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Antigens of *Actinobacillus actinomycetemcomitans* Recognized by Patients with Juvenile Periodontitis and Periodontally Normal Subjects

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Most juvenile periodontitis patients respond to infection by *Actinobacillus actinomycetemcomitans* by producing serum antibodies. Specific antigens inducing the humoral immune response have not been identified, nor has the role of the resulting antibodies in disease progression been determined. Adsorbed and unadsorbed sera from juvenile periodontitis patients and normal subjects were analyzed by enzyme-linked immunosorbent assay and Western blots (immunoblots), using digested and undigested bacterial sonicates and French pressure cell fractions to determine the biochemical class, cross-reactivity, and cellular location of the antigens in different *A. actinomycetemcomitans* serotypes. Antigens detected by using high-titer sera included the following: (i) serotype-specific nonprotein material located on the cell surface, (ii) soluble-fraction proteins showing highly variable antibody binding, (iii) cross-reactive proteins, and (iv) a protein present in soluble and cell wall fractions and immunopositive for all sera tested. In addition, one apparently nonprotein component that was enriched in the cell wall fraction was observed. Sera with high immunoglobulin G titers to one, two, three, or none of the three *A. actinomycetemcomitans* serotypes were observed. There was a high degree of variation from one patient to another in the humoral immune response to serotype-specific and cross-reactive antigens. As demonstrated by whole-cell adsorption experiments, the serotype-specific surface antigen accounted for approximately 72 to 90% of the total antibody-binding activity for sera with titers greater than 100-fold above background, while cross-reactive antigen accounted for <28%. Antibody binding the whole-cell sonicate for high-titer sera was inhibited 90% by lipopolysaccharide from the same serotype, strongly suggesting that lipopolysaccharide is the immunodominant antigen class.

The microbial etiology of periodontitis in humans and other animals is now firmly established, and several species and serotypes of putative periodontal pathogens have been identified (13, 23, 24, 28–30, 32, 34, 39, 41–44, 46, 47). *Actinobacillus actinomycetemcomitans*, especially serotype b and to a somewhat lesser extent serotype a, has been closely linked to patients with localized and generalized juvenile periodontitis (JP) (46). Sensitive solid-phase assays such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay have been used to demonstrate elevated levels of serum antibodies reactive with antigens of *A. actinomycetemcomitans* in most although not all JP patients (6–9, 12, 19, 26, 35, 36, 38). The role such antibodies play in the course of these diseases remains unknown, in major part because the antigens that induce the humoral immune response have not been identified and characterized, and the biological effectiveness of the induced antibodies has not been demonstrated.

Most of the serum and gingival fluid antibody studies have used ELISA or radioimmunoassay with intact or disrupted whole cells, rather than purified components, as the solid-phase antigen. Consequently, the data have provided little information about the biochemical identity or relative importance of the dominant antigens. Limited studies have been performed with fractions of *A. actinomycetemcomitans*, including serotype-specific polysaccharide (44, 45), leukotoxic protein (21, 38, 46), envelope proteins (5), and lipopolysaccharide (LPS) (10, 15, 17, 22, 25), but with minor exceptions, the antigenic properties of these fractions have not been compared. Recently, Califano et al. (3) used Western blots (immunoblots) to establish the importance of nonprotein antigens of *A. actinomycetemcomitans* serotype b in patients with high serum antibody titers. However, whether this nonprotein antigen is the mannose-rich serotype-specific polysaccharide purified and described by Zambon et al. (44, 45), the fucose-rhamnose serotype-specific polysaccharide described by Amano et al. (1), or LPS remains an unanswered question.

The long-term goal of our research is to identify, purify, and characterize the major antigen(s) of *A. actinomycetemcomitans* recognized by antibodies in the sera of patients with JP and high-titer normal control subjects. Our approach has been to assess antibody binding to components of whole-cell sonicates and crude cell wall and soluble fractions by using ELISA and Western blots of whole-cell adsorbed and unadsorbed sera to determine the degree of serotype specificity and location of the major antigen(s) and subsequently to focus upon the most highly reactive fractions for further purification and characterization. At present we report results demonstrating that the immunodominant antigen class of *A. actinomycetemcomitans* is serotype-specific nonprotein material on the surface of the cells and that it is present in the purified *A. actinomycetemcomitans* LPS fraction.

MATERIALS AND METHODS

Patients and subjects. Studies were performed with sera from 19 patients diagnosed as having JP by standard criteria

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including radiographic evidence of alveolar bone loss and pocket depths of 5 mm or greater around the first permanent molars or incisors, with onset in the circumpubertal period. Patients varied in age from 10 to 34 years. Samples were also obtained from 19 subjects judged to be periodontally normal by radiographic and clinical examination, ranging in age from 5 to 38 years, one of whom was a sibling of a JP patient. Serum from clotted venous blood or, in a few cases, plasma containing 1.0 IU of heparin per ml was harvested by centrifugation and stored at −80°C until tested.

Bacteria and culture conditions. Cultures of *A. actinomycetemcomitans* obtained from the American Type Culture Collection were ATCC 43717, ATCC 43718, and ATCC 43719, which represent serotypes a, b, and c, respectively. In some experiments, *A. actinomycetemcomitans* strains Aa 75, Y-4, and 67, provided by J. J. Zambon (State University of New York, Buffalo) were used to confirm the results observed with the ATCC strains. Organisms were cultured to the mid-logarithmic stage of growth in enriched Trypticase soy broth (33) in an anaerobic glove box. Cells were harvested by centrifugation at 10,000 × *g* for 10 min, washed twice in phosphate-buffered saline (PBS) and once in distilled water, and then immediately stored at −70°C until used. The protein content of cells and fractions was determined by the method of Lowry et al. (20).

Antigen preparations. Lyophilized bacteria were suspended in 2.0 ml of distilled water at 10 mg/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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), ELISA, or immunoblot assay, as described below.

Cell wall and cytosol fractions were prepared from intact *A. actinomycetemcomitans* cells by the method of Inouye et al. (16). Frozen cells were suspended in 50 mM Tris buffer (pH 7.5) and broken, using a French pressure cell at 15,000 lb/in^2. Unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min. The cell walls were then pelleted from the supernatant at 20,000 × *g* for 20 min, and the supernatant containing the soluble fraction was harvested. The walls were washed three times with Tris buffer and once with PBS, and both fractions were lyophilized. LPS was prepared from both the crude culture supernatant and whole cells by the phenol-water extraction method of Westphal and Jann (40). Composition and purity were assessed as described below.

Gas-liquid chromatography was performed by the method of Bryn and Jantzen (2) to identify and quantitate the major sugar and fatty acid constituents of LPS of all three serotypes. Chromatography was performed with a Hewlett-Packard 5890 series II chromatograph equipped with a flame ionization detector and an HP-1 fused silica capillary column. Briefly, trifluoroacetyl derivatives of samples were prepared following methanolysis. The retention time patterns of chromatographically separated sugar and fatty acid standards were used to identify individual unknown peaks in the samples. The percentage dry weight of each major identified peak was calculated by comparing its integrated area, corrected for its response factor, with that of the known amounts of internal standards (xylose and pentadecanoic acid) which were added to samples prior to derivatization (see Table 5). In addition, total protein and DNA-RNA content were measured as described by Sims et al. (27), and the presence and location of LPS and absence of protein in isolated LPS separated by SDS-PAGE was demonstrated by silver staining (14) and Coomassie blue staining, respectively (data not reported here).

ELISA. Ultrasonically disrupted cells were coated onto the surface of polystyrene microtest plate wells (Flow Laboratories) at 1 μg of protein per ml in 50 mM carbonate buffer (pH 9.6) containing 20 mM $MgCl_2$ for 4 h at room temperature. Coating solutions were washed from the wells, and unbound sites on the plastic surface were blocked with 0.1% Tween 20 in 10 mM TES-buffered saline [0.85% NaCl, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5] (TBS), using a Titertek automatic plate washer. Serum (1:100) was diluted in TBS serially across rows of 12 wells, and the plate was incubated on a rotating platform (140 rpm) for 1 h. The plate was washed 10 times, goat anti-human immunoglobulin G (IgG) · Fab′ alkaline phosphatase conjugate (1:500 dilution in TBS) was added to the wells, and rotation was continued for 1 h. Washing was repeated, followed by rinsing with 50 mM carbonate–20 mM $MgCl_2$ buffer (pH 9.6), and *p*-nitrophenyl phosphate (200 μg/ml) in the same buffer was added to each well. UV A_{405} was then measured for each well after an incubation time of 30 min, using a Titertek Multiskan MC photometer. Dilutions of a standard patient serum pool, prepared by combining equal aliquots of 10 patient serum samples shown to be positive for one or more serotypes by Western blots, were included on each plate to control for interplate assay variation. Antibody titers for individual sera from comparable plates were calculated as the reciprocal of the highest dilution corresponding to an absorbance of 0.150 (roughly 10% of the standard curve maximum absorbance), as determined by linear interpolation between dilution points (4). The mean titer of the three lowest control sera was taken as the titer baseline used to calculate titer ratios. Titer values were log transformed to ensure equality of variance and normality of distribution, and differences between the mean and median titers of the patient and normal control groups were assessed by Student's *t* test and the Mann-Whitney test, respectively.

ELISA inhibition experiments. Plates were coated with ultrasonically disrupted cells and blocked with detergent at room temperature as described above. Inhibitors (LPS, cell sonicate, or ethanol precipitate of culture medium) were then added to coated wells in 50-μl aliquots at concentrations in blocking buffer that would give the indicated final inhibitor concentration (25 μg/ml unless otherwise stated), when combined with an equal volume of diluted serum. The serum was diluted 1:2,000 and added in 50-μl aliquots to wells already containing inhibitor and to control wells without inhibitor. Plates were then incubated for 1 h and further processed to assess specific IgG binding to the plate antigen as described for the standard ELISA above. Percentage inhibition (PI) due to each inhibitor at a given concentration was calculated as follows: PI = [1 − (ELISA absorbance with inhibitor/absorbance without inhibitor)] × 100.

Nitrocellulose immunoblots. Whole cells and cell wall and soluble fractions separated by SDS-PAGE (18) were electrophoretically transferred to sheets of nitrocellulose as described by Towbin et al. (37). 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. Samples (25 μl) were separated in 8% polyacrylamide gels (18). 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 a 7-V/cm electrical field for 1 h. The nitrocellulose sheets were

TABLE 1. Relative IgG binding activity of whole-cell sonicates from different *A. actinomycetemcomitans* serotypes as measured by ELISA

Patient serum	Patient titer[a]			Control serum	Control titer		
	A-75[b]	B-Y4	C-67		A-75	B-Y4	C-67
P0	25.3	51.0	149.2	C0	13.4	5.8	152.6
P1	413.5	856.8	32.9	C1	17.6	3.9	15.0
P2	63.3	12.5	17.4	C2	19.6	1.9	5.5
P3	28.5	119.7	14.9	C3	9.2	1.8	2.8[c]
P4	15.4	1.9	4.9	C4	24.9	12.8	20.8
P5	20.7	11.5	22.4	C5	11.8	4.3	7.7
P6	4.7	15.2	3.0	C6	30.5	5.8	18.5
P7	16.6	13.7	5.8	C7	14.7	7.0	15.5
P8	23.1	14.0	42.5	C8	120.6	26.4	317.6
P9	205.9	16.0	324.8	C9	11.8	3.9	7.7
P10	5.1	15.8	2.9	C10	38.9	25.9	30.5
P11	54.4	91.9	61.7	C11	15.7	9.6	7.8
P12	307.2	218.8	713.7	C12	18.6	6.6	12.2
P13	22.3	332.8	18.0	C13	3.9	1.8	4.0[c]
P14	94.1	492.1	53.2	C14	2.5[c]	1.1[c]	7.7
P15	425.3	1,024.0	104.7	C15	2.4[c]	0.8[c]	1.8[c]
P16	10.2	15.1	18.1	C16	3.7	1.2[c]	14.1
P17	128.0	48.4	30.5	C17	44.8	17.9	34.6
P18	54.5	450.4	50.8	C18	1.7[c]	5.7	9.7
Mean	100.9[d]	200.1[d]	88.0[e]	Mean	21.4	7.6	36.1
Standard	136.2	303.2	169.3	Standard	26.9	7.8	75.8
Median	48.4[d]	30.5[d]	26.5[e]	Median	14.7	5.7	12.2

[a] Calculated as the reciprocal of the serum dilution (10^2) that corresponded to an absorbance of 0.150 (roughly 10% of the maximum absorbance observed).
[b] A, B, and C designate *A. actinomycetemcomitans* sonicate ELISA plate antigens that were made from ATCC 43717, 43718, and 43719, respectively.
[c] Baseline titer values (mean ± standard deviation): 2.20 ± 0.44 for A-75, 1.03 ± 0.21 for B-Y4, and 2.87 ± 1.10 for C-67.
[d] $P < 0.05$ for Mann-Whitney or *t* test (for log-transformed titer values), indicating that the mean or median for patient titer is significantly greater than that for control titer.
[e] The mean or median is not significantly greater than that for controls.

washed 5 or 10 min with five changes of blocking buffer (10 mM TES, 1% gelatin, 0.1% Tween 20, pH 7.5), incubated in human serum diluted 1:500 or 1:1,000 in blocking buffer for 2 h on a rocking platform, washed again, incubated 2 h in goat anti-human IgG(γ) alkaline phosphatase conjugate (Sigma) diluted 1:500, washed again, and incubated in commercially prepared 5-bromo-4-chloro-3-indolyl-phosphate–Nitro Blue Tetrazolium phosphatase substrate solution (Kirkegaard and Perry, Gaithersburg, Md.) until antibody-bearing bands were visible.

Adsorption of sera with whole bacteria. Bacteria from a given serotype (50 μg [wet weight]) were suspended in 1.0 ml of serum that was diluted 1:50 in 10 mM TES at pH 7.5 and incubated at room temperature with constant gentle mixing on a rocker platform for 30 min. Control sera without bacteria were treated in the same way. The sera were centrifuged at 10,000 × *g* for 10 min to remove the bacteria and used without further treatment, as described for individual ELISA or immunoblot experiments.

RESULTS

Endpoint ELISA titer data for patient and control sera using whole-cell sonicates of the three *A. actinomycetemcomitans* serotypes are shown in Table 1. Eleven of nineteen serum samples from patients and 2 of 19 samples from control subjects had high titers (50-fold or more above background) for one or more *A. actinomycetemcomitans* serotypes. Mean and median titer values for patient sera for serotypes a and b (but not c) were significantly higher than for control sera ($P < 0.05$, Student's *t* test or Mann-Whitney test). While some of the patient serum samples manifested no elevated antibody reactivity to any of the three serotypes of *A. actinomycetemcomitans*, others had antibodies reactive with one, two, or three of the serotypes. Six patterns were observed when the patient ELISA endpoint titer data were sorted according to high and low values based on a cutoff of titers greater or less than 50-fold over background (Table 2). The most common pattern was when reactivity with no serotype was high (8 of 19; P2, P4 to P8, P10, and P16 [Table 1]), followed by the case in which reactivity with serotype b alone was high (5 of 19; P3, P11, P13, P14, and P18). High titers were observed as follows: to both a and c in one case (P9), to only a in one case (P17), to only c in one case (P0), to a and b in two cases (P1 and P15), and to all three serotypes in one case (P12). Of 19 serum samples from normal control subjects, 2 were positive. C0 was positive for serotype c, and C8 was positive for serotypes a and c. These analyses were not intended as an epidemiologic survey, but rather to serve as a guide for selection of representative high- and low-titer sera for further study.

Western blots of SDS-PAGE-separated bacteria of all three serotypes run side by side were prepared and incubated in selected sera as a means of assessing the relative contribution of different molecular species to the total antigen content of the cells. The protein components of *A. actinomycetemcomitans* could be separated into a large number of discrete bands (Fig. 1, gel lanes A, C, and B). As expected for strains from the same species, the patterns were very similar, although not identical. The Western blots revealed a diversity of patterns, and those shown in Fig. 1 are representative. All patient and control sera detected one band of approximately 28 kDa present in all three serotypes.

TABLE 2. Distribution of high IgG titers against different *A. actinomycetemcomitans* serotypes as measured by whole-cell sonicate ELISA

Subject	No. of sera with high titers[a] against the indicated sonicate[b] serotype(s):							
	a only	b only	c only	a and b	b and c	c and a	a, b, and c	None
Patients (n = 19)	1	5	1	2	0	1	1	8
Controls (n = 19)	0	0	1	0	0	1	0	17

[a] Endpoint titer values (see Materials and Methods) >50-fold higher than baseline (mean of the three lowest titers of 19 normal sera tested concurrently) (Table 1).

[b] ELISA plate coat antigen.

This binding was not artifactual since it was not observed in specimens incubated with conjugate alone. Patterns for patient sera shown to have a low titer by ELISA, represented here by P4, exhibited faint antibody binding to a large number of protein bands, which could be abrogated by using a more dilute serum concentration. Patterns for all of the high-titer sera were highly positive for antibody binding to high-molecular-mass components (>28 kDa) for one or more serotypes. Sera P3, P13, P14, and P18 were immunodominant for serotype b, P0 was immunodominant for serotype c, P1 was immunodominant for both serotypes a and b, and P12 was immunodominant for a, b, and c. Intense staining in the high-molecular-mass region of the blots correlated with the ELISA data, indicating that the high-molecular-mass material represents the serotype-specific antigen.

The putative serotype-specific material appeared as a smear throughout the high-molecular-mass region of the blots. However, only discrete bands were observed in gels stained with Coomassie blue to show protein (Fig. 1). Except for sera P13 and P14, the relative binding to protein components other than those of the immunodominant serotype(s) as assessed by ELISA was slight. While sample P14 was immunodominant for serotype b, it also detected a low-molecular-mass component in serotypes a and c (subsequently found to be in the region of cell wall component 6) that was not observed in the serotype b lane. In contrast, with sample P13, the same component was detected in serotype b as well as in a and c, and a large number of other high- and low-molecular-mass bands were detected in all three serotypes. A shared component subsequently shown to be soluble-fraction protein 8 was uniquely detected when serum P13 was used. Thus patient sera varied greatly with regard to recognition of both the diffuse putative serotype-specific material and the discrete antigen bands.

Blot patterns for low- and high-titer healthy control subject sera are also shown in Fig. 1. The patterns in lanes labeled C1 are typical of the low-titer samples, with faint staining of multiple protein bands in all three serotypes. Patterns for C0 and C8, sera immunodominant by ELISA for serotype c and for a and c, respectively, manifest intense antibody binding to the serotype-specific antigens. Thus, even with control sera, blot patterns correlated with ELISA data.

To further delineate the nature of the high-molecular-mass serotype-associated material and determine which antigens were located on the cell surface, we prepared Western blots using whole-cell sonicates digested or not digested with proteinase K and incubated them in sera that had been

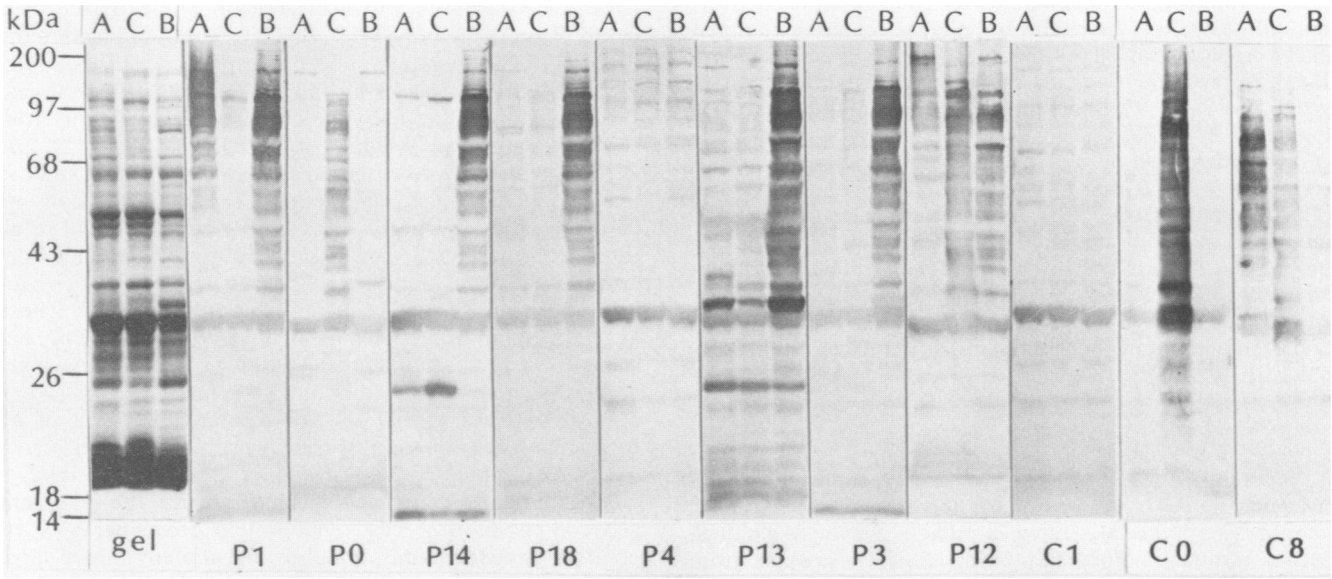

FIG. 1. Blot patterns for serum samples from patients and controls. Triplicate lanes labeled "gel" are SDS-PAGE patterns of the three *A. actinomycetemcomitans* serotypes stained with Coomassie blue to demonstrate protein components. The remaining lane sets are Western blots of the three serotypes developed with sera from eight patients and three control subjects, each sample diluted 1:1,000. Molecular mass markers are displayed to the left of the gel, and the serotype is indicated at the top of each lane. The "gel" patterns were not prepared in the same run as the blots, and migration was not as far. The gel patterns are aligned with the blots based on the intensely staining band at approximately 28 kDa.

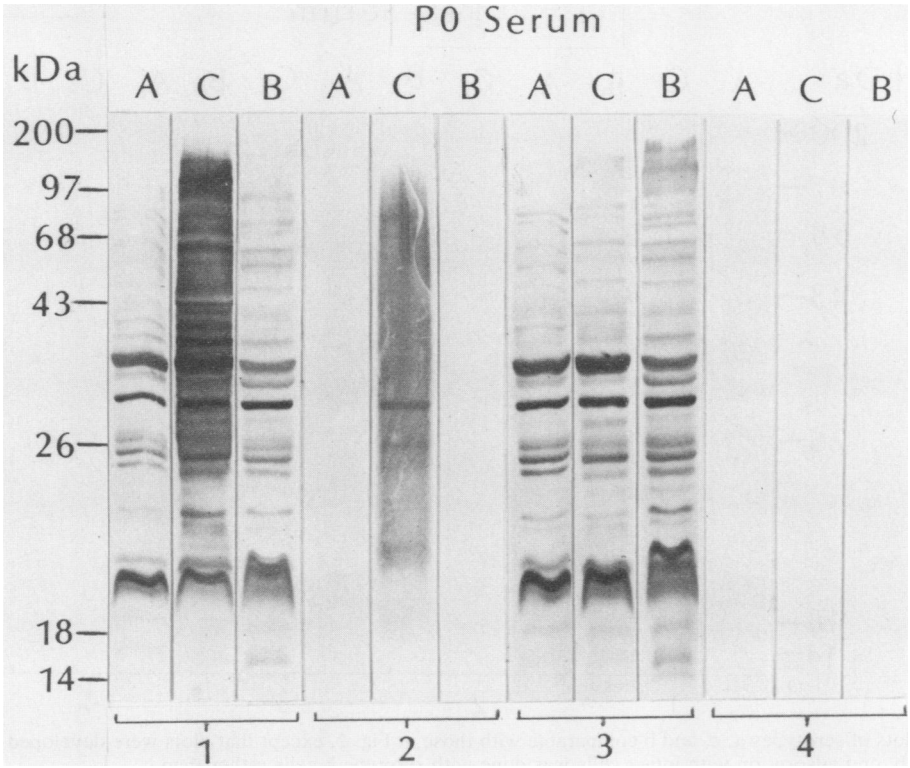

FIG. 2. Western blot of PAGE-separated components of *A. actinomycetemcomitans* serotypes a, c, and b (labeled A, C, and B), either digested with proteinase K for 60 min (panels 2 and 4) or undigested (panels 1 and 3), and stained to show IgG binding to bands after incubation with *A. actinomycetemcomitans* serotype c-positive serum at a 1:500 dilution, either adsorbed with saturating amounts of intact serotype c cells (panels 3 and 4) or unadsorbed (panels 1 and 2). The molecular masses of protein standards are shown at the left (in kilodaltons).

adsorbed or not adsorbed with intact *A. actinomycetemcomitans* cells. To assure the detection of all antigenic components, the SDS-PAGE sample load was increased from 1 to 2 mg/ml, and the blots were incubated with a serum dilution of 1:500, rather than 1:1,000. SDS-polyacrylamide gels stained with Coomassie blue showed no detectable protein in the digested samples (data not shown). As shown in lane set 1 (Fig. 2) containing undigested sonicates of serotypes a, c, and b, and developed using unadsorbed serum immunodominant for serotype c, protein bands are antibody positive for all three serotypes, and the lane containing the serotype c material exhibits an intensely staining smear in the high-molecular-mass portion of the pattern. In contrast, proteinase K-digested material separated and developed in the same way (lane set 2) revealed no antibody binding in the position of protein bands, but persistent staining was observed for the diffuse type-specific material. Lane set 4 containing digested serotype a, c, and b material, respectively, and developed with the same c-adsorbed serum, revealed no staining. In contrast, lane set 3 representing the same serotypes not digested but also developed with c-adsorbed serum revealed staining of proteins for all three serotypes but absence of binding against the serotype-specific material.

The results of an experiment identical to that described above but developed using adsorbed and unadsorbed serum P18 immunodominant for serotype b (Table 1, Fig. 1) are shown in Fig. 3, and they confirm the above observations. Again, the serotype-specific antigen was not removed by proteinase K digestion (Fig. 3, lane sets 1 and 2); antibodies binding to the serotype-specific antigen were removed by whole-cell adsorption (lane sets 3 and 4). In addition, virtually all of the protein components of all three serotypes were detected with adsorbed and unadsorbed sera even though the serum was immunodominant for serotype b only (lane set 3). Comparable experiments using sera immunodominant for serotype a only could not be performed, because we had only one such serum sample in our collection and it had all been used.

To assess the relative importance of soluble and insoluble antigenic components, bacteria were fractionated into cell wall and soluble cytosol fractions and separated in neighboring gel lanes. A typical Coomassie blue-stained gel pattern for the cell wall and soluble fractions is shown in Fig. 4. Distinct single bands or band pairs or clusters were observed in the cell wall fraction. These were assigned positions 1 through 7 on the basis of their distance of migration. Components at positions 5 and 6 were quantitatively dominant as estimated by staining intensity. Protein patterns for cell walls from the other two serotypes were very similar but not identical to the pattern observed for serotype b (data not shown). Twelve distinct bands or band groups were identified in patterns of the soluble fractions (Fig. 4), and these were also assigned positions on the basis of distance of migration.

Gels containing serotype b cell wall and soluble fractions in adjacent lanes were blotted and incubated in some of the same patient and control sera used for the experiments shown in Fig. 1. Notably, the smear material observed in blots of whole sonicates (Fig. 1) was absent from both the cell wall and soluble-fraction patterns, suggesting that much

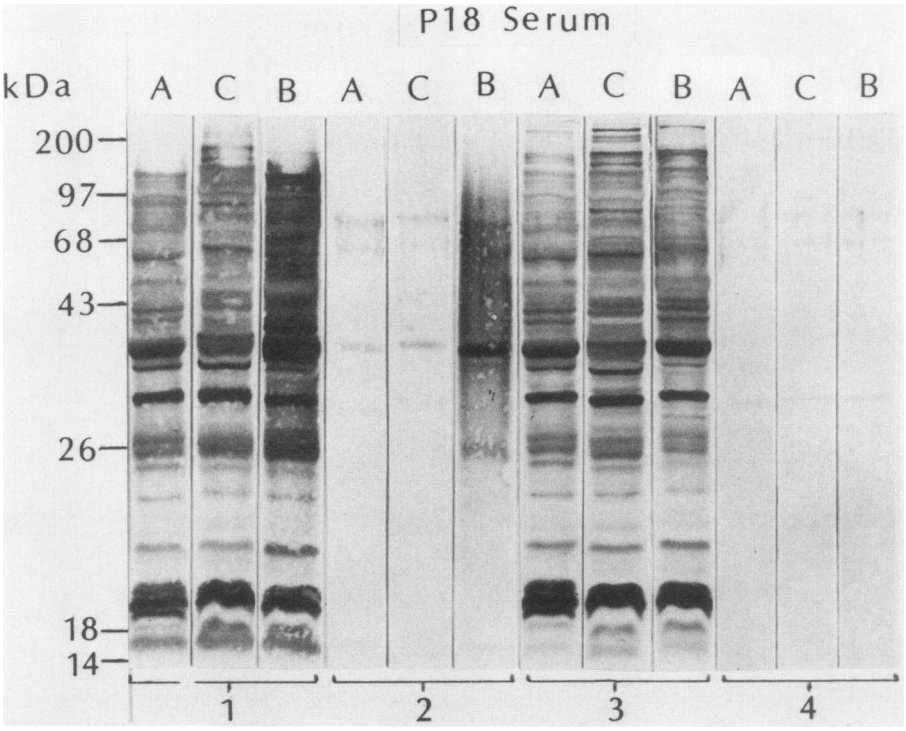

FIG. 3. Western blots of serotypes a, c, and b comparable with those in Fig. 2, except that blots were developed in serum (1:500) with a high titer to serotype b, and adsorption with intact cells was done with serotype b cells rather than c.

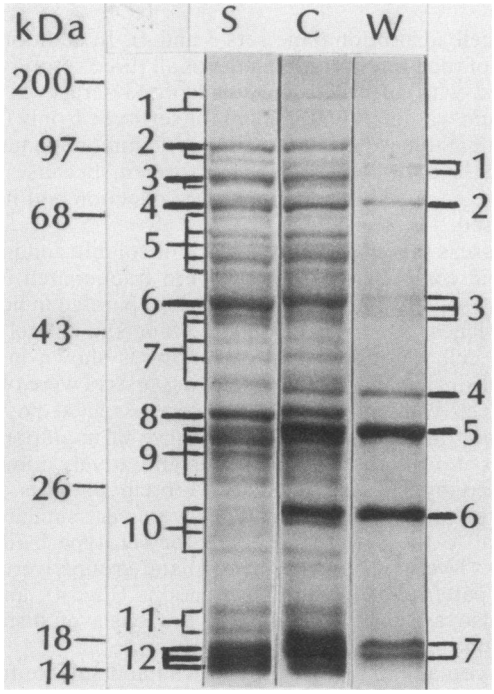

FIG. 4. SDS-PAGE patterns of whole-cell sonicate (lane C), cell walls (lane W), and soluble cytosol fraction (lane S) for serotype b cells. Visible protein components have been numbered on the basis of their distance of migration. Molecular mass standards are provided to the left of the gel (in kilodaltons).

of the type-specific smear material was eliminated during the cell wall washing step.

In lanes containing cell wall material, all five patient serum samples immunodominant for serotype b (P1, P3, P13, P14, and P18) detected five IgG-binding components (corresponding to Coomassie blue-detected protein bands 1 to 5, respectively) and one component migrating between proteins 2 and 3 that did not correspond to any protein band (Fig. 5, arrow). In some runs, faint binding to wall component 7 could be detected. Serum P0, immunodominant for serotype c, detected only a component in the position of cell wall protein 5; the same was true for serum P4, which exhibited no serodominance, and for the C1 control serum. None of the serum samples detected cell wall protein 6, one of the two quantitatively dominant proteins in the cell wall fraction (Fig. 4).

For lanes containing the soluble fraction, the IgG binding patterns were much more complex and variable than those for cell wall (Fig. 5). With some notable exceptions, the IgG-binding bands in blots developed with sera immunodominant for serotype b corresponded well with the protein bands demonstrated by Coomassie blue staining in Fig. 4. IgG binding to the soluble fraction using sera C1 and P0, neither of which was immunodominant for serotype b, was slight. As observed using whole-cell sonicates (Fig. 1), sample P4 which showed a very low titer by ELISA exhibited faint staining to multiple components in the soluble fraction. The most intense IgG binding observed was to a component in the position of soluble-fraction protein 8 using serum P13 (Fig. 1 and 5). This component, which was not recognized by any other patient or control serum sample, was also detected in the cell wall fraction, but the staining intensity was much lower.

Whole-cell adsorption experiments were also done to determine the proportions of serotype-specific antibodies

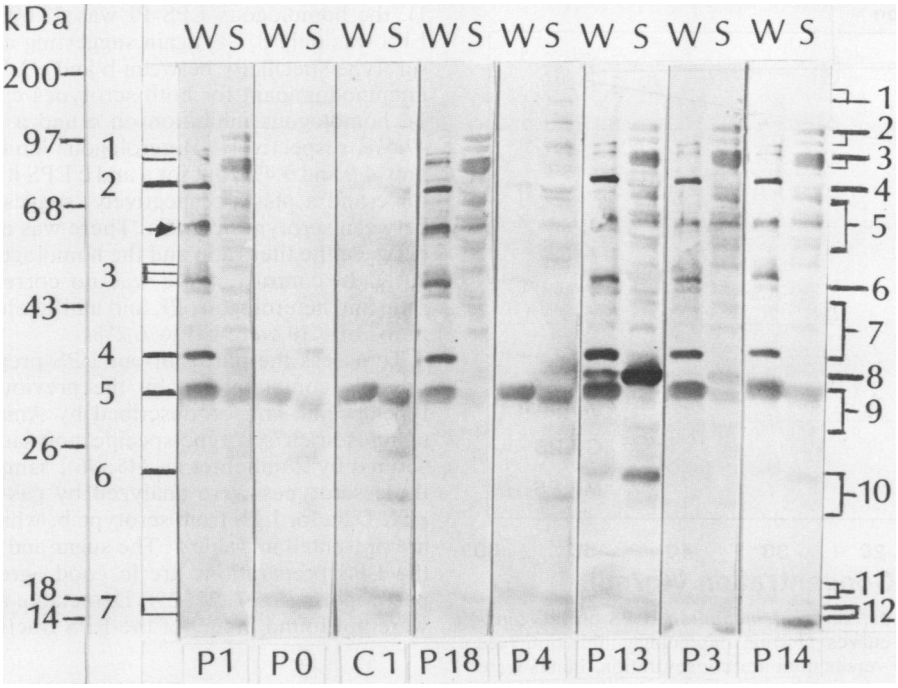

FIG. 5. Western blots of cell wall (W) and soluble (S) fractions from *A. actinomycetemcomitans* serotype b developed with patient and control sera (diluted 1:1,000) as indicated. Protein component designations noted on Fig. 4 for cell wall components are shown at the left of the gel, and designations for the soluble fraction are shown to the right of the gel. Serum P12 was not included in this experiment. The arrow indicates an immunopositive band in lane W for P1 not seen in gels stained for protein.

and cross-reactive antibodies in five high-titer serum samples, using ELISA plates coated with whole-cell sonicates (Table 3). Samples of each serum were adsorbed or not adsorbed with either serotype a, b, or c cells. The relative degree of removal of IgG against each serotype was then determined by comparing the A_{405} in ELISA in wells containing adsorbed samples (diluted 1:800) with that in unadsorbed controls. The resulting data (expressed as PI) are ranked in Table 3 on the basis of decreasing titer-to-control baseline ratio. Results of adsorbing each sample with heterologous cells (those from a serotype other than that of the plate sonicate) were compared with those for cells that were homologous relative to the plate antigen. There was a significant negative correlation between baseline titer ratios and heterologous PI values ($P < 0.01$), but there was no correlation between titer and homologous inhibition levels. For high-titer sera with titer baseline ratios of 53 to 611, only one PI value was above the range of 1.2 to 28%. In contrast, most of the heterologous PI values for low-titer sera (with ratios less than 24) were above 80%. Furthermore, for all low-titer sera tested, both homologous and heterologous PI values were high and approximately the same for a given plate antigen, regardless of the serotype used for adsorption, indicating that the activity was mainly against cross-reactive cell components. In contrast, most of the antibody in high-titer sera reflect a high degree of serotype specificity, as indicated by the difference between homologous and heterologous inhibition levels.

Because LPS comigrated in Western blots with the serotype-specific material demonstrated here and in previous work (27) and because serotype-specific high titers in the ELISA correlated well with the type-specific antibody binding activity in Western blots, additional ELISA inhibition

TABLE 3. Relationship between magnitude of IgG titer against *A. actinomycetemcomitans* sonicate antigen and degree of inhibition by adsorption with whole cells from homologous and heterologous serotypes

Serum	Plate antigen	Baseline titer ratio[a]	PI[b]			
			Homologous	Heterologous		
				a	b	c
P1	b	832	90.3	11.1		14.7
P18	b	437	84.1	8.3		1.9
P1	a	187	79.1		10.7	14.2
P9	c	113	90.2	28.1	1.2	
P9	a	94	90.5		6.3	43.4
C0	c	53	72.2	1.8	8.2	
P0	c	52	55.5	15.9	7.2	
P0	b	50	72.4	18.2		35.0
P18	a	24	72.4		70.9	68.6
P18	c	18	86.1	84.2	80.9	
P1	c	12	92.4	80.3	79.0	
P9	b	16	90.0	90.0		89.7
P0	a	11	60.0		67.9	58.6
C0	a	6	86.0		85.1	83.3
C0	b	6	85.5	87.9		90.4
Pearson's correlation			0.26[c]	−0.50[c]	−0.76[c]	−0.71[d]

[a] Baseline titer ratio = titer to indicated serotype/mean of three lowest titers for the same serotype for normal control sera. All titers used to calculate the ratios are shown in Table 1.

[b] PI = 100 × [1 − (ELISA A_{405} for serum diluted 1:800 preadsorbed with intact cells from the indicated serotype/absorbance for the same serum without adsorption)].

[c] $P > 0.05$, correlation not significant.

[d] $P < 0.01$, significant negative correlation.

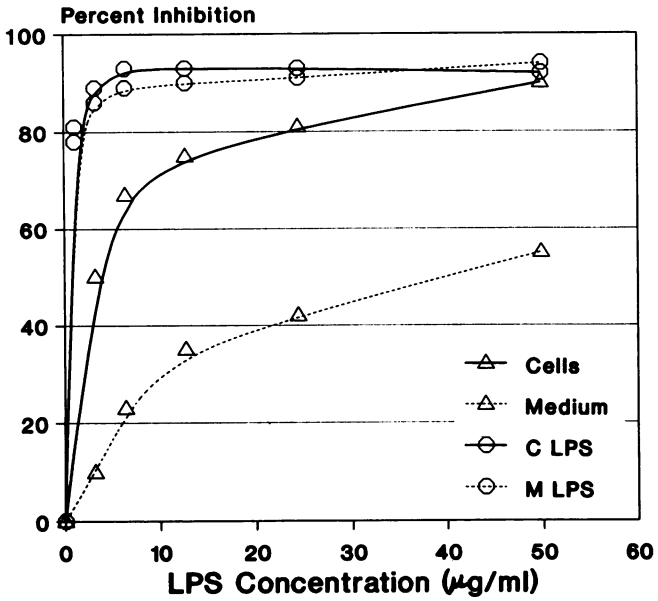

FIG. 6. Effect of *A. actinomycetemcomitans* LPS on IgG binding. ELISA inhibition curves plotting percentage inhibition (see Materials and Methods) versus inhibitor concentration. Plates were coated with serotype b whole-cell sonicate and developed using a serum immunodominant for serotype b with and without inhibitors at various concentrations. Inhibitors used were ethanol precipitate of the culture medium (Medium), whole-cell sonicate (Cells), and LPS isolated from the culture supernatant (M LPS) or from the cells (C LPS). Endpoint titers were calculated at an absorbance cutoff of 0.350 optical density units and were used to find the PI due to each inhibitor at each concentration as follows: PI = 100 × [1 − (titer with inhibitor/titer without inhibitor)].

experiments were done to determine what proportion of the antibody binding activity could be accounted for by LPS. Wells were coated with sonicate of serotype b and developed with a serum with a high titer versus b in the presence or absence of potential inhibitors of binding. As shown in Fig. 6 in which PI is plotted versus inhibitor concentration, antibody binding was inhibited in a dose-dependent manner up to 50% by adding an ethanol precipitate of the culture supernatant and up to 90% by 50 μg of whole-cell serotype b sonicate per ml. In contrast, LPS isolated either from *A. actinomycetemcomitans* culture medium or from the cells by the phenol-water method of Westphal and Jann (40) inhibited antibody binding greater than 90% at approximately at 4 μg/ml.

To confirm the above observation and determine whether the inhibitory activity of LPS was serotype specific, additional inhibition experiments were performed using LPS isolated from all three serotypes and representative sera with high and low titers against sonicate antigens. The results are shown in Table 4. For three sera with a very high titer against b sonicate and a Western blot pattern dominant for b (P14, P18, and P13 [Fig. 1]) homologous LPS strongly inhibited antibody binding against b sonicate, giving PI values of 89.4, 90.6, and 84.4%, respectively. In contrast, inhibition due to heterologous LPS at the same concentration was much lower: 3.1 to 9.2% for a LPS, and 7.1 to 21.9% for c LPS, clearly indicating that LPS was the major sonicate component recognized and that the epitopes recognized by these sera were highly serotype specific. Similarly for P0, a serum with a c-dominant blot and titer pattern (Fig. 1, Table 1), the homologous LPS PI was 57.6%, while the PI for b LPS was only 8.7%, again suggesting a very high degree of serotype specificity between b and c LPS. Using serum C8, immunodominant for both serotypes c and a (Fig. 1, Table 1), homologous inhibition on c and a plates was 57.0 and 47.5%, respectively. Heterologous inhibition by b LPS was only 4.9 and 9.4%, but for a and c LPS it was 35.2 and 38.4%, for c and a plates respectively, suggesting cross-reactivity between serotypes c and a. There was a positive correlation between the titer ratio and the homologous PI ($r = 0.90$, $P < 0.01$). In contrast, there was no correlation between titer ratio and heterologous PI, and all PI values corresponding to ratios of <10 were below 6.2%.

To assess the purity of our LPS preparations and detect possible contamination by the previously described serotype-specific antigen described by Amano et al. (1) or the mannose-rich serotype-specific polysaccharide antigen described by Zambon et al. (45, 46), samples of LPS from all three serotypes were analyzed by gas-liquid chromatography. Data for LPS from serotype b, which is representative, are presented in Table 5. The sugar and fatty acid profiles of the LPS preparations are in good agreement with several past reports (2, 17, 22, 25). Detectable amounts of mannose were not found in any of the LPS fractions.

DISCUSSION

Numerous reports have demonstrated that sera of many patients with JP contain high antibody titers against antigens of *A. actinomycetemcomitans* (7–9, 11, 19, 21, 26, 30, 31, 35, 46). However, which *A. actinomycetemcomitans* antigens are immunodominant has not been determined. Previous studies have focused upon specific cell surface components without taking other antigenic components or total antigenic reactivity into account (3, 8a, 44, 45). We assessed total anti-*A. actinomycetemcomitans* antibody in high-titer patient and control sera against unfractionated *A. actinomycetemcomitans* cells using ELISA and against separated *A. actinomycetemcomitans* antigens using whole-cell Western blots to determine the contribution of each component to the total. Whole sonicates were separated into soluble cytosol and cell wall fractions to better resolve protein components and determine the solubility of the serotype-specific nonprotein antigens. We assessed antigenic cross-reactivity among serotypes by cross-adsorbing sera with whole cells and testing them with both blots and ELISA, we assessed the role of nonprotein antigens in general by using blots of proteinase K-digested cells, and we assessed the role of LPS in particular by using ELISA inhibition experiments.

The following four types of antigen were identified by Western blots: (i) serotype-specific nonprotein material that was located on the cell surface, migrated over a large area of the gels, and was immunodominant in both high-titer patient and high-titer control subject sera (Table 3; Fig. 1 to 3); (ii) two soluble-fraction proteins, one at position 8 and another in cluster 10 (Fig. 1, P13), that showed a high degree of variability in antibody binding, regardless of antibody titer; and (iii) cross-reactive proteins, the antibodies for which were not removed by whole-cell adsorption and for which antibody titers were low. These were observed as faint bands in lanes of blots in Fig. 1 other than those binding serotype-specific antibodies and as darker bands in blots in lane sets 1 and 3 of Fig. 2, and lane sets 1 and 3 of Fig. 3 where the assay sensitivity was higher; and (iv) a band of approximately 28 kDa observed in both the soluble and cell wall fractions in the position of cell wall protein 5 and soluble

TABLE 4. Relationship between magnitude of IgG titer against *A. actinomycetemcomitans* sonicate antigen and degree of inhibition by LPS isolated from homologous versus heterologous serotypes

Serum	Sonicate antigen on plate	Dark blot lane[a]	Baseline titer ratio[b]	LPS PI[c]			
				Homologous	Heterologous		
					a	b	c
P14	B	+	478	89.4	8.4		21.9
P18	B	+	437	90.6	9.2		19.8
P13	B	+	323	84.4	3.1		7.1
C8	C	+	111	57.0	35.2	4.9	
C8	A	+	54	47.5		9.4	38.4
P0	C	+	52	57.6	19.6	8.7	
P0	B	−	50	28.5	11.1		10.6
P14	A	−	42	24.2		8.4	9.5
C8	B	−	26	19.8	9.2		8.5
P18	A	−	24	21.6		12.9	16.5
P14	C	−	19	14.5	5.3	8.5	
P18	C	−	18	8.7	14.8	7.5	
P0	A	−	11	12.7		3.3	7.5
P13	A	−	10	16.8		3.5	10.4
C1	A	−	8	2.4		6.2	4.1
P4	A	−	7	3.0		5.4	3.7
C1	B	−	6	7.6	1.4		6.1
P13	C	−	5	6.8	6.0	2.8	
C1	C	−	5	4.5	2.5	4.9	
P4	C	−	2	4.5	4.8	4.9	
P4	B	−	2	7.4	4.1		4.2
Pearson's correlation coefficient				0.90[d]	0.003[e]	0.25[e]	0.36[e]

[a] See Fig. 1.
[b] Ratio = titer to indicated serotype/mean of three lowest titers for same serotype for normal control sera.
[c] PI = 100 × [1 − (ELISA absorbance with LPS/absorbance without LPS)].
[d] $P < 0.01$, significant positive correlation with titer ratio.
[e] $P > 0.05$, not significant.

cluster 9 (Fig. 5) that was immunopositive for all patient and control sera tested. In addition, we observed a single immunopositive band in the cell wall fraction (arrow, Fig. 5, P1) that did not stain with Coomassie blue (Fig. 4). Although the antigens of category 1, which clearly correlated with high ELISA titers, appear to be immunodominant as defined by having induced a high titer in patient or control sera, antigens in the other categories may be of equal or greater biological importance.

The most prominent feature of the Western blots shown in Fig. 1 was the high degree of smeared staining of many high-molecular-mass components observed for the serotypes that were immunodominant by ELISA (Table 1), with only slight reactivity detected for blots of low-titer serotypes. This observation was unexpected, since the three serotypes have almost identical protein profiles, as assessed by SDS-PAGE patterns stained for proteins in which the bands were discrete and without evidence of the diffusely migrating antigen seen in blots (Fig. 1). We suspected that the smear material was nonprotein serotype-specific antigen comigrating with and coating the protein bands and thereby either blocking antibody binding to them, in the case of sera with no serotype-specific activity, or causing them to be falsely positive, in the case of sera with high titers of type-specific antibodies.

By increasing the serum concentration and the sample load in the SDS-PAGE Western blot assay, staining of protein bands that were only faint under less sensitive conditions (Fig. 1) became more intense (Fig. 2 and 3). This observation is consistent with the idea that some protein bands represent background antigens recognized only by antibodies of low titer, i.e., those only detected at high serum concentrations. Most of the proteinase-sensitive components held in common by the serotypes bound antibody, regardless of the serodominance of the serum used, espe-

TABLE 5. Major sugar and fatty acid constituents of lipopolysaccharides isolated from *A. actinomycetemcomitans* Y4 culture medium or cells

Constituent	% Composition[a] (dry weight) of LPS derived from:	
	Medium	Cells
Rhamnose	3.74	3.47
Fucose	5.13	5.45
Galactose	3.16	2.76
Mannose	ND[b]	ND
Glucose	29.16	26.38
Glucosamine[c]	3.56	3.01
Heptulose[d]	5.15	4.35
KDO[e]	ND	ND
C14:0[f]	3.23	2.80
3-OH-C14:0[g]	8.96	7.82

[a] Determined by gas-liquid chromatography and calculated as described in Materials and Methods.
[b] ND, Not detected.
[c] Glucosamine values may be overestimated as a result of comigration of a fatty acid peak.
[d] Total LD-glyceromannoheptose and DD-glyceromannoheptose.
[e] KDO, 2-Keto-3-deoxyoctanoate.
[f] Myristic acid.
[g] 3-Hydroxymyristic acid.

cially after removal of the serotype-specific antibody by whole-cell adsorption, indicating that these were cross-reactive proteins. This binding was not artifactual, since incubation with alkaline phosphatase-conjugated anti-IgG alone did not label the proteins (data not shown). The fact that the type-specific smear persisted at a higher serum dilution in blots is consistent with the ELISA data (Table 1), showing that type-specific activity was detected at extremely high serum dilutions.

The whole-cell adsorption experiments shown in Fig. 2 and 3 demonstrated clearly that the serotype-specific material seen in blots was located on the cell surface. The type-specific material appeared to be carbohydrate since it was not destroyed by proteinase K digestion, and earlier work showed it to be sensitive to oxidation by periodic acid (27). Furthermore, the observation made in a previous study (27) that purified LPS stained for antibody binding as a diffuse smear in Western blots in the same location as the type-specific antigen observed here (Fig. 2 and 3) and the fact that LPS inhibited the type-specific antibody binding in ELISA up to 90% (Fig. 6, Table 4), strongly suggests that the immunodominant antigen is LPS.

We assumed that the high-molecular-mass type-specific antigen was a part of the cell walls and would be enriched in the cell wall fractions. Surprisingly, Western blots of French pressure cell soluble cytosol and cell wall fractions revealed this material was missing from both fractions. Loss of the material probably resulted from the multiple washing steps needed to remove soluble components from the cell walls. Because the PAGE patterns were spread over two lanes rather than one, improved resolution of discrete antigen bands was achieved. A careful comparison of the blots developed with serotype b-dominant sera in Fig. 5 with the Coomassie blue-stained protein components of serotype b in Fig. 4 reveals that virtually all of the components larger than cell wall protein 5 are antibody positive. Cell wall proteins 1 to 5 were distinctly antibody positive, and 7 was faintly so, while wall protein 6, one of the two quantitatively major components as assessed by protein staining, was antibody negative in all cases and may represent a sequestered or nonimmunogenic protein. In contrast, the cell wall antigen migrating between protein 2 and cluster 3 had a high level of antibody binding but did not stain for protein. It may be a quantitatively very minor but highly immunogenic protein or may not be a protein.

The responses observed in the Western blots to antigens other than the serotype-specific antigen(s) varied greatly from one patient serum to another and further documented the individual nature of the pattern of response (Fig. 1). Of the eight sera studied by Western blots, soluble fraction protein 8 was detected in only one (P13), and the component in the position of cell wall protein 6 was detected in only two (P13 and P14). These two components were not recognized by P1 or P12, which recognize the serotype-specific antigens of two and three serotypes, respectively. P13 also recognized a number of low-molecular-mass components not only for serotype b but also for a and c (Fig. 1). P14, serodominant for b, recognized the component in the position of cell wall protein 6 for serotypes a and c but not for b, while P13, also serodominant for b, recognized this component for b as well as for a and c (Fig. 1).

Another prominent feature of the Western blots in Fig. 1 and the ELISA data in Table 1 was the presence of high antibody titers to more than one serotype in some samples. This observation contrasts with a previous report (44), but is in agreement with the report of Gmur et al. (11).

Assessment of serospecificity and cross-reactivity of *A. actinomycetemcomitans* sonicate antigens from different serotypes by ELISA using plates coated with whole-cell sonicates and high-titer sera adsorbed with intact cells from different serotypes (Table 3) clearly shows that high, immunodominant IgG titers against *A. actinomycetemcomitans* are due mainly to serotype-specific antigens in both patient and control sera.

The negative correlation between titer baseline ratios and PI values for sera adsorbed with heterologous serotype cells is statistically significant, whereas no such correlation was observed when the adsorption was performed with homologous cells (Table 3). When the data were separated into high- and low-titer groups with the cutoff set at titers more than 50-fold above baseline, the mean PI for heterologous adsorption was only 12.4 (± 11.4) for high-titer sera but 73.1 (± 20.5) for low-titer sera. In contrast, adsorption by cells that were homologous relative to the plate antigen resulted in PI values of 80.3 (± 12.9) for high- and 80.6 (± 11.1) for low-titer sera. These data support the idea that the antibody reactivity of low-titer sera in ELISA consists mostly of cross-reactive antibodies, whereas the high-titer sera contain mostly serotype-specific antibodies with only a small proportion that cross-react. Our choice of a cutoff of 50-fold above background was somewhat arbitrary. The data suggest the existence of an intermediate zone. For example, serum P0 with a titer ratio of 36 was inhibited by 35% by adsorption with serotype c cells when tested on b plates. Likewise, sample P9 with a titer ratio of 94 was inhibited 43.4% by adsorption with serotype c cells and tested on a plates.

The observations that whole-cell adsorption removes the type-specific smear material detected visually by Western blots (Fig. 2 and 3) and that the presence of the type-specific smear correlated strongly with high-titer values (Table 1) suggests that the smear material is in fact the immunodominant antigen. The ELISA inhibition experiments shown in Fig. 6 and Table 4 provide evidence that the immunodominant antigen resides in the isolated LPS fraction. When the data in Table 4 were assessed in terms of titer ratios of >50, as was done in the whole-cell adsorption experiments (Table 3), mean PI by homologous LPS was 71.1% (± 19.1%), but for heterologous LPS only 15.5% (± 11.7%). For samples with titer ratios of <50 and not manifesting serodominance (Fig. 1) for the plate sonicate antigen, mean PI values were only 12.20% (± 8.3%) for homologous LPS and 7.8% (± 4.1%) for heterologous LPS. These observations are consistent with the fact that the type-specific material migrates in SDS-PAGE over the same area as isolated LPS (Fig. 2 and 3) and with Western blots demonstrating that the isolated LPS binds antibody from high-titer sera over the same migration range (see lanes F, f, G, g in Fig. 4 of reference 27). The material is proteinase resistant (Fig. 2 and 3), and we have shown previously that it is sensitive to periodate oxidation (27), suggesting that the immunodominant epitopes are carbohydrate. Nevertheless, binding to the LPS could be accounted for by contamination by the serotype-specific mannose-rich polymer described by Zambon et al. (45, 46) or the type-specific fucose-rhamnose polymer characterized by Amano et al. (1). Such contamination seems unlikely. The Westphal and Jann (40) purification procedure is effective in separating LPS from soluble polysaccharides. The sugar and fatty acid composition of our LPS as assessed by gas-liquid chromatography is typical of purified *A. actinomycetemcomitans* LPS (Table 5) and comparable with published data (2, 17, 22, 25). The mannose-rich polymer was not present

since, in agreement with previous reports (2, 17, 22, 25), we did not find detectable amounts of mannose by gas-liquid chromatography. We cannot at this time rule out contamination by the fucose-rhamnose polymer described by Amano et al. (1), since these sugars are constituents of the LPS preparation.

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