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Immunodominant Antigen of *Actinobacillus actinomycetemcomitans* Y4 in High-Responder Patients

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This study was undertaken to look for characteristics of the immunodominant antigen(s) of *Actinobacillus actinomycetemcomitans* Y4 that might help explain the high antibody titers in periodontitis patients. Radioimmunoassays (RIA) were performed on sera from 481 patients; sera from the 32 patients with the highest anti-Y4 titers (above 128,000 RIA U/ml) were further analyzed. Y4 antigen was boiled for 45 min or treated with papain, and antibody responses were analyzed by RIA and Western blotting (immunoblotting). In addition, carbohydrate was purified from Y4 and examined by Western blotting. The results indicated that the immunodominant antigen of Y4 in high responders was stable after papain treatment or boiling for 45 min. Papain or boiling eliminated protein bands but a large diffuse band persisted on Western blots. With increasing dilutions of sera, bands on Western blots corresponding to protein antigens disappeared, while the large diffuse band resembling that of carbohydrate persisted. Partially purified Y4 carbohydrate contained the large diffuse band. Double-immunodiffusion analysis indicated that rabbit serotype b-specific antiserum and patient sera recognized the same antigen. When the carbohydrate extract was passed over a lipid A-binding column to remove lipopolysaccharide, the smear corresponding to the immunodominant antigen was still present on Western blots. The immunodominant antigen of Y4 in high-responder individuals appears to be a carbohydrate and is possibly the capsular polysaccharide.

Juvenile periodontitis is characterized by rapid destruction of the periodontal tissues. Disease often commences around puberty and results in severe loss of tooth support before age 30. *Actinobacillus actinomycetemcomitans*, a gram-negative organism frequently isolated from lesions of juvenile periodontitis (1, 12, 17, 28), is thought to be a major etiologic agent of this form of periodontal disease (21). *A. actinomycetemcomitans* Y4 has been shown to elicit remarkably high serum antibody titers in juvenile periodontitis patients (4, 14, 16, 18, 22, 24). In contrast, other periodontitis-associated organisms, such as *Fusobacterium nucleatum*, elicit comparatively low titers (22, 23), even though they represent a larger proportion of the flora for most patients (17). This suggests that antigens of Y4 have unusual characteristics that make them potent immunogens. We therefore sought to determine the nature of the immunodominant antigens of Y4 in this study. Radioimmunoassay (RIA) and a modification of the Western blot (immunoblot) technique were used to identify and characterize the immunodominant antigen of Y4. Our data indicate that the immunodominant antigen of Y4 is the serotype b-specific antigen and that it is papain and heat resistant. This stability of the antigen and its appearance on Western blots before and after partial purification are consistent with the characteristics of a carbohydrate.

MATERIALS AND METHODS

Human subjects. Subjects included 481 clinically characterized patients (227 blacks, 249 whites, 3 Asians, and 2 Indians) at the Medical College of Virginia/Virginia Commonwealth University Clinical Research Center for Periodontal Diseases, Richmond. These patients were characterized as follows: (i) juvenile periodontitis (n = 76)—subjects of 30 years or less with a localized pattern of severe periodontal destruction limited to first-molar or incisor teeth and up to two additional teeth; (ii) severe periodontitis (n = 72)—subjects of 35 years or less with a generalized pattern of severe destruction, with attachment loss of at least 5 mm on eight or more teeth, at least three of which were not first molars or incisors; (iii) adult periodontitis (n = 103)—subjects over 35 years of age with chronic periodontitis generalized to all four quadrants; and (iv) nonperiodontitis (n = 230)—subjects of any age with no evidence of attachment loss, except recession on the buccal surface of anterior teeth at no more than one site and pockets no greater than 3 mm.

Bacteria and antigen preparation. *A. actinomycetemcomitans* Y4 and *Bacteroides gingivalis* D43B-4 were grown in mass culture by W. E. C. Moore and L. V. H. Moore of Virginia Polytechnic Institute and State University. The bacterial strains were centrifuged, frozen, shipped to the Medical College of Virginia/Virginia Commonwealth University, sonicated, and used to coat polystyrene RIA strips or run on Western blots. Protein concentrations in the sonicates were determined by the method of Lowry et al. (15). Tetanus toxoid (TT), used as a control, was the generous gift of Lederle Laboratories.

RIA. Details of the solid-phase RIA technique used for detection and quantitation of specific antibody have been described elsewhere (23). Briefly, 200 μl of sonicated bacteria or TT (25 to 100 μg/ml in carbonate buffer, pH 9.6) was placed in each well of plastic assay strips (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.). The strips were then incubated at 4°C for 16 h and washed in tap water 20 times. Sera were serially diluted (in the range of 1:50 to 1:102,400) in diluent (equal volumes of distilled water containing 10% nonfat dry milk and phosphate-buffered saline [PBS], pH 7.2). Each dilution of serum was added to two antigen-coated wells and two uncoated wells. The final quantity of serum in the well varied from 4 μl (1/250 ml) in the first well to 0.002 μl (1/512,000 ml) in the final well. The

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uncoated wells served as a background control. After the incubation and washing procedures described above, 200 μl of iodinated (specific activity, 25 μCi/μg) goat anti-human immunoglobulin G (Fc fragment specific) in diluent was added per well. After incubation and washing of wells, radioactivity was measured to within an accuracy of 10% (1282 Compugamma Counter; LKB Instruments, Inc., Rockville, Md.). The difference in counts per minute (delta counts per minute) between coated and uncoated wells was the basic datum unit. A plot of delta counts per minute versus serum dilution was prepared. A cutoff was selected (generally 3,000 to 10,000 delta cpm) that intersected the curve at the end of the linear portion of the curve or point of inflection. This was designated the endpoint. The titer of the serum antibody was the inverse of the dilution at the endpoint.

Western blot analysis. Antigen preparations were diluted to the appropriate concentration for the particular antigen (*A. actinomycetemcomitans*, 5 μg/50 μl; *B. gingivalis*, 20 μg/50 μl; TT, 20 μg/50 μl) in sample buffer (0.0626 M Tris, 10% glycerol, 2.3% sodium dodecyl sulfate [SDS] [pH 6.8]) and were boiled for 90 s. The running buffer was 0.025 M Tris containing 0.192 M glycine and 0.1% SDS. Linear gradient (5 to 20%) polyacrylamide gels were run at 30 mA per gel until the smallest prestained molecular-weight marker (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.) reached the bottom of the gel (after approximately 5 h). An SE600 vertical gel apparatus (Bio-Rad Laboratories, Richmond, Calif.) and a Bio-Rad power supply model 500/200 were used. Following electrophoresis, the antigens were blotted to nitrocellulose in a Bio-Rad transblot cell for 5 h at 200 mA. The blots were incubated in equal volumes of distilled water containing 10% nonfat dry milk and PBS (pH 7.2) for 1 h to block nonspecific binding (10). The blots were then cut into individual lanes to allow treatment of replicate lanes with different dilutions of serum (diluted in equal volumes of distilled water containing 10% nonfat dry milk and PBS, pH 7.2). Each lane was incubated in 4 ml of diluent containing 0.5 ml of serum at the lowest dilution and 0.01 μl (1/1,024,000 ml) of serum at the highest dilution. The separate nitrocellulose lanes were then incubated with patient serum for 16 h, followed by four 10-min washes with TTBS (Tween 20 plus Tris-buffered saline). The 16-h incubation was important to improve sensitivity. The lanes were then exposed to a 1/1,000 dilution of alkaline phosphatase-labeled goat anti-human immunoglobulin G (H & L; KPI Laboratories, Inc., Gaithersburg, Md.) for 4 h. This was followed by four more washes with TTBS. The blots were developed with the BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate–Nitro Blue Tetrazolium) phosphatase substrate system. Some of the SDS-polyacrylamide gels were also stained with Coomassie brilliant blue (Bio-Rad, Rockville Center, N.Y.) or silver (silver stain kit; Bio-Rad).

Papain digestion. Proteolytic digestion was accomplished by adding 200 μl of papain (Sigma Chemical Co., St. Louis, Mo.) solution per well (0.1 mg/ml in 0.1 M phosphate, 0.001 M Cys, 0.002 M ETDA buffer [pH 6.2]) to antigen-coated plates and incubating them for 4 h at 37°C (25). Both papain and pepsin were used in preliminary studies, and both produced the same results. Papain was selected because it has a less selective cleavage site, resulting in increased degradation of each protein molecule. The antigen was digested after coating of microdilution plates to prevent competition for binding sites on the plates by papain. The plates were then washed 20 times in tap water. For Western blots, papain solution was prepared as described above with 7.5 mg of papain per ml, and the appropriate antigen was added to a concentration of 1 mg/ml. This mixture was then incubated for 4 h at 37°C. Following the incubation, the mixture was concentrated to 0.5 ml with a Centriprep concentrator (Amicon Corp., Scientific Systems Div., Danvers, Mass.) and then diluted to the same concentration used for undigested antigen.

Heat treatment. Antigen preparations (*A. actinomycetemcomitans*, *B. gingivalis*, or TT) were placed in a boiling water bath for 45 min. Preliminary experiments had indicated that boiling for 45 min was necessary to virtually eliminate reactivity with TT and *B. gingivalis* controls.

Carbohydrate purification. Carbohydrate was partially purified from *A. actinomycetemcomitans* Y4 by extraction with phenol and water, followed by precipitation of the carbohydrate from the aqueous phase with cold ethanol (8). One milliliter of Y4 whole cells (75.5 mg/ml) was pelleted in an Eppendorf Microfuge. The pellet was then resolubilized in 23 ml of 0.05 M Tris—0.001 M EDTA—50 μg of lysozyme per ml (pH 7.5) and incubated for 15 min at 37°C. SDS was then added to a concentration of 1.5% to gently lyse the cells. Phenol (46 ml) was added, and the mixture was shaken for 15 min. The mixture was then centrifuged at 5,900 × *g* for 10 min at 4°C. The aqueous layer was removed, and 2 volumes of cold 95% ethanol were slowly added. The mixture was then centrifuged at 5,900 × *g* for 10 min at 4°C. The precipitate was dissolved in 23 ml of 20 mM phosphate buffer, RNase (40 μg/ml) and DNase (40 μg/ml) were added, and the mixture was incubated for 1 h at 37°C. The extracted carbohydrate was concentrated to 0.5 ml with a Centriprep concentrator (Amicon) and then diluted in sample buffer one-sixth of the original volume. In some experiments, low-molecular-weight material was removed by concentrating the extracted carbohydrate with a Centriprep concentrator having a 30-kilodalton (kDa) cutoff. This was accomplished with three cycles of concentration and resuspension in 10 ml of deionized water. In some experiments, lipopolysaccharide (LPS) was removed from the carbohydrate extract with a lipid A-binding affinity column (Detoxi-Gel; Pierce Chemical Co., Inc., Rockford, Ill.). The column was equilibrated with 3 bed volumes of PBS (pH 7.5). Carbohydrate extract (20 μl) was then added to the column which represented microgram amounts of LPS. According to the manufacturer, each column has a binding capacity of 2 mg of LPS. Six bed volumes of PBS were then added. Six bed volumes of effluent were collected and concentrated with a Centriprep concentrator.

LPS purification. *A. actinomycetemcomitans* LPS (a kind gift of Frank Nichols, University of Connecticut School of Dental Medicine) was purified by the method of Westphal and Jann (26). Analysis of the phenol-water LPS extract on Coomassie-stained SDS-polyacrylamide gels revealed several protein bands. This Y4 LPS preparation was therefore further treated with papain as described above to digest contaminating protein.

Double immunodiffusion. Double-immunodiffusion precipitin reactions were performed in agar gels (Ouchterlony technique) by using the slide micromethod (18). The well cutters were selected to produce wells of 10-μl capacity, one in the center and six located circumferentially. To the center well 40 μl of *A. actinomycetemcomitans* Y4 carbohydrate extract was added in 10-μl increments. The peripheral wells received 5 μl of a 1/2 dilution of human serum or rabbit serotype-specific antiserum. After the precipitin pattern developed, the gels were washed to remove excess protein and

dried and stained at room temperature in a 0.5% solution of Coomassie blue R-250 in 10% acetic acid–25% isopropanol–water. Excess stain was removed by washing the slides in a 10% acetic acid solution. Rabbit serotype-specific antisera were the kind gift of Joseph Zambon.

Immunoelectrophoresis. Immunoelectrophoresis was conducted in a 1.0% agarose gel in 0.02 M Tris barbiturate (pH 8.6) by using a Pharmacia Flat Bed Apparatus with cooling plate (Pharmacia, Inc., Piscataway, N.J.). Y4 sonicate (10 μl of sonicate containing 43 μg of protein per ml) or Y4 carbohydrate extract (10 μl) was added to the wells and electrophoresed for 60 min at 10 V/cm while being cooled to 10 to 14°C. The troughs were then filled with 100 μl of a 1/8 dilution of human serum and incubated at room temperature for 18 h. The gel was then pressed, washed in PBS (pH 7.4) for 18 h followed by a 60-min wash in deionized water, dried, and stained with Coomassie blue.

RESULTS

To identify subjects with high anti-*A. actinomycetemcomitans* Y4 antibody titers, sera from 481 patients were assayed. Sera from 32 subjects with the highest anti-Y4 antibody titers (i.e., above 128,000 RIA U/ml) were selected for further analysis. These samples represented less than 10% of the total, with 26 being from blacks, 4 from whites, and 2 from Asians. All but 2 of these subjects had early-onset forms of periodontitis: 17 had juvenile periodontitis, 13 had severe periodontitis, 1 had adult periodontitis, and 1 had nonperiodontitis.

To determine the nature of the immunodominant antigens of Y4, antigen preparations were first subjected to heat treatment or papain digestion. Antibody titers to these altered antigens were then determined by RIA. Representative plots of delta cpm versus reciprocal dilutions for Y4 as well as for TT and *B. gingivalis*, which served as controls, are shown in Fig. 1. For TT (Fig. 1a), the titer for untreated antigen was 16,000 RIA U/ml. Boiling the antigen decreased the titer to 2,000 RIA U/ml, and papain digestion reduced the titer to 750 RIA U/ml. Similarly, for *B. gingivalis* (Fig. 1b), a titer of 16,000 RIA U/ml for untreated antigen was decreased to 4,000 RIA U/ml by papain and to less than 500 RIA U/ml by boiling. In contrast, a titer of 256,000 RIA U/ml for *A. actinomycetemcomitans* (Fig. 1c) was unaffected by papain and only modestly decreased by boiling for 45 min.

The mean antibody titers for all 32 serum samples are summarized in Fig. 2. These results indicate that the antibody titer for TT (this control was used for 4 of the 32 subjects) was virtually eliminated both by papain treatment and by boiling. In contrast, antibody titers against Y4 were unaffected by papain digestion and only modestly decreased by boiling.

Western blots were prepared to more directly examine the antigens responsible for the antibody responses. As a control for the effectiveness of enzyme digestion, *B. gingivalis* antigen was examined by Western blot analysis with and without papain treatment. At low dilution of patient serum there were over 30 discrete bands for *B. gingivalis* (Fig. 3, lane Bg). With papain digestion, the protein bands were eliminated, leaving the characteristic "stepladder" effect commonly associated with LPS (Fig. 3, lane Bg pap). When the *A. actinomycetemcomitans* Y4 antigen preparation was analyzed by using 0.5 ml of patient serum in the incubation mixture (Fig. 3, lane Aa), about 50 discrete bands were present. In addition to these bands, there was a diffuse band

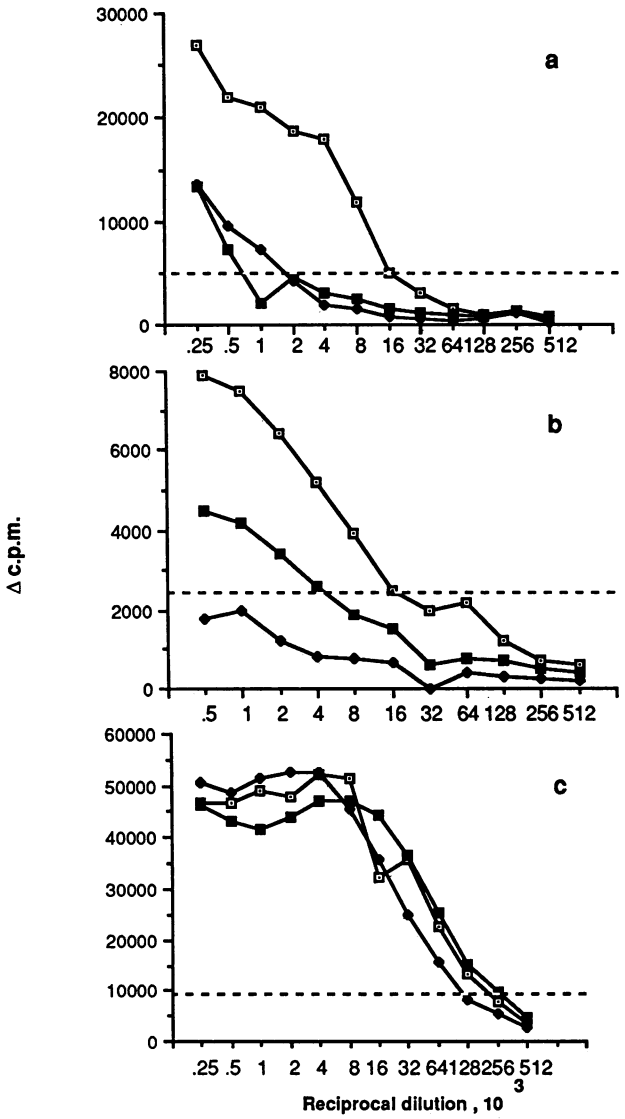

FIG. 1. Typical RIA curves showing the effect of boiling (◆) and papain digestion (■) of antigen on antibody titers compared with untreated antigen (⊡). Panels a, b, and c represent the antigens TT, *B. gingivalis*, and *A. actinomycetemcomitans* Y4, respectively. The dotted line locates the cutoff for each curve. The cutoff marks the point in the linear portion of each curve slightly before the inflection point in the lower portion of the curve. The antibody titer is defined as the inverse of the serum dilution at the endpoint.

or "smear" in the upper portion of the lane. Although the locations of the protein bands were highly reproducible from gel to gel, this diffuse smear was not. Generally the smear occupied the upper half of the gel beginning at the origin, but sometimes it did not begin until the level of the 200-kDa marker. If the Y4 antigen was boiled for 45 min before Western blot analysis, then many of the discrete protein bands disappeared but the smear persisted (Fig. 3, lane Aa boil). With papain pretreatment of antigen, all but two low-molecular-weight bands disappeared, leaving only the smear in the upper portion of the lane (Fig. 3, lane Aa pap). The presence of the smear on Western blots before or after boiling and papain digestion suggests that the antigen persisted after treatment rather than becoming aggregated or unveiled.

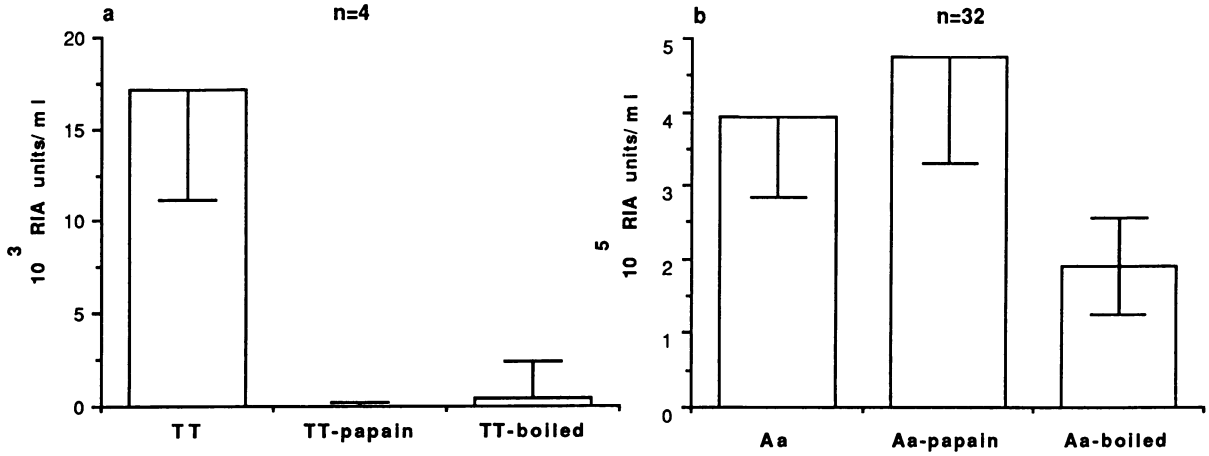

FIG. 2. Compilation of RIA data for all patients having a high antibody titer to *A. actinomycetemcomitans* Y4, showing the effect of boiling and papain digestion on antibody titers. TT, Tetanus toxoid; Aa, *A. actinomycetemcomitans*. The error bars indicate the standard errors of the means.

To determine which bands represent the immunodominant antigen for Y4, Western blots were run, and replicate lanes were stained with progressively less serum (from 1/250 to 1/1,024,000 ml) (Fig. 4). With increasing dilution, all the protein bands were eliminated, leaving the smear in the upper portion of the lane when only 1/256,000 ml of serum was used. In the original Western blot, a faint smear was visible even at 1/1,024,000 ml. To determine the immunodominant antigens for the group of high-responder patients, 1/100,000 ml of serum from 31 subjects was incubated with a single lane. The results of this assay are summarized in Table 1. Note that sera from all 31 of these high-responding patients reacted with the smear in the upper portion of the lane. Other bands are present for many of the patients at 1/100,000 ml of serum; however, the discrete bands with the highest prevalence (100, 98, and 85 kDa) were found in only 34% of the subjects. For every patient (five subjects) whose serum was serially diluted beyond 1/100,000 ml, only the smear was detected.

The properties of the "smear" antigen are consistent with those of a carbohydrate. To test this more directly, *A. actinomycetemcomitans* Y4 sonicate was subjected to a carbohydrate purification procedure involving extraction

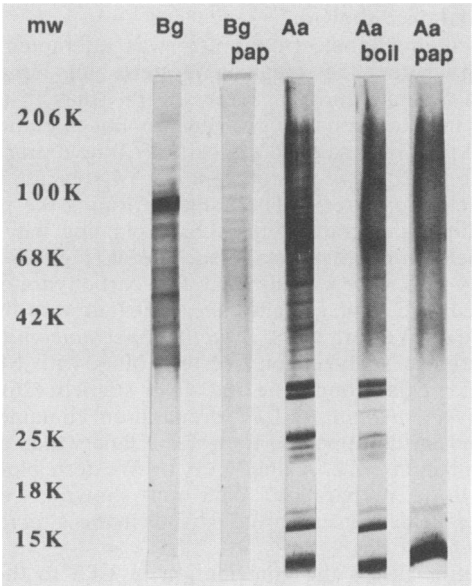

FIG. 3. Western blot with a low dilution (0.5 ml) of patient serum, showing the effects of boiling and papain (pap) digestion on *A. actinomycetemcomitans* Y4 (Aa). The blot for *B. gingivalis* (Bg) was run as a control for papain digestion.

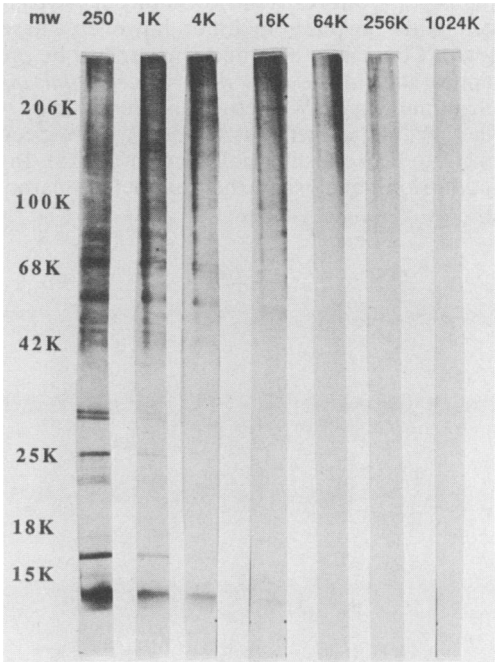

FIG. 4. Typical Western blot of *A. actinomycetemcomitans* Y4, illustrating the immunodominant antigens. Replicate lanes of Y4 were run on polyacrylamide gel and transferred to nitrocellulose. Decreasing amounts of patient serum (1/250 to 1/1,024,000 ml) were used in the incubation mixture for each lane to allow visualization of the immunodominant antigen with subsequent staining. Note that the discrete bands disappeared with decreasing amounts of serum but that the smear was still visible in the upper portion of the Western blot for a serum dilution of 1/256,000 ml. In the original Western blot, the smear was also visible at 1/1,024,000. mw, Molecular weight.

TABLE 1. Prevalence of individual bands on Western blots for *A. actinomycetemcomitans* Y4

Band[a] (kDa)	No. of individuals responding[b] (%)
Smear	31 (100)
100	10 (34)
98	10 (34)
85	10 (34)
99	9 (28)
80	9 (28)
68	8 (25)
75	7 (21)
87	6 (18)
205	5 (15)
180	3 (9)
160	3 (9)
60	3 (9)
58	2 (6)
18	2 (6)
15	1 (3)
25	1 (3)
210	1 (3)
90	1 (3)

[a] Bands appearing on Western blots when stained with 1/100,000 ml of serum in 4 ml of diluent.

[b] The serum of 1 subject of the 32 was unavailable for Western blotting.

with phenol-water followed by precipitation with cold ethanol. This preparation was run on a Western blot and stained with 0.5 ml of patient serum. The appearance was much like that observed with papain digestion of Y4 (Fig. 5). Interestingly, the two papain-resistant low-molecular-weight bands copurified with the carbohydrate; however, these bands stained with Coomassie blue and appeared to be protein.

The appearance of the *A. actinomycetemcomitans* immunodominant antigen on Western blots prompted us to determine whether this antigen was the serotype b-specific antigen thought to be a capsular polysaccharide (28). In double-immunodiffusion analysis, the extracted carbohydrate

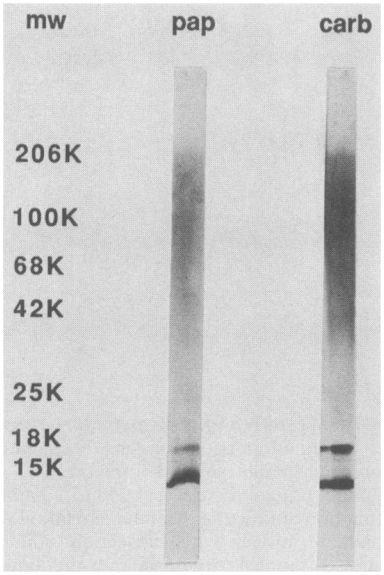

FIG. 5. Comparison of papain (pap)-digested *A. actinomycetemcomitans* Y4 antigen with Y4 carbohydrate (carb) on Western blots. Patient sera were used at a low dilution (1/2, or 0.5 ml) to reveal any antigens in the purified material. mw, Molecular weight.

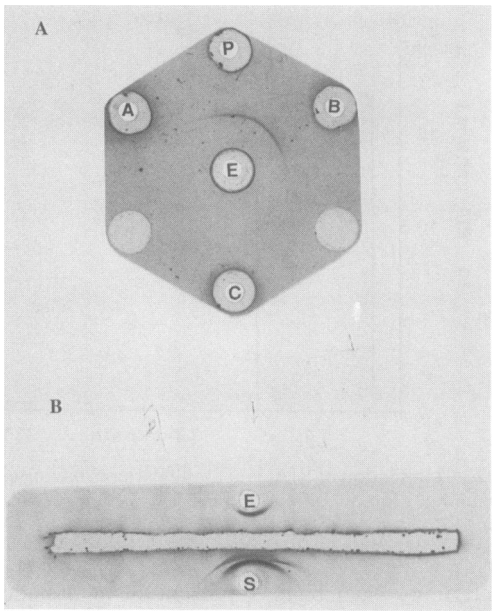

FIG. 6. (A) Double-immunodiffusion analysis (Ouchterlony technique) showing that rabbit serotype b-specific antiserum and patient serum recognize the same antigen. Peripheral wells P, B, A, and C contained patient serum (P) and *A. actinomycetemcomitans* serotypes B-, A-, and C-specific antisera, respectively (5 μl each of a 1/2 dilution). The center well (E) contained Y4 carbohydrate extract (40 μl). (B) Immunoelectrophoresis of the immunodominant antigen at pH 8.6. Well E contained 10 μl of Y4 carbohydrate extract, and well S contained 10 μl of Y4 sonicate. Two precipitin bands were noted when using the whole sonicate and a single band was noted with the carbohydrate extract.

preparation showed a line of identity with patient serum and rabbit serotype b-specific antiserum (Fig. 6A). No reaction was seen with serotype a- or c-specific antisera. In addition, Western blots with the rabbit serotype b-specific antiserum revealed a smear typical of that seen when using human serum (data not shown).

Crossed immunoelectrophoresis was attempted to help establish whether the smear on Western blots actually represented a single antigen. After several trials, it appeared that the immunodominant antigen was not migrating at the required pH. We analyzed the carbohydrate extract as well as whole *A. actinomycetemcomitans* Y4 sonicate by using immunoelectrophoresis. The antigen forming the precipitin bands did not migrate at pH 8.6, explaining why crossed immunoelectrophoresis was unsuccessful (Fig. 6B).

It was considered likely that the carbohydrate extract contained LPS which might have contributed to the smear observed on Western blots. *A. actinomycetemcomitans* LPS was therefore analyzed on Western blots with SDS-polyacrylamide gels (Coomassie and silver stained) (26). The *A. actinomycetemcomitans* LPS preparation contained a diffuse band in the upper portion of the lane which appeared very similar in silver-stained gels or Western blots to the carbohydrate preparation (data not shown). Coomassie staining revealed several protein bands in the *A. actinomycetemcomitans* LPS.

To better assess the contribution of LPS to the diffuse band in Western blots, the carbohydrate was further enriched. Two phenol-water extractions and cold ethanol precipitations were carried out on the Y4 sonicate. Further purification was accomplished by using a concentrator with

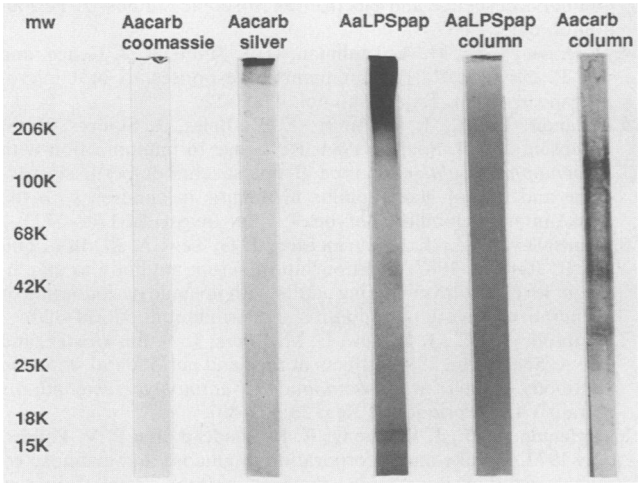

FIG. 7. Persistence of the immunodominant antigen in the carbohydrate preparation after removing protein and LPS. The carbohydrate preparation was enriched by two cycles of phenol-water extraction followed by precipitation with cold ethanol. Low-molecular-weight materials were removed by using a concentrator with a 30-kDa filter. LPS was used as a control to establish that the lipid A-binding column would remove *A. actinomycetemcomitans* LPS. SDS-polyacrylamide gels were stained with Coomassie blue or silver or analyzed on Western blots. Lanes: Aacarb coomassie, SDS-polyacrylamide gel of Y4 carbohydrate extract stained with Coomassie blue; Aacarb silver, SDS-polyacrylamide gel of Y4 carbohydrate extract stained with silver; AaLPSpap, SDS-polyacrylamide gel of Y4 LPS that was digested with papain stained with silver; AaLPSpap column, SDS-polyacrylamide gel of Y4 LPS that had been digested with papain and passed over an LPS-binding affinity column stained with silver; Aacarb column, Western blot (with a 1/50 dilution of patient serum) of Y4 carbohydrate extract that had been passed over an LPS-binding affinity column. Although not apparent in the photograph, a single discrete band was detectable in the original Western blot at 15 kDa. It appears that enough protein persisted, after all the purification steps, to be detected by the very sensitive Western blot. mw, Molecular weight.

a 30-kDa exclusion to remove low-molecular-weight material. Finally, this preparation was passed over a lipid A-binding column to remove LPS. Results of the analysis of this preparation on SDS-polyacrylamide gels are presented in Fig. 7 (lanes Aacarb coomassie and Aacarb silver). This demonstrated the extracted-antigen preparation before passage over the column. Note that the low-molecular-weight protein bands seen in Fig. 5 are not evident in the Coomassie-stained gel. Discrete bands are also absent from the silver-stained gel; however, the smear or diffuse band is present in the upper portion of the lane (Fig. 7). The smear appears dark only at the top of the gel in this photograph, but the typical smear pattern was apparent to the middle portion of the gel in the original. The carbohydrate extract was then passed over a lipid A-binding affinity column to remove LPS and analyzed on SDS-polyacrylamide gels. *A. actinomycetemcomitans* LPS which was papain treated was used as a control. Silver staining of the *A. actinomycetemcomitans* LPS SDS gels before and after passage over the column indicated that the column bound the LPS effectively (Fig. 7, lane AaLPSpap and AaLPSpap column). However, Western blotting of the carbohydrate extract passed over the affinity column still revealed a smear in the upper portion of the lane, suggesting that this antigen was not LPS (Fig. 7, Aacarb column).

DISCUSSION

Analyses of the antibody reactive with *A. actinomycetemcomitans* sonicate by RIA and Western blot analysis strongly suggest that the immunodominant antigen for Y4 is a carbohydrate. By immunodominant antigen, we mean the antigen that is responsible for the observed antibody titer in the high-responder subjects. When sera are used at high dilution, only high-titer antibody is measurable. The antigen detectable at the highest serum dilution on the Western blot corresponds to the antigen responsible for the endpoint or antibody titer in the RIA. This antigen was heat stable and resistant to proteolytic digestion, purified with carbohydrate, and resembled carbohydrate on Western blots. This antigen preparation contains the serotype b-specific antigen, as demonstrated in double-immunodiffusion analysis. It has been suggested that the type-specific antigen of Y4 represents capsular carbohydrate, as it is a surface antigen readily detectable on whole cells by immunofluorescence studies (28). A lipid A-binding affinity column which bound *A. actinomycetemcomitans* Y4 LPS failed to retain the smear antigen found on Western blots of Y4 carbohydrate extract, further supporting the idea that the immunodominant antigen may be capsular polysaccharide.

Carbohydrate, which is known to be associated with the cell surface of *A. actinomycetemcomitans* and may represent capsule, could be a virulence factor for Y4 as it is for several other organisms. Purified surface carbohydrate from *A. actinomycetemcomitans* serotype c is the serotype-specific antigen and has been identified as mannan (28). Capsular polysaccharide is known to inhibit phagocytosis of many organisms (e.g., *Streptococcus pneumoniae*). In part, this appears to be the result of inhibition of the alternative complement pathway and decreased opsonization (9, 11). Mannans isolated from *Saccharomyces cerevisiae* have been shown to inhibit the respiratory burst and release of myeloperoxidase in phagocytes (27).

Pneumococcal polysaccharide has been shown to persist for long periods in mice; such polysaccharides have a half-life of up to 50 days (20). If the *A. actinomycetemcomitans* Y4 carbohydrate is as resistant to degradation in vivo as it is in vitro, it could also persist for long periods within the periodontal tissues. Bacteria (13, 19) and antigens of Y4 (2, 3) are observable within the periodontal tissues of juvenile periodontitis subjects. If the carbohydrate of Y4 persists in the tissues, then it might stimulate repeated cycles of antibody synthesis. Thus, long-term persistence might explain the high titer of antibody reactive with this antigen in periodontitis patients.

If the immunodominant antigen is in fact a carbohydrate, the very issue of its potential immunogenicity is quite interesting. A T-cell-dependent (immunoglobulin G) response is not typically seen with a carbohydrate antigen. The T-cell help could be obtained through presentation of the carbohydrate in the context of Ia. This has yet to be demonstrated for a carbohydrate antigen. Alternatively, one of the two proteins that copurify with the carbohydrate may be attached to the carbohydrate, allowing Ia binding. Another possibility would be the availability of T-cell help through polyclonal T-cell activation in the periodontal tissues or lymph nodes. Future studies are planned to examine the behavior of the Y4 carbohydrate antigen in different antigen presentation systems to determine the mechanism of production of the immunoglobulin G response.

High antibody titers to Y4 have been associated with localization and decreased severity of disease in juvenile

periodontitis (6, 18). Future studies will examine the relation of antibody response to the immunodominant antigen as a function of disease severity and extent to determine whether antibody directed against this antigen is protective. Levels of antibody directed against the immunodominant antigen may also correlate with disease severity. If this is the case, then measuring antibody for the most-relevant antigen may decrease the false-positive results due to cross-reactive antigens.

Our previous data indicated an effect of race on antibody response to *A. actinomycetemcomitans* Y4 (7). These data suggested that more black individuals have elevated levels of antibody to Y4 than do white individuals, and while most localized juvenile periodontitis patients are black, the general pattern is more evenly split between the black and white populations (7). In the present study, 81% of the high responders were black, and the ratios for high responders by race were 26/227 (11%) for blacks, 4/249 (1.6%) for whites, and 2/3 (66%) for Asians. Therefore, it appears that blacks as a group respond differently than do whites to Y4. It is interesting to note that all these individuals responded to the carbohydrate antigen. A similar effect of race has been shown for the antibody response to carbohydrate antigens of *Haemophilus influenzae*, which has been attributed to an immune-response gene associated with the Km_1 immunoglobulin light-chain allotype (5). An immune-response gene such as the one associated with the κ light-chain allotype may also explain the effect of race on the antibody response to Y4. In this study we have identified the immunodominant antigen for patients with the highest antibody titers to Y4. As previously mentioned, most of these patients are black. The dominant antigen for other patients responding to Y4 at a somewhat lower level may not be the same antigen, especially in the white population.

We conclude that the immunodominant antigen for *A. actinomycetemcomitans* Y4 is heat and papain stable. The characteristics of this antigen in Western blot analysis before and after phenol-water extraction and ethanol precipitation are consistent with those of a carbohydrate. The data also indicate that the immunodominant antigen contained in the carbohydrate extract is the type-specific antigen. In future work this carbohydrate will be characterized chemically, and we will examine the relation between antibody response to the immunodominant antigen and disease severity and extent.

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