass material was observed in fractions 69 to 74.

Column effluent fractions were pooled into six fractions and in later runs into four fractions (Fig. 4), thoroughly dialyzed, and lyophilized for further analysis. As shown in Fig. 7, dot blot analysis revealed that fractions 2 and 3 contained most of the antibody binding activity, with immune staining still apparent at dilution step 12, where dots contained only 0.35 ng of carbohydrate. Lesser amounts of activity were observed in fractions 1 and 4, with still less in fraction 5 and very little activity in fraction 6.

Fractions 1 through 6 were subjected to analysis by GLC (Fig. 8). Fraction 1, which contained very little antibody binding activity, was rich in rhamnose and glucose with lesser amounts of fucose and galactose. Markers for LPS, including heptulose and hydroxy fatty acids, were not detected. The 16:0 fatty acid, which indicates phospholipids, accounted for 9.34 mol% of the recovered sugars and fatty acids. The composition of pools 2 and 3 was very similar. Almost 50 mol% of the recovered material was accounted for by rhamnose and roughly 20 mol% was accounted for by fucose. Glucose and galactose together accounted for about

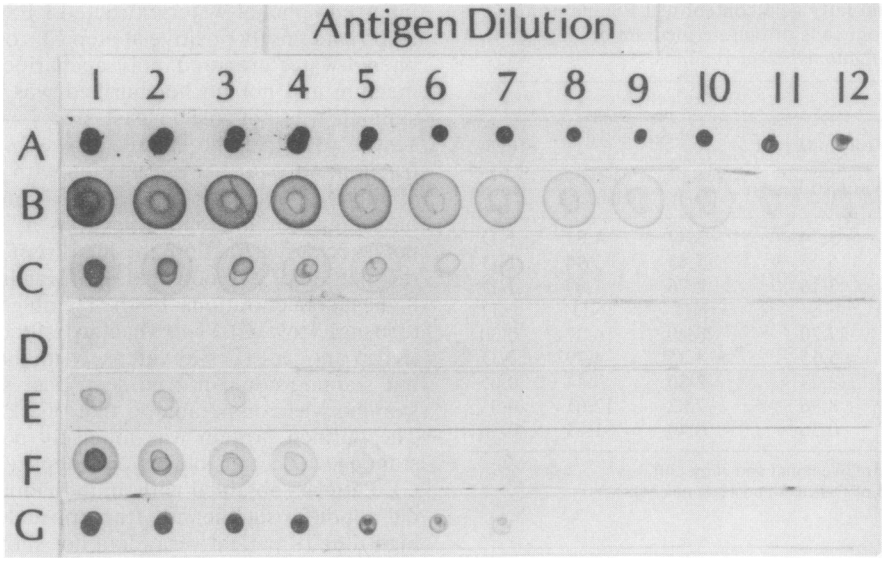

FIG. 3. Dot blot assays developed with a 1:500 dilution of pooled high-titer JP patient sera. The beginning antigen concentration was 1 mg/ml; 1:1 dilutions were made at each of the 12 steps, and 4 μl was applied at each step. All rows contained material derived from serotype b organisms. Rows: A, whole-cell sonic extract; B, LPS prepared from cells by the phenol-water method of Westphal and Jann (30); C, unpurified polysaccharide prepared by the phenol method from spent culture medium; D, phenol polysaccharide from spent culture medium purified by polymyxin B-agarose chromatography; E, medium-derived polysaccharide prepared by the method of Zambon et al. (36); F, rhamnose-fucose serotype antigen prepared by Amano et al. (1); G, purified protein fraction.

15 mol%, and glucosamine was present. Heptulose was not detected, but the 14:0 and 3:OH, 14:0 fatty acids accounted for about 3 and 5 mol%, respectively. Pools 5 and 6 were of very similar composition, and they differed greatly from pools 2 and 3. Rhamnose and fucose were not detected; glucose and galactose accounted for about 11 and 1.5 mol%, respectively. Heptulose accounted for about 6.5 mol%, the 14:0 fatty acid accounted for about 28 mol%, and the 3:OH,14:0 fatty acid accounted for 49 mol% of the moles recovered. Pool 4 differed from the other fractions in that it contained 36 mol% glucose, 25 mol% rhamnose, and ~20 mol% hydroxy and other fatty acids. Its composition, therefore, was somewhat intermediate between that of fractions 2 and 3 and that of fractions 5 and 6.

ELISA inhibition of LPS subfractions. Since the compositions of fractions 2 and 3 in previous column runs were very similar, as were those of fractions 5 and 6, for all subsequent column runs the fractions were combined into four pools and designated as A through D (Fig. 4). After thorough dialysis to remove deoxycholate and salts, the fractions were lyoph-

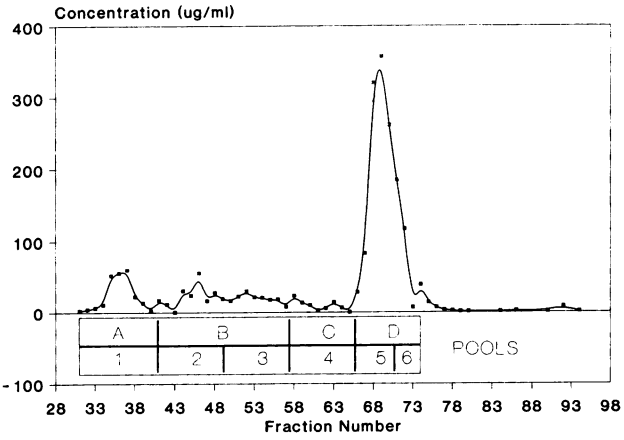

FIG. 4. Representative chromatogram of the fractionation of LPS purified by the method of Westphal and Jann (30) from whole cells on Sephacryl S-300. The effluent was monitored by the sugar content measured by the method of Dubois et al. (5). In early runs, fractions were combined as illustrated in the bars labeled 1 through 6; because the compositions of fractions 2 and 3 and fractions 5 and 6 were very similar, subsequent runs were combined as designated by the bar labeled A through D.

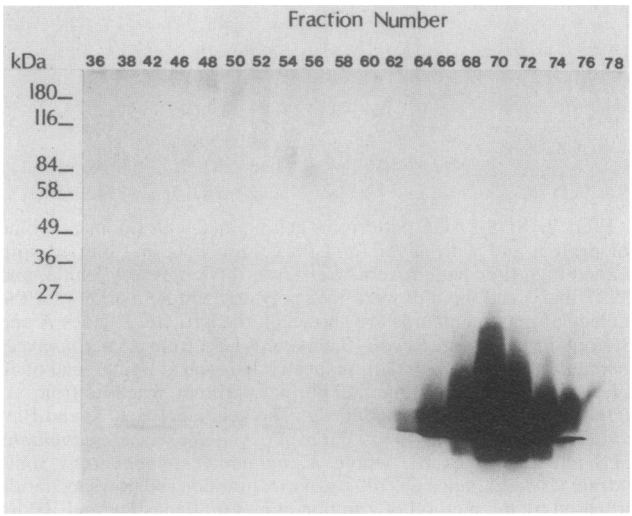

FIG. 5. SDS-PAGE analysis of fractions obtained from a Sephacryl S-300 column run of LPS purified from whole cells by the method of Westphal and Jann (30) and stained with silver by the method of Hitchcock and Brown (12).

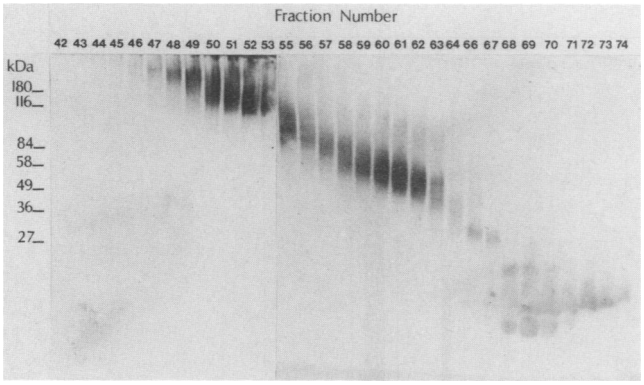

FIG. 6. Western blot and SDS-PAGE pattern of fractions obtained from a Sephacryl S-300 column run of LPS developed with pooled high-titer JP patient serum.

ilized and tested by ELISA inhibition with high-titer pooled JP sera. The results obtained with plates coated with either LPS or whole-cell sonic extract are reported as inhibitor activity units calculated as explained in Materials and Methods. The inhibition by purified LPS with plates coated with LPS as the antigen is set at 100 activity units (Fig. 9). Notably, fraction B was highly inhibitory, with values of >90 activity units for plates coated with LPS and ~130 activity units for those coated with whole-cell sonic extract. Fraction C was weakly inhibitory. In marked contrast, inhibition was negligible for fractions A and D as well as for the Koga antigen preparation (1) and for polysaccharide prepared by the method of Zambon et al. (36). Inhibition by our protein fraction and by an unrelated *Salmonella* LPS were also negligible.

The column fractions were retested along with LPSs from *A. actinomycetemcomitans* and *E. coli* as inhibitors, and plates were coated with LPS and column fractions B and D. High-titer sera from three individual patients were tested, and typical results are shown in Fig. 10. Inhibition by fractions A and D and by unrelated LPS was negligible. Fraction B was highly inhibitory with values greater than 200 inhibitor activity units for plates coated with LPS and greater than 125 activity units for plates coated with fraction B. As noted in the previous experiment, fraction C was considerably less inhibitory than fraction B. Interestingly, fraction D manifested negligible inhibition on plates coated with fraction D, most likely because the amount of antibody binding to such plates in the absence of inhibitor was exceedingly small. Thus, most of the inhibitory activity observed can be accounted for by fraction B, which contains 19.4% of the dry weight material recovered from the column.

Our final set of experiments was aimed at determining the relationships among the various antigen preparations by using dot blots developed with the Y4 serotype-specific antiserum generated in rabbits by Zambon et al. (35). As shown in Fig. 11, the results are very similar to those of dot blots developed with pooled patient sera (Fig. 3). The whole *A. actinomycetemcomitans* sonic extract and phenol-water-purified LPS continued to stain at step 12, where the dots contain only 1.95 ng of material. In contrast, polysaccharide prepared by our method and known to be contaminated with LPS, stained through dot 6, containing 125 ng of material. The polysaccharide fraction from spent culture medium prepared by the method of Zambon et al. (36) and the Koga antigen stained only through dot 4, containing 0.5 μg of material.

DISCUSSION

We separated components of *A. actinomycetemcomitans* serotype b cells into protein, LPS, and polysaccharide fractions. We characterized the fractions by SDS-PAGE and used Coomassie blue and silver staining to detect protein and LPS, respectively, GLC to assess carbohydrate and fatty acid composition, and Western blots and dot blots to quali-

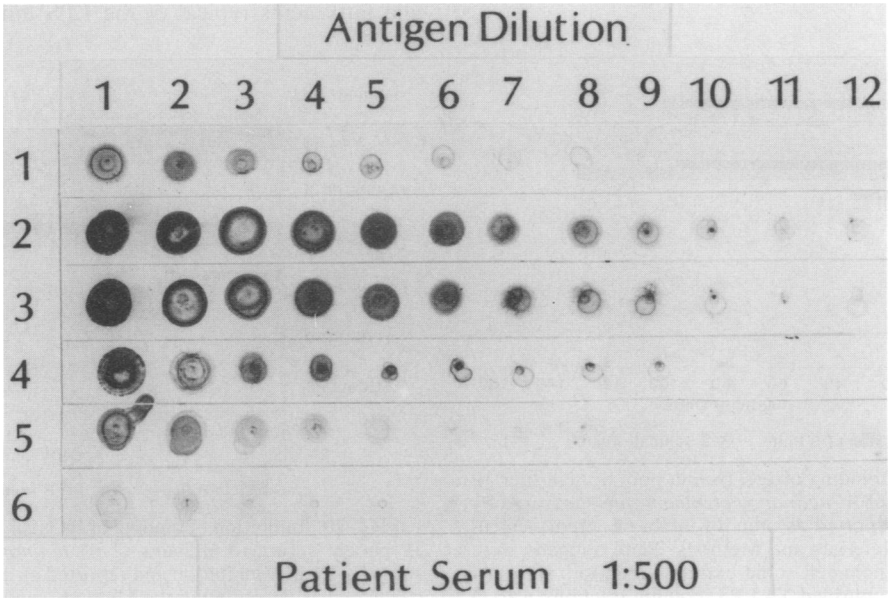

FIG. 7. Dot blot assay of pooled fractions of LPS obtained from chromatography of a column of Sephacryl S-300. Fractions extend from 1 (largest molecular mass) to 6 (smallest molecular mass) (Fig. 4). Dots contained 720 ng for dot 1 and 0.35 ng for dot 12, as measured by the phenol-sulfuric acid sugar assay (5).

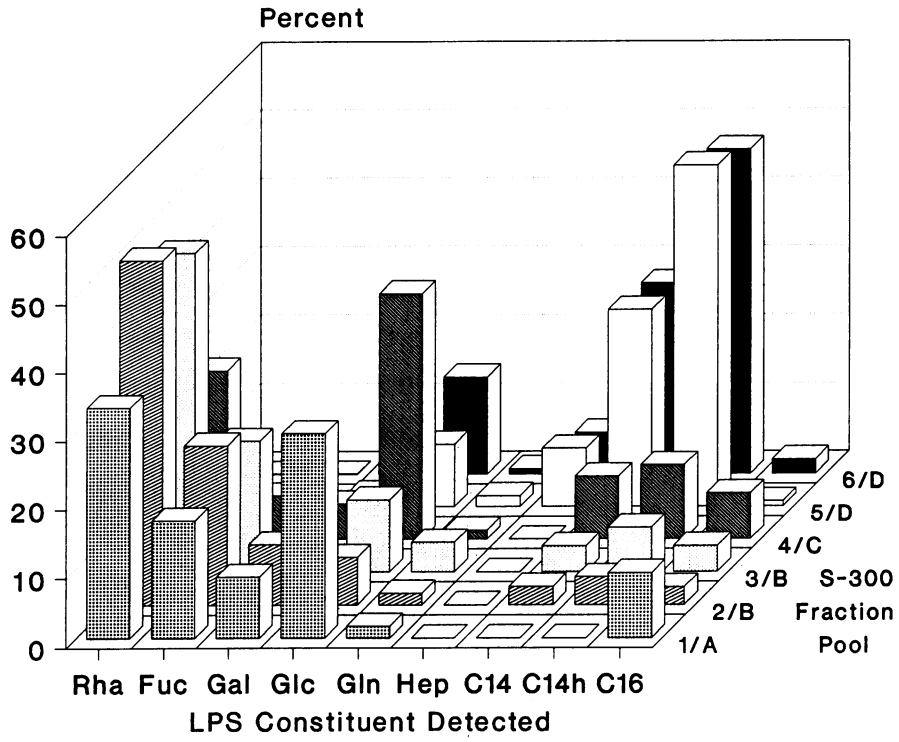

FIG. 8. GLC analysis of Sephacryl S-300 fraction pools used in the dot blot assay. Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; Gln, glucosamine; Hep, heptulose; C14, 14:0 fatty acid; C14h, 3-OH,14:0 fatty acid; C16, 16:0 fatty acid. Fractions are labeled 1 through 6 as well as A through D (Fig. 4).

tatively and quantitatively assess relative binding of antibodies from high-titer sera from patients with JP. Our data and a previous report (22) show that the purified LPS fraction contains a serotype-specific antigen and quantitatively binds more antibody than do the other fractions. Significant binding also occurs to the protein fraction, although such binding predominates only in low-titer sera and is cross-reactive among the serotypes (22). We separated LPS into high- and low-molecular-mass material by column chromatography. The high-molecular-mass fraction accounted for most of the antibody binding activity and was highly enriched in sugars. In contrast, the low-molecular-mass material accounted for very little antibody binding activity and consisted of sugars and fatty acids typical of the LPS inner core and lipid A.

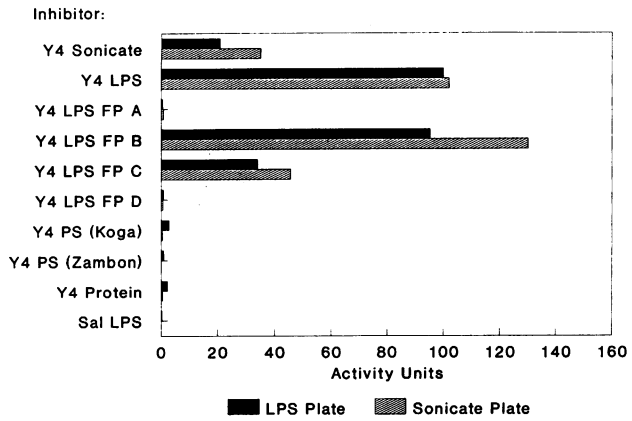

FIG. 9. Inhibition of binding of IgG from a pool of high-titer JP patient sera to antigens of *A. actinomycetemcomitans* measured by ELISA inhibition and reported as units of inhibitor activity calculated as described in Materials and Methods. Plate antigens were purified LPS and the whole-cell sonic extract. Inhibitors were Y4 whole-cell sonic extract, purified Y4 LPS, column fractions A to D (Fig. 4) (FPA to FPD), Y4 polysaccharide (Y4 PS Koga) (1), Y4 polysaccharide (36) (Y4 PS Zambon), Y4 protein, and *Salmonella enteritidis* LPS (Sal LPS) (Sigma; catalog no. L-6011).

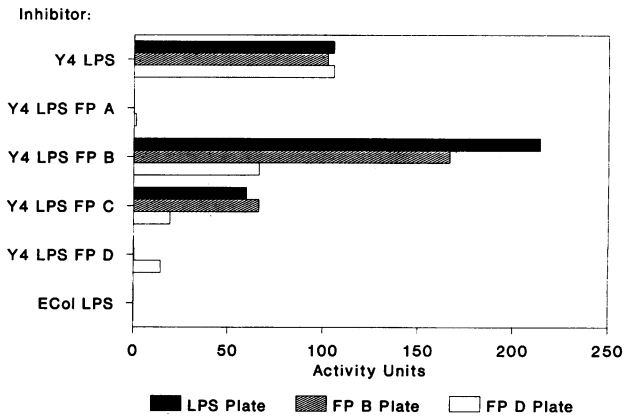

FIG. 10. Inhibition of binding of IgG from an individual high-titer JP patient serum to antigens of *A. actinomycetemcomitans* measured by ELISA inhibition and reported as units of inhibitor activity calculated as described in the Methods section. Plate antigens were purified LPS (30) and column fractions FPB and FPD (Fig. 4). Inhibitors were Y4 LPS, LPS column fractions FPA through FPD (Fig. 4), and LPS from *E. coli* (ECol LPS).

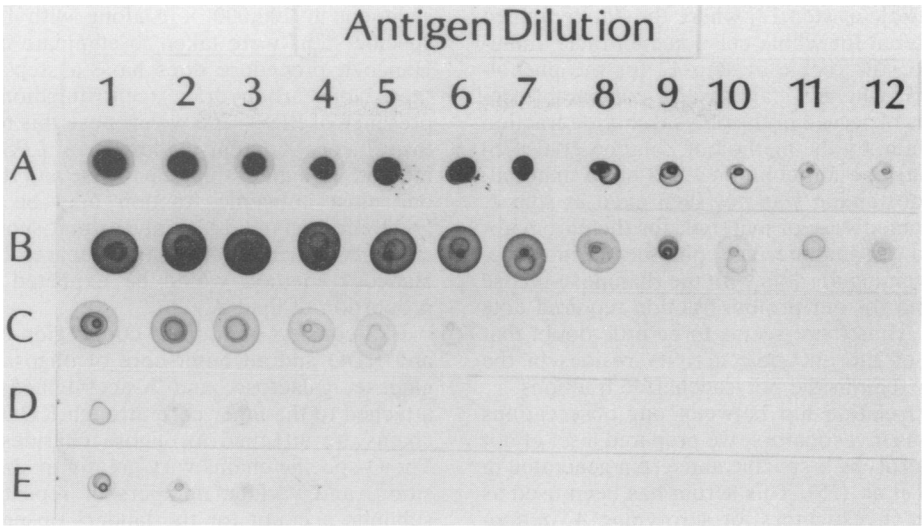

FIG. 11. Dot blot assay prepared with anti-serotype b rabbit serum diluted with 1:500. The beginning antigen concentration was 1 mg/ml; 4-μl samples were applied with a 1:1 dilution at each of 12 steps. All rows contained serotype b-derived material. Rows: A, whole-cell sonic extracts; B, LPS prepared from cells by the phenol-water method of Westphal and Jann (30); C, unpurified polysaccharide prepared by the phenol method from spent culture medium; D, medium-derived polysaccharide prepared by the method of Zambon et al. (36); E, rhamnose-fucose serotype antigen prepared by Amano et al. (1).

These observations are consistent with the conclusion that lipid A-bound polysaccharide, rather than free polysaccharide, is the immunodominant antigen of *A. actinomycetemcomitans* serotype b. Our observations differ significantly from those in previous reports (1, 3, 36, 37).

A serotype-specific antigen of *A. actinomycetemcomitans* was first purified from serotype c by Zambon et al. (36), who used DEAE-Sepharose and gel filtration chromatography of ethanol precipitates from spent culture medium; the antigen was shown by GLC to consist of 87.3% mannose and 16.3% glucose. More recently, serotype-specific antigens were also purified from *A. actinomycetemcomitans* serotypes a and b with the same methods (37). The serotype b-specific antigen was too large to enter 5% SDS-PAGE gels, and it contained 81.7% mannose, 6.6% galactose, and 11.7% glucose. Antigens of a different composition were obtained chromatographically from ethanol precipitates after the mannan-like antigens were removed by immunoabsorption (37). These antigens were greater than 2,000 kDa in size and contained 3.2 mol% rhamnose, 1.0 mol% mannose, 1.5 mol% galactose, and 2.1 mol% glucose, with 9.3% of the dry weight accounted for by amino acids. These antigens, too, were shown to be serotype specific. The polysaccharide fraction we obtained from the spent culture medium with the method of Zambon et al. (36) was similar in that it was too large to enter SDS-PAGE gels and it contained 48% mannose, 3% glucose, and 2.5% galactose (Fig. 1A). Our preparation, however, contained relatively little antigen as assessed by binding of antibody from high-titer sera on dot blots (Fig. 3).

GLC analysis of the whole-cell sonic extract revealed a carbohydrate and fatty acid composition typical of gram-negative bacteria (Fig. 1B), but mannose was not detectable. We extracted polysaccharide directly from the cells and found it to consist of 11% fucose, 9% rhamnose, 5% glucose, and 2.8% galactose; mannose was not detected. Thus the cells did not appear to be the source of the mannose-containing polymers obtained from spent culture medium. We analyzed virgin culture medium, since it contained a yeast extract additive. The dry weight composition of the virgin medium was 15% mannose, 5% glucose, 2.8% galactose, 1% fucose, and 0.3% rhamnose (Fig. 1A). It seems likely that the mannose-containing polymer present in polysaccharide fractions obtained from spent culture medium derives in part or in total from components of the virgin culture medium rather than from the bacterial cells. We did not examine plate-grown cells for slime or capsule production.

Polysaccharide fractions prepared from the spent culture medium by phenol-water extraction before polymyxin B chromatography contained 38% mannose, 25% glucose, lesser amounts of galactose, fucose, and rhamnose, and small amounts of hydroxy fatty acids, indicating LPS contamination (Fig. 1A), and they bound antibody to approximately the same extent as did the material prepared by the method of Zambon et al. (36) (Fig. 3). After polymyxin B affinity chromatography, these markers, including 3-hydroxy 14:0 and 14:0 fatty acids, were not detected; heptulose accounted for only 0.3% of the dry weight, and antibody binding was negative (Fig. 3). Thus antibody binding to crude polysaccharide fractions appears to be accounted for by contaminating LPS.

A serotype-specific antigen not containing mannose has been obtained from *A. actinomycetemcomitans* serotype b by autoclaving and then chromatographing on DEAE-Sephadex A-25 and Sephacryl S-300 (1). It was composed of 43.9% L-rhamnose, 49.1% D-fucose in repeating disaccharide units, 2.7% fatty acid, and less than 1% peptide. This preparation manifested more antibody binding activity in the dot blot assay than did the polysaccharide we prepared by the method of Zambon et al. (36) and slightly less activity than did the crude phenol polysaccharide fraction we obtained directly from the cells (Fig. 3).

A quantitative assessment of the amount of antigen-binding activity per dry weight unit of the various purified fractions was obtained by using dot blots developed with either single or pooled high-titer JP sera (Fig. 3). Immuno-

staining was still visible at step 12, where the dot contained only 1.95 ng of material for whole-cell sonic extract. Immunostaining was also still visible at step 12 for the phenol-water-prepared LPS, whereas staining at a comparable degree of intensity was observed in the polysaccharide fraction prepared from medium by the method of Zambon et al. (36) only to step 5, where the dot contained 250 ng of material. Comparable immunostaining was not seen even at step 1, where the dot contained 4 μg of material, for the chromatographically purified medium-derived polysaccharide fraction. Comparable staining for dots with the rhamnose-fucose Koga antigen (1) and for our protein fraction required dots containing 62.5 ng. Thus there seems to be little doubt that the major portion of the antigenic activity resides in the purified LPS rather than in the polysaccharide fractions.

To establish the relationship between our observations and those of previous investigators, we prepared a set of dot blots by using the serotype b-specific antiserum generated in rabbits by Zambon et al. (35). This serum has been used as a standard by many investigators for serotyping *A. actinomycetemcomitans*. The results of these experiments demonstrated clearly that this antiserum recognizes a component in purified LPS rather than antigens in the polysaccharide fraction. To detect binding to the polysaccharide prepared by the method of Zambon et al. (36), more than 250- to 500-fold more material was required in the dot than was the case for purified LPS.

Our observations also differ from those reported by Califano et al. (3). These investigators studied binding of antibodies specific for *A. actinomycetemcomitans* in high-titer JP patient sera to an antigen obtained from serotype b organisms by ethanol precipitation of a cold phenol-water extract of cells previously treated with lysozyme and SDS. After the removal of RNA and DNA by digestion, the preparation was passed over a proprietary affinity column (Pierce Co., Rockford, Ill.) in an effort to remove contaminating LPS. Califano et al. suggested that the immunodominant polysaccharide thus obtained might be the mannan-containing serotype-specific antigen described by Zambon et al. (36, 37). However, the material migrated on linear gradient SDS-PAGE gels (5 to 20% polyacrylamide) in the range of 42 to 206 kDa, whereas the material prepared by the method of Zambon et al. does not migrate in 5% polyacrylamide gels. The serotype-specific staining observed on Western blots is located in the region occupied in gels by high-molecular-mass LPS. The chromatographic procedure used to remove the LPS may not have been effective, and the serotype-specific staining observed on Western blots could have resulted from antibody binding to contaminating LPS rather than to non-LPS polysaccharide, as suggested by Califano et al. Notably, the antigen preparation was not biochemically characterized, and the extent of LPS contamination was not assessed directly.

It should be noted that the percentage of glucose in different LPS samples isolated from *A. actinomycetemcomitans* varied greatly (18 to 58%) from one serotype to another (data not shown) and was quite variable, depending on whether the LPS was from cells or culture medium (26 to 49% glucose) (Fig. 1). Furthermore, the Y4 LPS prepared by the method of Darveau and Hancock (4) had a much lower glucose percentage than did the phenol-water-extracted (30) LPS (Table 1). Although one explanation for the variation is real differences in the LPS preparations per se, it is also very possible that the glucose content is made artificially high by high-molecular-mass ($>10^6$ Da) carbohydrates such as glycogen or other carbohydrates from the cells that would sediment at 200,000 × *g* along with LPS micelles unless specific steps were taken to eliminate it. The Darveau and Hancock procedure does have a step that would remove these large carbohydrates (centrifugation at 50,000 × *g* in the presence of SDS), and we propose this to be the reason why the glucose content is lower in LPS prepared by that method. In light of this, the fucose and rhamnose contents in our most antigenic fraction may be an underestimated (~50% lower) in GLC analyses that show very high glucose contents. Furthermore, LPS prepared by the Darveau and Hancock method would be expected to retain a higher proportion of lipid A.

LPS consists of an inner core region containing heptulose and KDO and an outer core of oligosaccharides including glucose, galactose, and *N*-acetylglucosamine. Lipid A is attached to the inner core through KDO, and the O-specific chains are attached to oligosaccharides of the outer core. The O-specific chains vary greatly in size and sugar composition, and various numbers of repeating oligosaccharide subunits account for the ladders observed on SDS-PAGE gels (31). We and others have not found significant amounts of KDO in the LPS isolated from *A. actinomycetemcomitans*. KDO is present, however. We have detected trace amounts in some GLC runs, and we concur with Bryn and Jantzen (2), who suggest that the KDO of *A. actinomycetemcomitans* may not be derivatized in the procedure we used.

Our GLC data are consistent with the conclusion that the low-molecular-mass material obtained with column chromatography (containing the lipid A and core oligosaccharides, specifically heptulose) is relatively nonimmunogenic, whereas the high-molecular-mass moiety (consisting of a very small amount of core carbohydrate and lipid A and enriched with fucose and rhamnose) appears to be the immunodominant fraction. Our data are consistent with the view that the antigen described by Califano et al. (3) was, in fact, lipopolysaccharide rather than capsular polysaccharide and that the Koga antigen (1) is a portion of the O-specific carbohydrate derived from LPS, as they suggested.

Two papers that have appeared since our manuscript was submitted bear on our conclusions. Although Wilson and Schifferle (32) used a somewhat different approach, they arrived at the same conclusions as we did, e.g., that the polysaccharide moiety of LPS contains the serotype b antigenic determinants of *A. actinomycetemcomitans*. On the other hand, Ebersole et al. (7) reported that the predominant humoral immune response induced by *A. actinomycetemcomitans* infection in patients with various kinds of periodontitis is IgG1, followed by IgG3 rather than IgG2. Since IgG1 responses are generally induced by protein antigens, and carbohydrates such as saccharide polymers induce a predominantly IgG2 response, Ebersole et al. suggest that the primary serotype-specific antigen is likely to be a protein rather than a carbohydrate. Notably, this evidence is indirect in contrast to the direct evidence provided here and by Wilson and Schifferle (32). Furthermore, in contrast to the results of Ebersole et al. (9), we have observed a predominantly IgG2 response in early-onset periodontitis patients (13a). Thus, unresolved questions remain that can only be answered by additional research.

The evidence we report here and in a previous publication (22) supports the conclusion that the quantitatively dominant antigen of *A. actinomycetemcomitans* serotype b is the serotype-specific antigen located on the surface of the cells. Up to 90% of the antibody binding activity observed in the ELISA to plates coated with whole sonic extracts of sero-

type b cells with high-titer sera dominant for serotype b organisms can be accounted for by binding to LPS (22). The data indicate that antibody binding to some polysaccharide fractions may be a consequence of LPS contamination. Antibody binding to LPS appears to be through the O-antigen carbohydrate chains, in contrast to the core oligosaccharide and lipid A. Clearly binding also occurs to proteins, but this binding is mostly to epitopes shared by the serotypes and is small quantitatively for high-titer sera (22).

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