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Immunodominant Antigens of Actinobacillus Actinomycetemcomitans in Antibody Positive Subjects

Joseph V. Califano

School of Basic Health Sciences
Virginia Commonwealth University

This is to certify that the dissertation prepared by **Joseph V. Califano, D.D.S.** entitled *Immunodominant Antigens of Actinobacillus actinomycetemcomitans in Antibody Positive Subjects* has been approved by his committee as satisfactory completion of the disseration requirement for the degree of Doctor of Philosophy.

[redacted]

Director of Thesis

[redacted]

Committee Member

[redacted]

Committee Member

[redacted]

Committee Member

[redacted]

Committee Member

[redacted]

School Director of Graduate Study

[redacted]

Department Chairman

[redacted]

School Dean

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Date

Immunodominant Antigens of Actinobacillus actinomycetemcomitans in Antibody Positive Subjects

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

Joseph V. Califano, D.D.S.
B.A., Hofstra University May 1979
D.D.S., Medical College of Virginia/Virginia Commonwealth University
May 1984

Director: John G. Tew, PhD.
Professor, Microbiology and Immunology

Virginia Commonwealth University
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List of Abbreviations

Aa	*Actinobacillus actinomycetemcomitans*
AaY4	*Actinobacillus actinomycetemcomitans* strain Y4
AP	adult periodontitis
Bg	*Bacteroides gingivalis*
JP	juvenile periodontitis
LPS	lipopolysaccharide
NP	non-periodontitis
RIA	radioimmunoassay
SP	severe periodontitis
TT	tetanus toxoid

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ABSTRACT

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Joseph V. Califano, D. D. S.

Virginia Commonwealth University

Advisor: Dr. John G. Tew

This study was initiated to look for characteristics of the immunodominant antigen(s) of *Actinobacillus actinomycetemcomitans* (Aa) that might help explain the high antibody titers reactive with this organism in periodontitis patients. Radioimmunoassays (RIA) and limiting dilution analysis on western blots were used to identify and characterize the immunodominant antigens of Aa in high responder patients (i.e. patients with high antibody titers). Additional experiments focused on specific responding subjects with a range of antibody titers to serotype b (AaY4). This group included both white and black subjects. Similar studies then examined the immunodominant antigens of Aa serotypes a and c. The Aa serotype specificity of the high responders was determined and further work was confined to individuals responding specifically to serotypes a or c. The results indicated that the immunodominant antigen of AaY4 in all high responders was heat and papain stable. Limiting dilution analysis on western

blots showed that a large diffuse band resembling carbohydrate was the immunodominant antigen. Partially purified AaY4 carbohydrate also contained the large diffuse band. Double immunodiffusion (Ouchterlony) tests indicated that the dominant antigen recognized in the carbohydrate extract by the patients' sera was the same antigen recognized by serotype b specific rabbit antiserum which has been previously shown to react with the serotype specific carbohydrate of AaY4. When the subjects with specific antibody responses covering a range of positive antibody titers were examined, 95% of the subjects were responding to the smeared serotype specific carbohydrate antigen on western blots. For serotype-a, no immunodominant antigen was common to the majority of high responding subjects. For serotype-c two antigens were immunodominant in the majority of the high responding patients: 92% had an antigen with a diffuse smeared appearance on western blots typical of a carbohydrate antigen and 67% had an approximately 15Kd antigen. The smeared antigen gave a reaction of identity with patient serum and serotype c specific rabbit antiserum in double immunodiffusion assays. These findings indicated that the immunodominant antigen for serotypes-b and-c in high responding subjects was the serotype specific carbohydrate. The same antigen was immunodominant for subjects with a range of antibody titers to AaY4. The dominant antigen was highly variable for serotype-a. These antigens were highly immunogenic. This immunogenicity may explain the enormous antibody titers observed for Aa in response to what appears to be a small quantity of antigen.

INTRODUCTION

Periodontal disease is characterized by loss of the supporting tissues of teeth. It has been shown that the tissue destruction associated with the disease only occurs in the presence of bacterial plaque. As the disease progresses the normal relationship of the gingiva, gingival connective tissue, and bone, with the tooth is lost. Normally, teeth are attached to supporting bone by periodontal ligament fibers which extend from the cementum of the tooth into the alveolar bone. The gingiva in a similar manner is attached to the tooth by connective tissue fibers. In health they attach to a small amount of root surface that is not within the alveolar bone. Therefore, the gingival attachment extends coronally to the enamel (i.e. the cementoenamel junction). Periodontal disease begins as the gingival fibers detach from the root surface. This is followed by loss of alveolar bone and periodontal ligament fibers. This attachment loss can become extreme resulting in tooth loss. There are three major types of periodontal disease: adult periodontitis, severe periodontitis (generalized juvenile periodontitis or rapidly progressive periodontitis), and juvenile periodontitis (localized). Adult periodontitis is characterized by comparatively slow loss of attachment with active and stable periods throughout a patient's life. Severe periodontitis is a type of early onset periodontitis where all or many of the patient's teeth are affected by rapid attachment loss. Individuals with this disease are clinically identified by their young age and rapid rate of periodontal destruction. Severe periodontitis has similar characteristics to juvenile periodontitis and,

therefore, is sometimes referred to as generalized juvenile periodontitis. Localized juvenile periodontitis is another form of early onset periodontitis that usually begins during puberty. It has a localized pattern of disease affecting first molars and incisors preferentially. Additional teeth may become affected as the individual becomes older. Older individuals that have a generalized pattern of attachment loss where an earlier localized pattern of disease can be documented are often classified as juvenile periodontitis patients. The bony lesions in these subjects are typically deep vertical defects with the adjacent tooth unaffected. Early onset forms of periodontal disease have a familial tendency which suggests a genetic basis to these diseases. In addition, juvenile periodontitis patients often have defects in neutrophil chemotaxis. It is also interesting to note that a similar immune defect can be found in juvenile diabetic patients with early onset periodontal disease (15).

There has been considerable debate concerning the specific and non-specific plaque hypothesis. The specific plaque hypothesis suggests one organism is the primary etiologic agent for periodontal disease. This hypothesis would then support the idea that Koch's postulates could be satisfied for periodontal disease. The non-specific plaque hypothesis suggests that periodontal disease is a function of increased bacterial load with the same species in the same relative amounts as found in health. While this issue is still a matter of debate, it appears that the non-specific plaque hypothesis does not describe the flora found in diseased individuals. Periodontal patients not only have an increase in total numbers of bacteria, but the composition of the flora is altered (19). There is a shift from a flora where gram positive cocci predominate to one where gram negative anaerobic bacilli predominate. It may be that this general shift to gram negative organisms results in disease. In this case a mixed infection of predominantly gram negative anaerobic rods

would result in disease in susceptible individuals. It still remains a possibility that one or only a few specific organisms are responsible for disease. Three to four hundred organisms have been identified in sites affected with periodontal disease. Many of these organisms are difficult to culture. Utilizing the most effective techniques available, it is still not possible to culture all of the organisms present in the periodontal lesion. An important pathogen may be missed (possible example: the "large Treponeme") (19). Some of the four hundred bacterial species have strong association with periodontal disease even though they have yet to satisfy Koch's postulates.

Actinobacillus actinomycetemcomitans (Aa), a gram negative organism frequently isolated from lesions of juvenile periodontitis (1,13,19,30), is thought to be a major etiologic agent of this form of periodontal disease (21). There has been some disagreement on the degree of association with disease and the proportion of the flora in diseased sites. Some investigators have indicated that this organism is found in large numbers in almost all juvenile periodontitis lesions (32,1). Others have found the organism associated with diseased sites less frequently and in a smaller proportion of the flora (19). *Actinobacillus actinomycetemcomitans* (AaY4) has been shown to elicit remarkably high antibody titers in juvenile periodontitis (4,16,18,20,25,26). This antibody response has been shown to be protective in patients with early onset periodontits (6). Individuals that find the organism frequently in high numbers cite this data as evidence that the organism is indeed highly prevalent among juvenile periodontitis patients. They suggest that the high antibody titers are the result of large numbers of Aa organisms being present in the periodontal pocket (30,32). However, other periodontitis associated organisms such as *Fusobacterium nucleatum* elicit comparatively lower titers

(24,25) even though they represent a larger proportion of the flora for most patients (19).

Rational For This Study

This study was initiated to examine the immunodominant antigens of *Actinobacillus actinomycetemcomitans*. It was reasoned that the immunodominant antigens of Aa might have unique characteristics that might make them potent immunogens. This would explain the enormous antibody titers reactive with this organism that have been observed. Identifying the immunodominant antigens might also provide information regarding a patient's prognosis as antibody to this organism has been shown to be protective (6). The immunodominant antigens might also make good candidates for vaccines that could elicit a protective response.

The results of this study showed that the immunodominant antigen for Aa serotype-a was highly variable from patient to patient. In contrast, most patients with high antibody titers to serotype-c responded to a 15Kd protein antigen and/or a smeared antigen. The immunodominant antigen for Aa serotype b (AaY4) in both high responders and specific responders over a range of antibody titers was also a smeared antigen. This smeared antigen recognized by patient serum was also recognized by serotype specific rabbit antiserum. These data support the conclusion that the smeared immunodominant antigen was the serotype specific carbohydrate which appears to be part of the Aa capsule (30,31,32). The carbohydrate antigens therefore appear to be highly immunogenic and may be important antigens for Aa serotypes-b and-c. This potent immunogenicity may explain the enormous antibody titers that are found even when AaY4 is found in low numbers.

MATERIALS AND METHODS

Human Subjects

1. Immunodominant Antigen for *Actinobacillus actinomycetemcomitans* Strain Y4 in High Responder Patients

Subjects for these studies included 481 clinically characterized patients {227 black, 249 white, 3 Asian, 2 Indian (Asian)} at the Medical College of Virginia/Virginia Commonwealth University Clinical Research Center for Periodontal Diseases. These patients were diagnosed as follows: 76 juvenile periodontitis, 72 severe periodontitis, 103 adult periodontitis, and 230 non-periodontitis.

2. Immunodominant Antigens of *Actinobacillus actinomycetemcomitans* Serotype b in Early Onset Periodontitis Patients

Subjects for these studies were selected from a group of 99 early onset periodontitis patients at the Medical College of Virginia/Virginia Commonwealth University Clinical Research Center for Periodontal Diseases. The group of 99 subjects included 47 localized juvenile periodontitis (JP) (39 Black, 9 white) and 52 generalized severe early onset periodontitis (SP) (28 black, 24 white) patients. Eleven AaY4 seropositive white, early onset periodontitis subjects with a range of antibody titers were selected for study (see Table 1). Black subjects were then selected who had antibody titers comparable to those found among the white subjects. Our study group included 18 black subjects and 11 white subjects. This

included 21 individuals with severe periodontitis (SP) and 8 individuals with juvenile periodontitis (JP).

3. Immunodominant Antigens of *Actinobacillus actinomycetemcomitans* Serotypes a and c in High responder Patients

Subjects for these studies included 150 clinically characterized patients at the Medical College of Virginia/Virginia Commonwealth University Clinical Research Center for Periodontal Diseases.

Specific criteria for each of these categories are the following:

(1) *Juvenile periodontitis* (JP): Subjects were 30 years of age or less with a localized pattern of severe periodontal destruction limited to first molar or incisor teeth and up to two additional teeth.

(2) *Severe periodontitis* (SP): Subjects were 35 years of age or less with a generalized pattern of severe destruction with attachment loss of at least 5 mm on 8 or more teeth, at least 3 of which were not first molars or incisors.

(3) *Adult periodontitis* (AP): Subjects were greater than 35 years of age with chronic periodontitis generalized to all four quadrants of the mouth.

(4) *Non-periodontitis* (NP): Subjects of any age who had no evidence of attachment loss, except recession on the buccal surface of anterior teeth, and no more than one site or pockets no greater than 3mm.

Bacteria and Antigen preparation

Actinobacillus actinomycetemcomitans serotypes a, b, and c (VPI strain numbers: 14554,13127,14573 respectively) and *Bacteroides gingivalis* strain 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, Richmond, 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 (17). Tetanus toxoid, used as a control, was the generous gift of Lederle, Inc.

Radioimmunoassay

Details of the solid-phase radioimmunoassay technique used for detection and quantitation of specific antibody have been described (24). Briefly, 200 microliters of sonicated bacteria or TT (25-100 micrograms per 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 hours and then 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 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 1/250 ml in the first well to 1/512,000 ml in the final well. The uncoated wells served as a background control. After incubation and washing as described above, 200 microliters of iodinated (specific activity, 25 uCi/ ug) goat anti-human IgG (Fc specific) in diluent was added per well. After incubation and washing, radioactivity was measured to an accuracy of 10%(LKB instruments Inc., Rockville, Md; 1282 Compugamma Counter). The difference in counts (delta cpm between coated and uncoated wells) was the basic data unit. A plot of delta cpm versus serum dilution was prepared. A cut-off was selected (generally 3000-10,000 delta cpm) that intersected the curve at the end of the linear portion of the curve or point of inflection. This was designated as 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 (Aa 5ug/50ul, Bg 20ug/50ul, Tetanus toxoid 20ug/50ul) in sample buffer (0.0626M Tris 10% glycerol 2.3% SDS pH 6.8) and boiled for 90 seconds. The running buffer was 0.025M Tris containing 0.192M Glycine and 0.1% SDS. 5-20% linear gradient polyacrylamide gels were run at 30 milliamperes per gel until the smallest prestained molecular weight marker (Bethesda Research Laboratories Life Technologies, Inc. Gaithersburg, MD.) reached the bottom of the gel (approximately 5 hours). A BIO-RAD SE600 vertical gel apparatus and a BIO-RAD power supply model 500/200 were used. Following electrophoresis the antigens were then blotted to nitrocellulose in a BIO-RAD transblot cell for 5 hours at 200 milliamperes. The blots were incubated in equal volumes of distilled water containing 10% nonfat dry milk and PBS, pH 7.2 for 1 hour to block non-specific binding (11). 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 1/1024,000 ml serum at the highest dilution. The separate nitrocellulose lanes were then incubated with patient serum for 16 hours (1-3 hours in some experiments) followed by 4 ten minute washes with TTBS (Tween-20 Tris Buffered Saline). The 16 hour incubation was necessary to improve sensitivity. The lanes were then exposed to a 1:1000 dilution of alkaline phosphatase labeled goat anti-human IgG (H & L)(KPI laboratories Inc., Gaithersburg, MD.) for 4 hours. This was followed by 4 more washes with TTBS. The blots were developed with the BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium) phosphatase

substrate system. Some of the SDS-page gels were also stained with Coomassie brilliant blue (Bio-Rad, 220 Maple Ave. Rockville Centre, N. Y. 11571) or Silver stain (Bio-Rad silver stain kit, Bio-Rad, 220 Maple Ave. Rockville Centre, N. Y. 11571) (the method used was that described in the Bio-Rad Trans-Blot and Immun-Blot instruction manuals included with the Trans-Blot unit).

Competition Assay

To determine the specificity of the antibody response for each patient the competition assay was developed. Four replicate nitrocellulose lanes (for each serotype) were stained (as described above in the western blot technique, except incubation with primary and secondary antibody was for one hour each). The patient serum used was either untreated or incubated overnight with serotype-a, -b, or-c antigen (see Fig. 1 for illustration). It is important to note that a large quantity of antigen was required to completely eliminate staining (i.e. completely eliminate reactivity from the serum) in some patients. To completely absorb antibody molecules reactive with the antigen, serum was incubated (diluted 1/250) overnight with 3000ug of antigen followed by an overnight incubation with antigen coated petri dishes. These petri dishes were coated with antigen in the same manner as microtiter plates in the RIA (24). The patients' sera were then centrifuged in a microfuge at 6000 X g for 10 minutes to remove any insoluble material.

Double Immunodiffusion

Double immunodiffusion precipitin reactions were performed in agar gels (Ouchterlony test), using the slide micromethod (20). The well cutters were selected to produce wells of 10ul capacity in the center and six circumferencial wells. The center well received 40ul of AaY4 carbohydrate extract added in 10ul increments. The peripheral well received 5ul 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, dried and stained at room temperature in a 0.5% solution of Coomassie blue R-250 in 10% acetic acid, 25% isopropanol in water. The excess stain was removed by washing the slides in a 10% acetic acid solution. The rabbit serotype specific antisera were the kind gift of Dr. Joseph Zambon of Buffalo, NY.

Immunoelectrophoresis

Immunoelectrophoresis was conducted in a 1.0% agarose gel in 0.02M TRIS-barbiturate, pH 8.6 using a Pharmacia Flat Bed Apparatus with cooling plate following the method described in the unit's instruction manual. (Pharmacia Inc., 800 Centennial Ave. Piscataway, N.J. 08854). AaY4 sonicate (10ul sonicate containing 43ug/ml protein) or AaY4 carbohydrate extract (10ul) were added to the wells and electrophoresed for 60 minutes at 10V/cm while cooled to 10-14^{o}C. The troughs were then filled with 100ul of a 1:8 dilution (as per instruction manual) of human serum and incubated at room temperature for 18 hours. The gel was then pressed, washed (in PBS pH 7.4 for 18 hours followed by a 60 min. wash in deionized water), and dried. The gel was then stained with Coomassie blue.

Papain Digestion

Proteolytic digestion was accomplished by adding 200ul of papain (Sigma) solution per well (containing 0.1mg /ml in 0.1M Phosphate,with 0.001M Cys 0.002 ETDA buffer pH 6.2) to antigen coated plates and incubated for 4 hours at 37^{o}C (25). The antigen was digested after coating of microtiter plates as it was reasoned this would prevent competition for binding sites on the plates by papain (also a protein). The plates were then washed 20 times in tap water. For western blots, papain solution was prepared as above with 7.5

mg/ml papain and the appropriate antigen was added to a concentration of 1 mg/ml. This mixture was then incubated for 4 hours at 37°C. Following the incubation, the mixture was concentrated to 0.5 ml with a Centriprep concentrator (Amicon Corp. Scientific Systems Division, Danvers. MA) and then diluted to the same concentration used for undigested antigen.

Protease K Digestion

Proteolytic digestion was accomplished by placing a nitrocellulose lane that had electrophoretically separated Aa serotype-c antigens electroblotted to it in sample buffer (0.026 M Tris, 10% glycerol, 2.35 sodium dodecyl sulfate [SDS] [pH 6.8]) containing 50 ug/ml protease K for 4 hours at 37°C. Proteolytic digestion of serotype-c sonicate (7ug protein/ml in PBS) was accomplished by adding protease K to a concentration of 200ug/ml and incubating overnight at 37°C.

Heat Treatment

Antigen preparations (Aa, Bg, or TT) were placed in a boiling water bath for 45 minutes. Various temperatures and times studied in preliminary experiments indicated that boiling for 45 minutes was necessary to virtually eliminate reactivity with tetanus toxoid and Bg controls.

Carbohydrate Purification

Carbohydrate was partially purified from Aa by extraction with phenol and water followed by precipitation of the carbohydrate from the aqueous phase with cold ethanol (9). One milliliter of AaY4 whole cells (75.5mg/ml) was pelleted in an Eppendorf Microfuge. The pellet was then resolubilized in 23ml of 0.05M Tris,0.001METDA, 50ug/ml lysozyme (pH 7.5), and incubated for 15 minutes at 37°C. SDS was then added to a concentration of 1.5% to gently lyse the cells. Forty six milliliters of phenol were added and the mixture shaken for 15 minutes. The mixture was then centrifuged at

5900 X G for 10 minutes at 4°C. The aqueous layer was removed and two volumes of cold 95% ethanol was slowly added. The mixture was then centrifuged at 5900 X g for 10 minutes at 4°C. The precipitate was then dissolved in 23ml of 20mM phosphate buffer. Ribonuclease (40ug/ml) and deoxyribonuclease (40ug/ml) were added and incubated for 1 hour at 37°C. The extracted carbohydrate was concentrated to 0.5 ml with a Centriprep concentrator (Amicon corp.-Scientific Systems Division, Danvers, MA) and then diluted in sample buffer one sixth the original volume. In some experiments, low molecular weight material was removed by concentrating the extracted carbohydrate with a Centriprep concentrator having a 30kd molecular weight cut off. This was accomplished with 3 cycles of concentration and resuspension in 10 ml deionized water. In some experiments, lipopolysaccharide was removed from the carbohydrate extract with a lipid A binding affinity column (Detoxi-Gel; Pierce, Inc. box 117 Rockford, Ill. 61105). The column was equilibrated with 3 bed volumes of PBS pH 7.5. Twenty microliters of carbohydrate extract was then added to the column which represented microgram amounts of LPS. According to the manufacturer, each column has a binding capacity of 2 milligrams of LPS. Six bed volumes of PBS were then added. Six bed volumes of effluent were collected and concentrated with a centriprep concetrator.

LPS Purification

Actinobacillus actinomycetemcomitans lipopolysaccharide (a kind gift of Dr. Frank Nichols, Univ. of Connecticut School of Dental Medicine) was purified by the method of Westphal (28). Analysis of the phenol/water LPS extract on Coomassie stained SDS-page gels revealed several protein bands. This AaY4 LPS preparation was therefore further treated with papain as described above to digest contaminating protein.

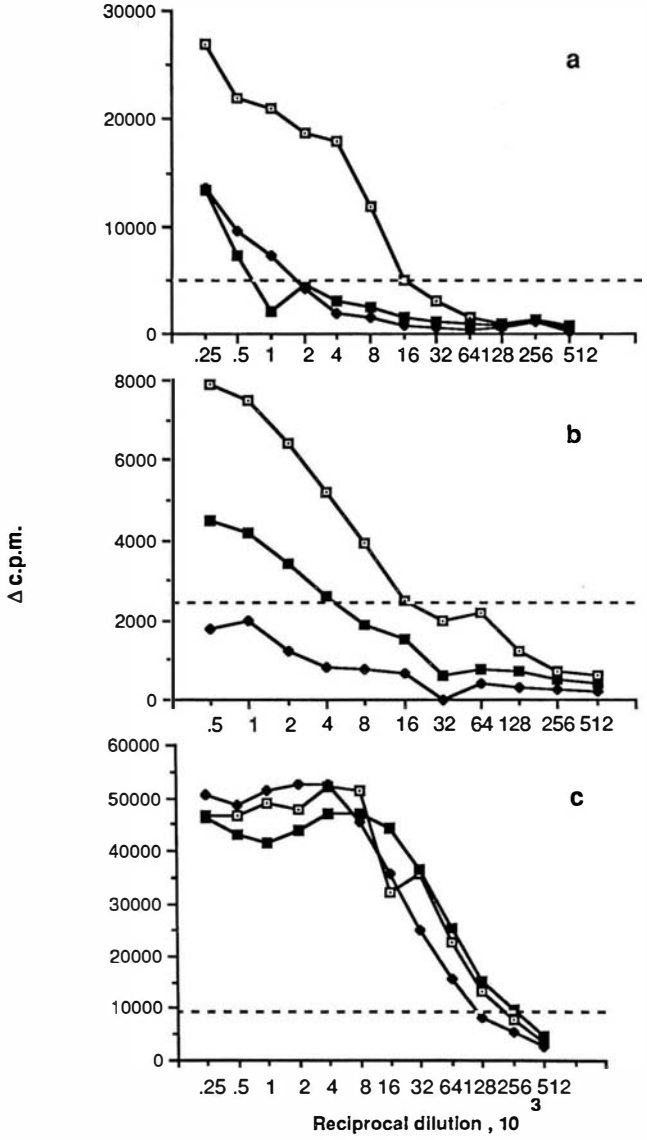
RESULTS

1. Immunodominant Antigen for *Actinobacillus actinomycetemcomitans* Strain Y4 in High Responder Patients

In order to identify subjects with high anti-AaY4 antibody titers, sera from 481 patients were assayed. Thirty-two subjects with the highest anti-AaY4 antibody titers (i.e. above 128,000 RIA units/ml) were selected for further analysis. This was less than 10% of the total group with 26 being black, 4 white, and 2 Asian. All but 2 of these subjects had early onset forms of periodontitis i.e., 17JP, 13SP, 1AP, and 1NP.

To determine the nature of the immunodominant antigens of AaY4, 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 dilution for AaY4 as well as TT and Bg which served as controls are shown in Figure 1. For tetanus toxoid (Fig. 1 panel A) the titer for untreated antigen was 16,000 RIA units/ml. Boiling the antigen decreased the titer to 2000 RIA units/ml and papain digestion reduced the titer to 750 RIA units/ml. Similarly for Bg (Fig. 1 panel B), a titer of 16,000 RIA units/ml for untreated antigen was decreased to 4,000 RIA units/ml by papain and less than 500 RIA units/ml for boiling. In contrast, a titer of 256,000 RIA units/ml for Aa (Fig. 1 panel C) was unaffected by papain and only modestly decreased by boiling for 45

FIG. 1. Typical RIA curves showing the effect of boiling (◆), and papain digestion (■) of antigen on antibody titers as compared to untreated antigen (❑). Panels a, b, and c, respectively illustrate the antigens TT, Bg, and AaY4. The dotted line locates the cut-off for each curve. The cut-off marks the point in the linear portion of the 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 end point.


30000
20000
10000
0
a
.25 .5 1 2 4 8 16 32 64128 256 512
8000
6000
4000
2000
0
b
.5 1 2 4 8 16 32 64 128 256 512
60000
50000
40000
30000
20000
10000
0
c
.25 .5 1 2 4 8 16 32 64128256 512
Reciprocal dilution , 10 3
Δ c.p.m.

minutes. The mean antibody titers for all 32 patients' sera is summarized in Figure 2. These results indicate that the antibody titer for TT (this control was applied to 4 of the 32 subjects) was virtually eliminated by both papain treatment and boiling. In contrast, antibody titers against AaY4 were unaffected by papain digestion and only modestly decreased by boiling.

To more directly examine individual antigens responsible for the antibody responses, westem blots were prepared. As a control for the effectiveness of enzyme digestion, Bg antigen was examined by westem blot analysis with and without papain treatment. At low dilution of patient serum, there were over 30 discrete bands for Bg (Fig. 3 "Bg"). With papain digestion, the protein bands were eliminated leaving the characteristic "step ladder" effect commonly associated with lipopolysaccharide (LPS) (Fig. 3 "Bg pap"). When the AaY4 antigen preparation was analyzed using 0.5 ml of patient serum in the incubation mixture (Fig. 3 "Aa"), about 50 discrete bands were present. In addition to these bands there was also a diffuse band or "smear" observed in the upper portion of the lane. Although the location 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 this smear did not begin until the level of the 200kd molecular weight marker. If the AaY4 antigen was boiled for 45 minutes before western blot analysis, many of the discrete protein bands disappeared but the smear persisted (Fig. 3 "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 persistence of the antigen after treatment is occurring rather than aggregation or unveiling of antigen.

FIG. 2. Compilation of RIA data for all patients having a high antibody titer to AaY4 showing the affect of boiling and papain digestion on antibody titers. Panel a is the tetanus toxoid positive control (4 subjects) and panel b is the data for AaY4 (32 subjects). The error bars indicate the standard error of the mean.

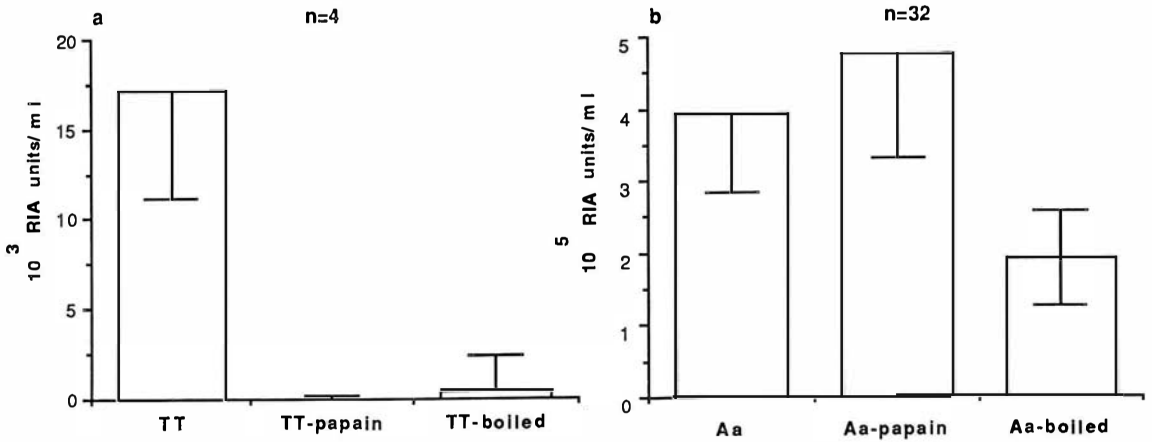
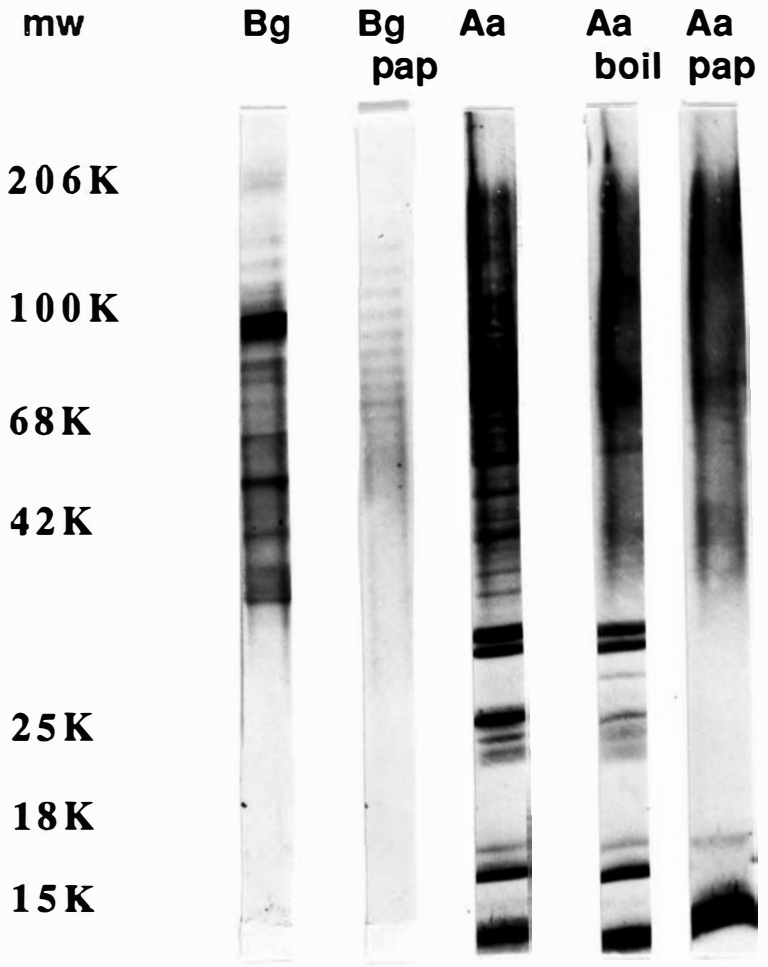

a
n=4
20
15
10
5
0
3
10 RIA units/ml
TT
TT-papain
TT-boiled
b
n=32
5
4
3
2
1
0
5
10 RIA units/ml
Aa
Aa-papain
Aa-boiled

FIG. 3. A western blot using a low dilution (1/2) of patient serum showing the effect of boiling and papain digestion on AaY4. Bg is run as a control for papain digestion.


mw
Bg
Bg pap
Aa
Aa boil
Aa pap
206K
100K
68K
42K
25K
18K
15K

To determine which bands represent the immunodominant antigen for AaY4, western blots were run and replicate lanes were stained with progressively less serum from 1/250 ml to 1/1024K ml (fig-4). With increasing dilution, all of the protein bands were eliminated leaving the smear in the upper portion of the lane when only 1/256K ml of serum was used. In the original western blot, a faint smear was visible even at 1/1024K. To determine the immunodominant antigens for the group of high responder patients, 1/100K 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 react with the smear in the upper portion of the lane. Other bands are present for many of the patients at 1/100K; however, the discrete bands with the highest prevalence (100Kd, 98Kd, 85Kd) were found in only 34% of the subjects. In every patient (5 subjects) where the serum was serially diluted beyond 1/100K ml, only the smear was detected.

The properties of the "smear" antigen are consistent with a carbohydrate (i.e. the antigen was heat and papain stable and did not focus into a discrete band on western blots). To test this more directly, AaY4 sonicate was subjected to a carbohydrate purification procedure involving extraction 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 AaY4 (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.

FIG. 4. A typical western blot of AaY4 illustrating the immunodominant antigens. Replicate lanes of AaY4 were run on polyacrylamide gel and transferred to nitrocellulose. Decreasing amounts of a patient's serum (1/250 ml-1/1024K 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 amount of serum, but the "smear" was still visible in the upper portion of the western blot with a serum dilution utilizing 1/256K ml of serum. In the original western blot, the smear was also visible at 1/1024K.

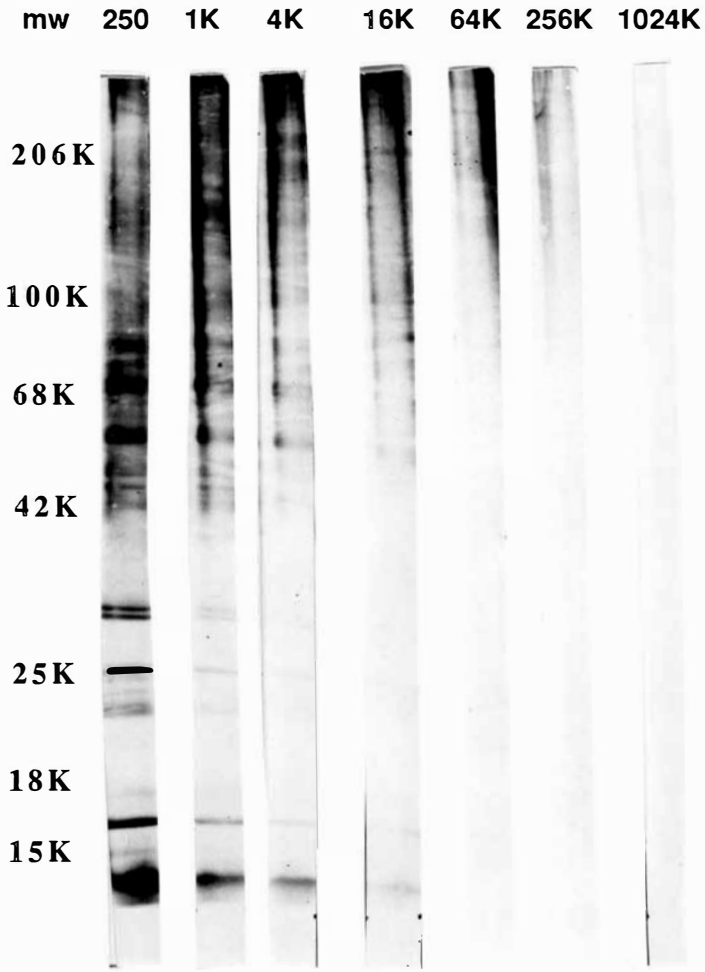

mw
250
1K
4K
16K
64K
256K
1024K
206K
100K
68K
42K
25K
18K
15K

FIG. 5. Comparison of papain digested AaY4 antigen with AaY4 carbohydrate on a western blot. Patients' sera are used at a low dilution (1/2 or 0.5ml) to reveal any antigens in the purified material.

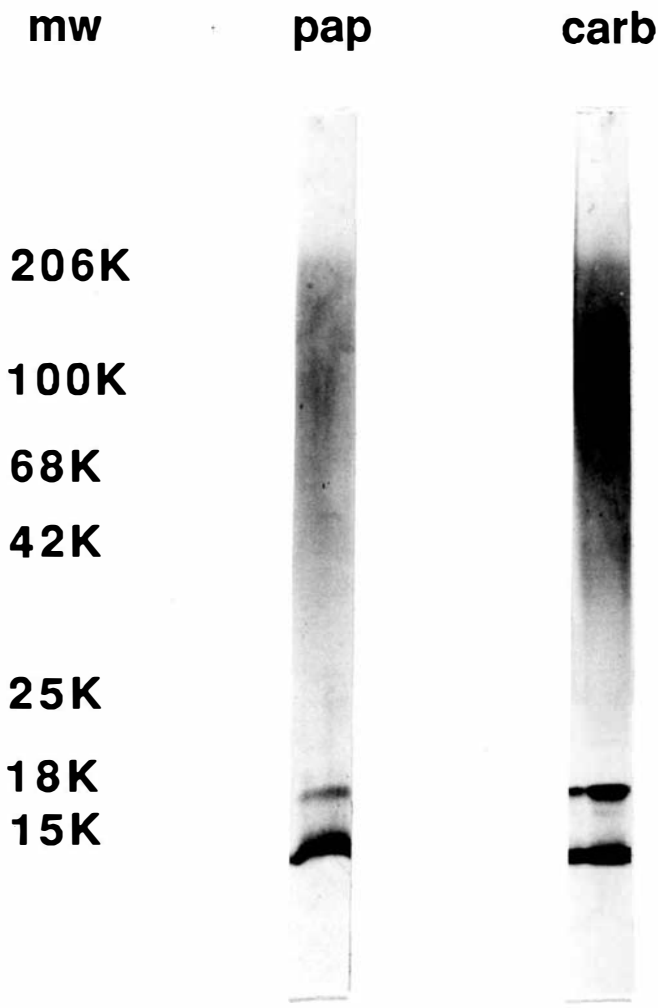

mw
pap
carb
206K
100K
68K
42K
25K
18K
15K

TABLE 1. Prevalence of individual bands on western blot for *Actinobacillus actinomycetemcomitans* strain Y4 in high responder patients

Number of individuals responding (%)[b]		Band[a]
31	100%	smear
10	34%	100Kd
10	34%	98Kd
10	34%	85Kd
9	28%	99Kd
9	28%	80Kd
8	25%	68Kd
7	21%	75Kd
6	18%	87Kd
5	15%	205Kd
3	9%	180Kd
3	9%	160Kd
3	9%	60Kd
2	6%	58Kd
2	6%	18Kd
1	3%	15Kd
1	3%	25Kd
1	3%	210Kd
1	3%	90Kd

a- Bands appearing on western blot when stained with 1/100,000 of a ml of serum in 4 ml of diluent

b- 1 subject of the 32 was unavailable for western blot

The appearance of the Aa immunodominant antigen on western blots prompted us to determine if this antigen was the serotype b specific antigen which is thought to be a capsular polysaccharide (30). In double immunodiffusion analysis, the extracted carbohydrate preparation showed a line of identity with patient serum and rabbit serotype b specific antiserum (Fig. 6 panel A). No reaction was seen with serotype a or c specific antisera. In addition, western blots using 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 blot actually represented a single antigen. After several trials, it appeared the immunodominant antigen was not migrating at the required pH. The carbohydrate extract as well as whole AaY4 sonicate was analyzed using immunoelectrophoresis. The antigen forming the precipitin bands did not migrate at pH 8.6 explaining why crossed immunoelectrophoresis was unsuccessful (Fig-6 panel B).

It was considered likely that the carbohydrate extract contained lipopolysaccharide which might contribute to the smear observed on western blots. *Actinobacillus actinomycetemcomitans* lipopolysaccharide (AaLPS) was therefore analyzed on western blots and SDS-page gels (Coomassie and silver stained) (28). The AaLPS 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 AaLPS.

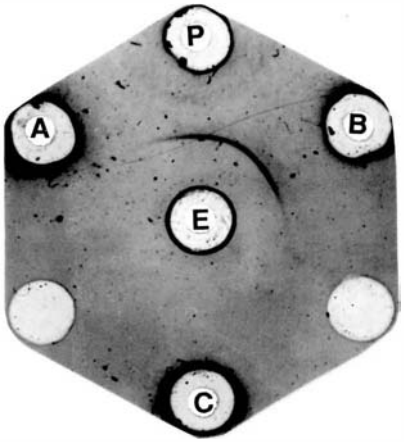
To better assess the contribution of LPS to the diffuse band in western blots, the carbohydrate was further enriched. Two phenol/water extractions

FIG. 6. Double immunodiffusion and immunoelectrophoresis showing that serotype-b specific rabbit antiserum and high responder patients recognize the same immunodominant antigen.

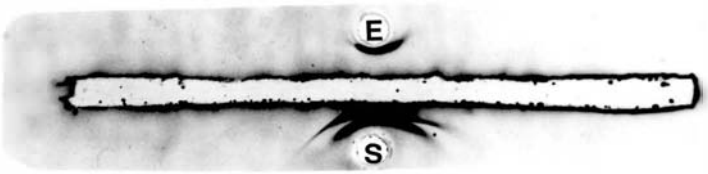
Panel A: A double immunodiffusion analysis (Ouchterlony technique) showing that rabbit serotype b specific antiserum and patient serum recognize the same antigen. Peripheral wells P, B, A, C contained: patient serum P, and Aa serotype B, A, C, serotype specific antisera respectively (5ul each of a 1:2 dilution). The center well (E) contained AaY4 carbohydrate extract (40ul)

Panel B: Immunoelectrophoresis of the immunodominant antigen at pH 8.6. Well E contained 10ul of AaY4 carbohydrate extract and well S contained 10ul of AaY4 sonicate. Two precipitin bands were noted when using the whole sonicate, and a single band with the carbohydrate extract.

A



B

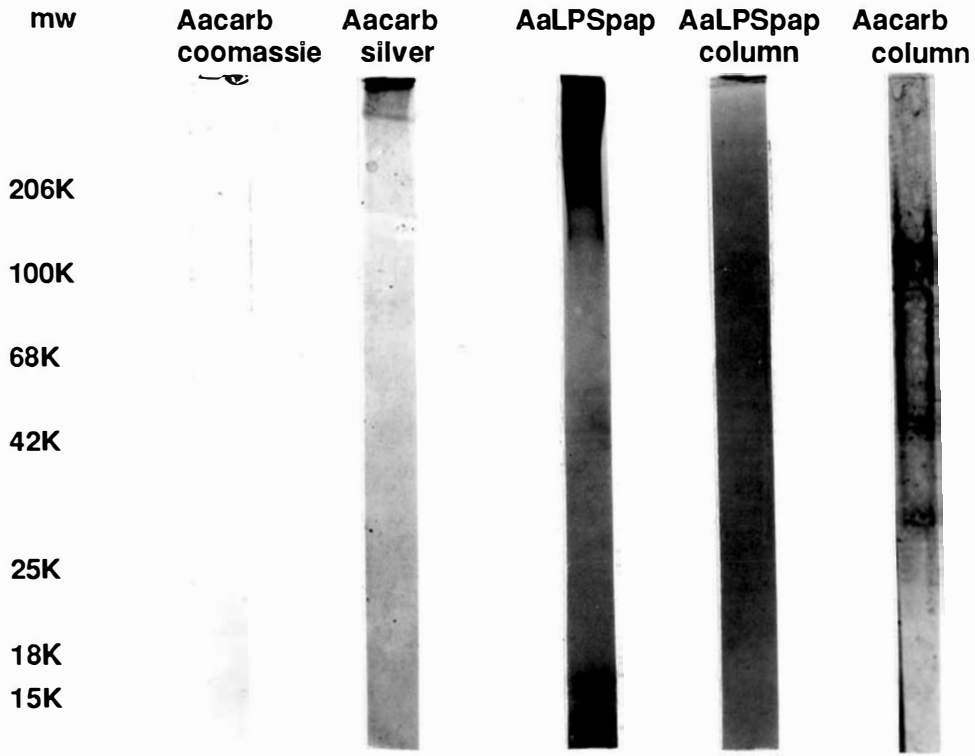


and cold ethanol precipitations were carried out on the AaY4 sonicate. Further purification was accomplished using a concentrator with a 30kd molecular weight exclusion to remove low molecular weight material. Finally this preparation was passed over a lipid A binding column to remove LPS. Analysis of this preparation on SDS-page gels is presented in Fig. 7 (Fig-7 "Aacarb coomassie" "Aacarb silver"). This demonstrates 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 up 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-page gels. *Actinobacillus actinomycetemcomitans* lipopolysaccharide, which was papain treated, was used as a control. Silver staining of the AaLPS SDS-gels before and after passage over the column indicated that the column bound AaLPS effectively (Fig. 7 "AaLPSpap" "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").

2. Immunodominant Antigens of *Actinobacillus actinomycetemcomitans* Serotype b in Early Onset Periodontitis Patients

In this set of experiments, the dominant antigens of early onset periodontitis patients with a range of antibody titers for Aa serotype b (including both black and white subjects) were examined. The objective of these experiments was to determine if the serotype

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 followed by cold ethanol precipitation. Low molecular weight materials were removed by using a concentrator with a 30Kd filter. LPS was used as a control to establish that the lipid A binding column would remove AaLPS. SDS-page gels were stained with Coomassie blue, silver or analyzed on western blot. Aacarb coomassie: SDS-page gel of AaY4 carbohydrate extract stained with coomassie blue, Aacarb silver: SDS-page gel of AaY4 Carbohydrate extract stained with silver, AaLPSpap: SDS-page gel of AaY4 LPS that was digested with papain stained with silver, AaLPSpap column: SDS-page gel of AaY4 LPS that has been digested with papain and passed over LPS binding affinity column stained with silver, Aacarb Column: western blot (using a 1/50 dilution of patient serum) of AaY4 carbohydrate extract that has 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 15kd. It appears that enough protein persisted, after all the purification steps, to be detected by the very sensitive western blot.


mw
Aacarb coomassie
Aacarb silver
AaLPSpap
AaLPSpap column
Aacarb column
206K
100K
68K
42K
25K
18K
15K

specific carbohydrate antigen that was immunodominant in high responding subjects (most were black) was also immunodominant in both black and white antibody positive subjects. Radioimmunoassays were completed on 99 early onset periodontitis patients (the early onset periodontitis subset of the 481 subject group) to find subjects with a range of antibody titers reactive with AaY4. Eleven white patients with a positive antibody titer, including very low titers, were selected. Black subjects with similar antibody titers to AaY4 were selected to compare with the white patients.

The specificity of the antibody responses of the 29 selected patients was then determined. Double immunodiffusion experiments were run to help assess the specificity of the antibody responses to AaY4. Serum from 18 of the 29 subjects produced a line of identity with an AaY4 serotype specific specific serum indicating that their responses were specific (Fig. 8). The specificity of the remaining subjects' antibodies was determined using the competition assay. Examples of the assay illustrating specific and nonspecific reactivities is shown in Figure 9. Heavily loaded western blots of whole AaY4 sonicate were used to allow all antigens to be represented in ample quantity. For each patient, four replicate lanes were stained using a patient's serum that was either untreated or preincubated with serotype -a, -b(AaY4), or -c antigen. In this example, only preabsorption of the specific responder's serum (subject A) with serotype-b antigen (lane b/b) completely eliminated the reactivity present in the control non-preabsorbed lane (lane b/-). Preabsorption with either of the other two serotypes eliminated only cross reactive antigens with most of the reactivity remaining in the

FIG. 8. Identity of the immunodominant antigen with the serotype specific antigen. Double-immunodiffusion analysis (ouchterlony technique) showing that serum from a high reponding patient previously shown to recognize the same antigen as the rabbit serotype-b specific antiserum and patient serum (from responders with a range of antibody responses) recognize the same antigen in serotype-b sonicate that has been treated with protease K. Peripheral wells P and PHS contained patient serum (from the subjects with a range of antibody titers) and High responder serum(20ul of undiluted serum for each). The center well S contained 5ul of serotype-b sonicate (originally having a protein concentration of 7ug/ml) that was treated with protease K.

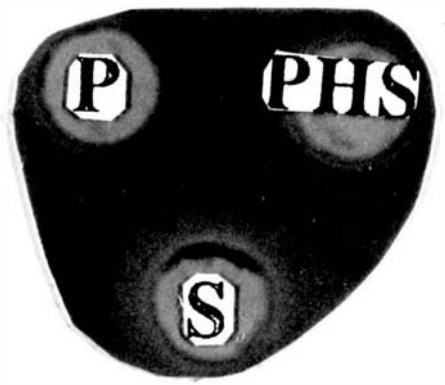
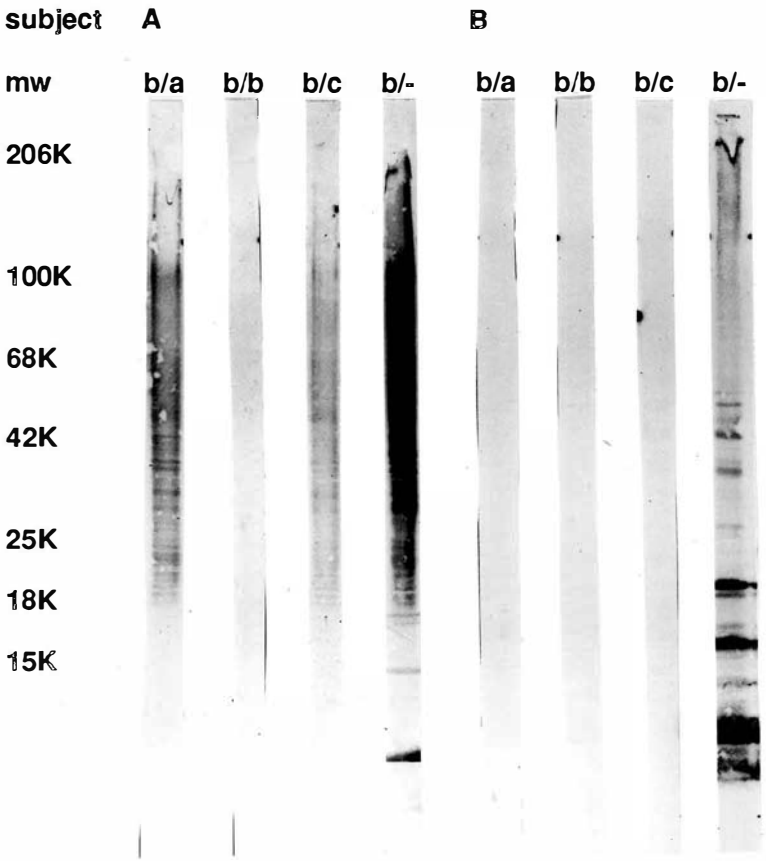

P
PHS
S

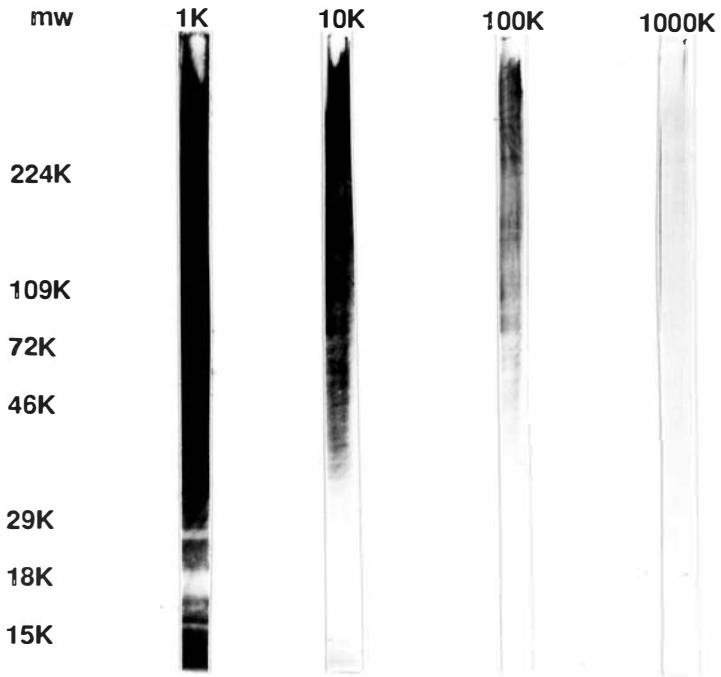
FIG. 9. Serological specificity of responses to Aa serotype b as determined by the competition assay using the western blot technique. Patient A: a specific responder to serotype-b, Patient B: a patient that responded nonspecifically to serotype-b, b/a: blot of serotype-b stained with serum preincubated with serotype-a antigen, b/b: blot of serotype-b stained with serum preincubated with serotype-b antigen, b/c: blot of serotype-b stained with serum preincubated with serotype-c antigen, b/-: blot of serotype-b stained with untreated serum.


subject
A
B
mw
b/a
b/b
b/c
b/-
b/a
b/b
b/c
b/-
206K
100K
68K
42K
25K
18K
15K

serum (lanes b/a, b/c). This result indicated that this patient was responding specifically. In subject B, a non-specific responder, preabsorption of the serum with any of the three serotype antigens (lanes b/a, b/b, b/c) eliminated all of the reactivity in the control lane (lane b/-). When this analysis was applied to the 11 subjects lacking precipitin lines in the double immunodiffusion experiment only 2 out of 11 were found to be responding specifically. Further examination of the data, showed that patients with an antibody titer of 43,000 units or greater demonstrated specific responses to serotype-b, while the lower responses tended to be against Aa common antigens This analysis allowed selection of 20 subjects from the original group having a specific response to AaY4. These included 6 white and 14 black subjects.

To determine which antigen in the bacterial sonicate was responsible for the antibody titer for each of the 20 patients, replicate western blot lanes were stained with increasing dilutions of patient serum in a limiting dilution analysis. This allowed visualization of the immunodominant antigen at the end point. This assay was analogous to the radioimmunoassay (RIA) that was used to determine the antibody titer. Each western blot lane corresponds to a well of a microtiter plate used in the RIA. An example of this analysis is shown in Figure 10. As replicate lanes were stained with progressively less serum (from 10^{-4} to 10^{-6} ml) most of the antigen bands disappeared leaving only the immunodominant antigen apparent at the highest dilution of serum (i.e. endpoint). In this case the immunodominant antigen at the endpoint was a smeared antigen in the upper range of the lane. The results of this analysis for all

FIG. 10. The immunodominant antigen of Aa serotype b. A typical western blot of Aa serotype-b illustrating the immunodominant antigen for a particular patient. Replicate lanes of Aa serotype-b were run on polyacrylamide gel and transferred to nitrocellulose. Decreasing amounts of a patient's serum (1/250 ml-1/1024K ml) were used in the incubation mixture for each lane. Note that the descrete bands have disappeared with the decreasing amount of serum but the smear was still visible in the upper portion of the lane at 1/100K ml of serum. In the original blot it was also visible at 1/1000K.


mw
1K
10K
100K
1000K
224K
109K
72K
46K
29K
18K
15K

the subjects is summarized in Table 2. The data indicated that 95% (19/20-total, 13/14 black, 6/6 white) of the specific seropositive subjects had a smeared antigen as the immunodominant antigen. One subject reacted with only a 72kd antigen and another reacted with both a 15kd and the smeared antigen as immunodominant antigens. All but these two patients gave a reaction of identity in double immunodiffusion experiments using serotype specific antiserum. The smear on western blot of the patient detecting the 15Kd antigen had the same appearance as that observed for all the other patients. I believe this is also a response to the serotype specific antigen; the antibody titer was too low for a visible reaction in double immunodiffusion analysis.

3. Immunodominant Antigens of *Actinobacillus actinomycetemcomitans* Serotypes-a and-c in High Responder Patients

Nineteen subjects with the highest anti-Aa serotype-a antibody titers and twenty one with the highest serotype-c antibody titers were selected from a group of 150 clinically characterized subjects. This was about 15% of the total group in each case. For serotype-a 11 were black and 8 white. For serotype-c 15 were black and 6 white. All but 4 of these subjects had early onset forms of periodontitis with 12JP, 6SP, and 1NP for serotype-a and 11JP, 8SP, and 2NP for serotype-c.

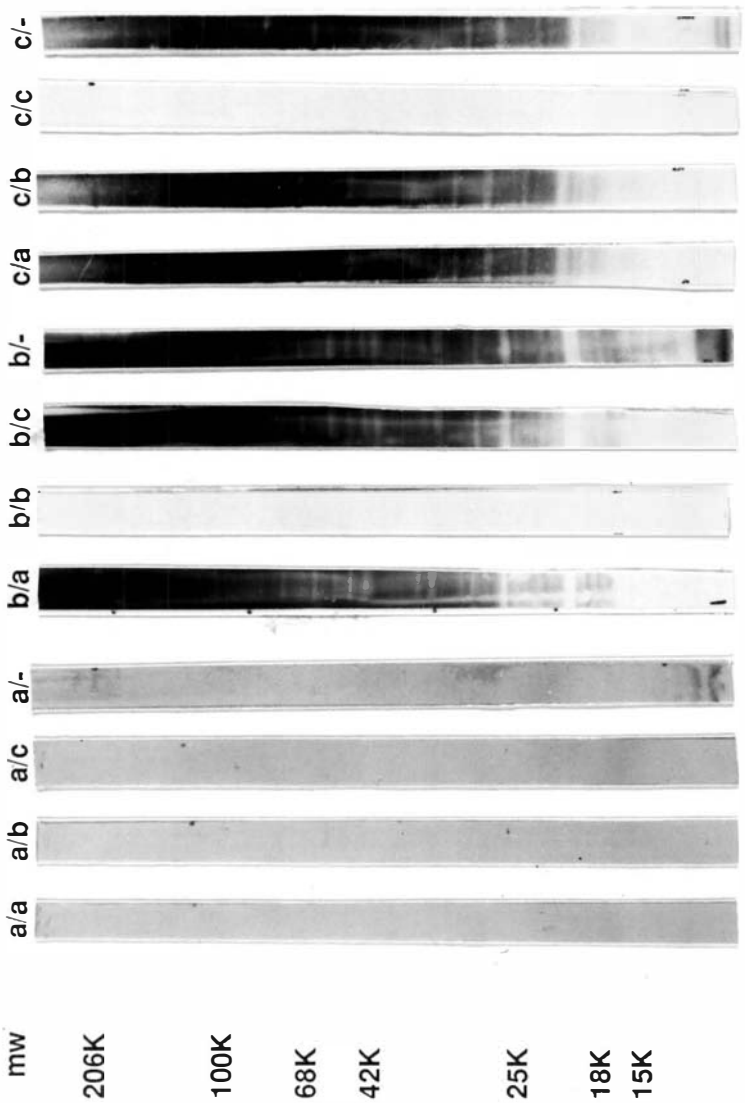
To determine which of these high responding patients were responding specifically to their respective Aa antigens, competition analysis was performed using all 3 Aa serotypes. The specificity of an individual patient's response is illustrated in Figure 11. Heavily loaded western blots of whole cell sonicate were used to allow all immunodominant antigens to be

TABLE 2. The immunodominant antigens of serologically specific early onset periodontitis patients for *Actinobacillus actinomycetemcomitans* serotype-b (AaY4)

Subject	Race	Titer(RIAunits)	Specific Response	Dominant antigen(s)[a]
1	W	940	-	nd
2	W	955	-	nd
3	W	1,400	-	nd
4	B	1,400	-	nd
5	B	1,800	-	nd
6	B	3,500	-	nd
7	B	6,000	+	SMEAR, 15Kd
8	W	10,500	-	nd
9	B	13,500	-	nd
10	B	18,700	+	72Kd
11	W	32,000	-	nd
12	B	42,600	+	SMEAR
13	W	64,000	+	SMEAR
14	B	64,000	+	SMEAR
15	B	122,300	+	SMEAR
16	B	128,000	+	SMEAR
17	B	149,500	+	SMEAR
18	W	192,000	+	SMEAR
19	W	196,300	+	SMEAR
20	B	198,400	+	SMEAR
21	B	230,400	+	SMEAR
22	B	230,400	+	SMEAR
23	B	243,200	+	SMEAR
24	B	254,700	+	SMEAR
25	B	254,700	+	SMEAR
26	W	402,900	+	SMEAR
27	W	506,800	+	SMEAR
28	B	732,200	+	SMEAR
29	W	793,600	+	SMEAR

a- Bands appearing on western blot when stained with decreasing amounts of serum (1/250 ml- 1/1024,000ml) in4 ml of diluent

FIG. 11. Competition assay using the western blot technique to determine the specificity of an individual patient's antibody response. Notice that preincubation of serum with antigen from any of the three serotypes removed antibody molecules reactive with that antigen on the blot (lanes a/a, b/b, c/c). In contrast, in the control when there was no preabsorbtion of sera, there was reactivity in each lane (lanes a/-, b/-, c/-). When antigen preparations from the remaining two serotypes were used to preabsorb the sera, the results indicated a specific response for serotypes -b and -c but not for -a. For serotypes -b and -c preincubation with either of the remaining serotype antigens resulted in elimination of reactivity for Aa common antigens but allowed staining of serotype specific antigens (lanes b/a, b/c, c/a, c/b). For serotype-a, preincubation of sera with antigen of any of the three serotypes completely eliminated reactivity with antigens present on the western blot (lanes a/a, a/b, a/c). These results indicated that this patient responded specifically to serotypes -b and -c but not to serotype-a. a/a: blot of serotype-a stained with serum preincubated with serotype-a antigen, a/b: blot of serotype-a stained with serum preincubated with serotype-b antigen, a/c: blot of serotype-a stained with serum preincubated with serotype-c antigen, a/-: blot of serotype-a stained with untreated serum, b/a: blot of serotype-b stained with serum preincubated with serotype-a antigen, b/b: blot of serotype-b stained with serum preincubated with serotype-b antigen, b/c: blot of serotype-b stained with serum preincubated with serotype-c antigen, b/-: blot of serotype-b stained with untreated serum, c/a: blot of serotype-c stained with serum preincubated with serotype-a antigen, c/b: blot of serotype-c stained with serum preincubated with serotype-b antigen, c/c: blot of serotype-c stained with serum preincubated with serotype-c antigen, c/-: blot of serotype-c stained with untreated serum.


mw
206K
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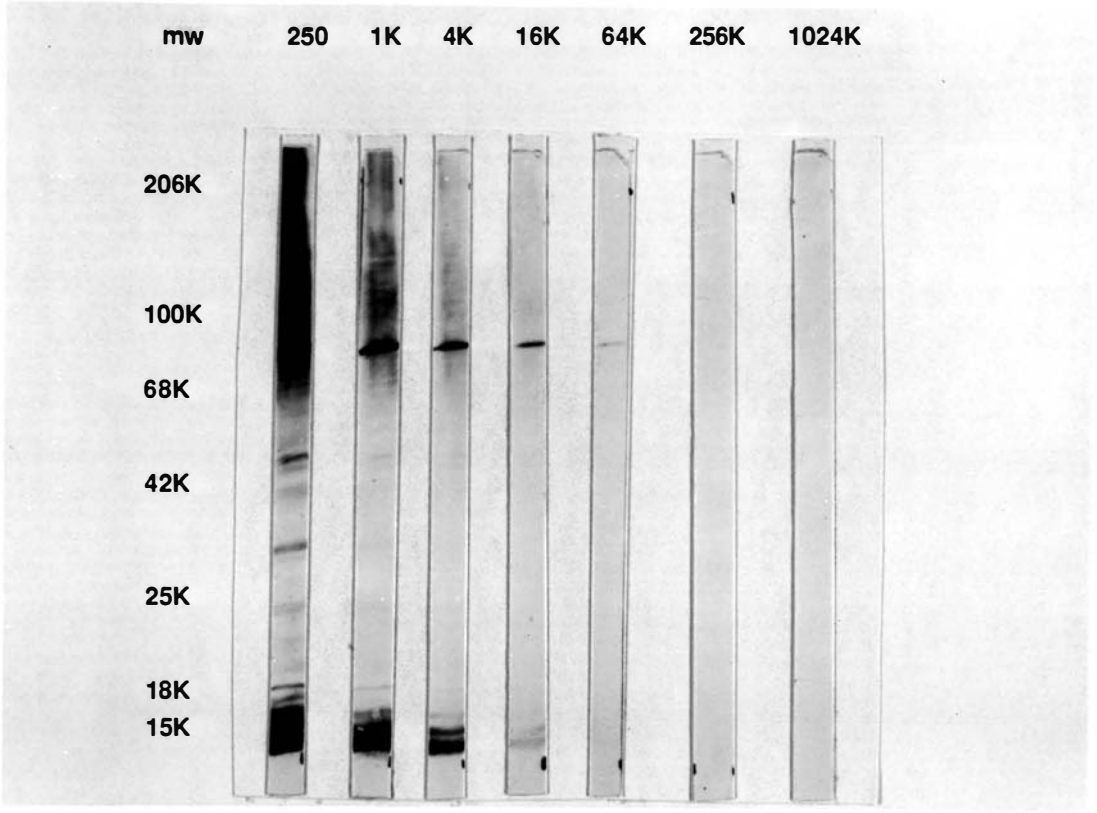
18K
15K
a/a
a/b
a/c
a/-
b/a
b/b
b/c
b/-
c/a
c/b
c/c
c/-

represented in ample quantity. Four replicate nitrocellulose lanes (a set of four for each serotype) were stained using patient serum that was either untreated or preincubated with serotype-a,-b, or-c antigen. For serotype-a, preincubation with antigen of any one of the three serotypes removed antibody molecules reactive with serotype-a antigen present on the blot (lanes a/a, a/b, a/c). Lane a/- was a control with no preabsorbtion of serum. The data indicated that this patient did not respond to serotype-a specifically because antigens from serotypes-b or-c could inhibit staining of a blot of serotype-a. Therefore, I concluded the response to serotype-a was only to cross-reactive antigens common between the three serotypes. For serotypes-b and-c, preincubation with serotype-b or-c antigen respectively (lanes b/b and c/c) removed all reactivity from the patient's serum. However, preabsorbtion with either of the other two remaining serotypes antigen (lanes b/a, b/c, c/a, c/b) eliminated only a small portion of the reactivity present in the patient's serum. Lanes b/- and c/- are controls with no preabsorbtion of serum. This indicated that the serum of this patient contained reactivity for serotype specific antigens of serotypes-b and-c. This patient therefore responded specifically to serotypes-b and-c but not specifically to serotype-a. This same analysis was used for all of the serotype-a and-c high responders to determine the specificity of each individual's response. Only about half of high responders were responding specifically to their respective antigen (9 of 19 for a and 12 of 21 for c). It was common to find individuals that responded specifically to only one serotype. In some cases, however, individuals responded specifically to more than one serotype as was shown in Fig. 11. For serotype-a, 5 were black and 4 were white. For serotype-c 9 were black and 3 were white. All but 2 of these subjects had early onset disease with 6JP, 3SP, 0NP for serotype-a and 5JP, 5SP, 2NP for serotype-c.

Those individuals that did not respond specifically to the appropriate antigen (a or c) often specifically responded to serotype-b. Only the patients responding specifically to the appropriate Aa serotype were used in the studies to determine the dominant antigens.

To establish the immunodominant antigens for serotypes-a and-c, I used limiting dilution analysis on western blots. Replicate lanes were stained with progressively less serum (from 1/250 to 1/1024,000 of a ml). With increasing dilution, most of the antigen bands disappeared leaving only the immunodominant antigen(s) apparent at the highest dilution of serum (i.e. endpoint). An example of this analysis is shown in Fig. 12. At a low serum dilution many discrete bands were present and a diffuse or smeared high molecular weight antigen was also present (lane 1/250). When the serum was further diluted to 1/1000 {1/1000th (10^{-3}) of a ml of serum}, many of the discrete bands disappeared. Therefore, the antibody titer reactive with these antigens was low. In this case, the smeared diffuse antigen was not the immunodominant antigen as the titer for this antigen was low. It was also of interest to note that some of the antigens that displayed intense staining at the lowest dilution (ex. the band at approximately 50Kd) disappeared at a serum dilution of 1/1000. The intensity of staining of individual bands at a low dilution of serum did not always correlate with the observed antibody titer for a given antigen when the antibody was diluted out further. For this patient, a serum content of 1/64K (1/64,000th of a ml of serum) resulted in a dominant antigen that was a discrete band having a molecular weight of 90kd. Although not apparent in the photograph, the 90Kd antigen was visible at a serum dilution of 1/256K (1/256,000th of a ml of serum). Similar analysis was completed for all the high responder patients. For some patients a single antigen was stained at the endpoint. For most two or three antigens

FIG. 12. A typical western blot of Aa serotype-a illustrating the immunodominant antigen for a particular patient. Replicate lanes of Aa serotype-a were run on polyacrylamide gel and transferred to nitrocellulose. Decreasing amounts of a patient's serum (1/250 ml-1/1024K ml) were used in the incubation mixture for each lane. Note that antigens that have elicited low titer antibodies are apparent only at low dilutions of serum while the 90Kd immunodominant antigen is still visible at a serum dilution of 1/64K (1/64,000 of a ml of serum). In the original western blot, the immunodominant antigen was also visible at 1/256K).


mw
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16K
64K
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206K
100K
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25K
18K
15K

remained at the endpoint with no individual having more than four. The data indicated that for serotype-a there were several different immunodominant antigens for different patients but none was present in more than 44.4% of the serotype-a high responders (table3). For serotype-c the immunodominant antigens included a number of discrete bands and a diffuse band typical of LPS or capsular polysaccharide (table4). Only two of these antigens were present in the majority of serotype-c high responders: 92% (11/12) had the diffuse or smeared antigen and 67% (8/12) had an approximately 15Kd antigen.

To determine if the 15Kd band was a protein antigen, two replicate lanes of a western blot of Aa serotype-c were stained using serum from a patient known to be responsive to this antigen. One of the lanes was left untreated while the other was exposed to protease K digestion. As shown in figure 13, protease K digestion eliminated the discrete low molecular weight bands in the 15Kd region of the blot. The enzyme did not appear to affect the smeared antigen. This result indicated that the 15Kd immunodominant band was a protein antigen. Competition assays revealed that the 15Kd antigen was a common antigen between the three serotypes while the smeared antigen was serotype specific. In Fig. 14, competition assays for serotype-c are shown for two patients: one that responded specifically to Aa serotype-c and one that did not. For the specific responder untreated serum stained both the smeared antigen as well as the 15Kd antigen (lane c/- patient A). The 15Kd antigen was eliminated from the blot by incubating the serum with antigen from any of the three serotypes (lanes c/a, c/b, c/c patient A). This indicated that this antigen was not serotype specific. In contrast, the smeared antigen was only eliminated when serum was incubated with serotype-c antigen (lane

TABLE 3. Prevalence of individual bands on western blot for *Actinobacillus actinomycetemcomitans* serotype-a

Number of individuals responding	(%)[b]	Band[a]
4	44.4%	smear
2	22.2%	83Kd
2	22.2%	15Kd
2	22.2%	14Kd
1	11.1%	103Kd
1	11.1%	90Kd
1	11.1%	57Kd
1	11.1%	28Kd

a- Bands appearing on western blot when stained with decreasing amounts of serum (1/250 ml- 1/1024,000ml)in 4 ml of diluent
b- 6 patients responded to a single antigen at endpoint, 3 responded to three antigens at endpoint

TABLE 4. Prevalence of individual bands on western blot for *Actinobacillus actinomycetemcomitans* serotype-c

Number of individuals responding (%)[b]		Band[a]
11	91.6%	smear
8	66.6%	15Kd
4	33.3%	14Kd
3	25.0%	57Kd
2	16.6%	170Kd
2	16.6%	160Kd
2	16.6%	78Kd

a- Bands appearing on western blot when stained with decreasing amounts of serum (1/250 ml- 1/1024,000ml) in 4 ml of diluent

b- 5 patients responded to two antigens at endpoint,5 patients responded to three antigens at endpoint,1 patient responded to four antigens at endpoint.

FIG. 13. A western blot using a low dilution (1/250 ml) of patient serum showing the effect of protease K digestion on the antigens of Aa serotype-c. Note that the 15Kd band was eliminated from the western blot by the protease while this treatment had no affect on the smeared antigen. Aa-c: western blot of untreated antigen, Aa-c protease: western blot of Aa-c that was digested with protease K before staining.

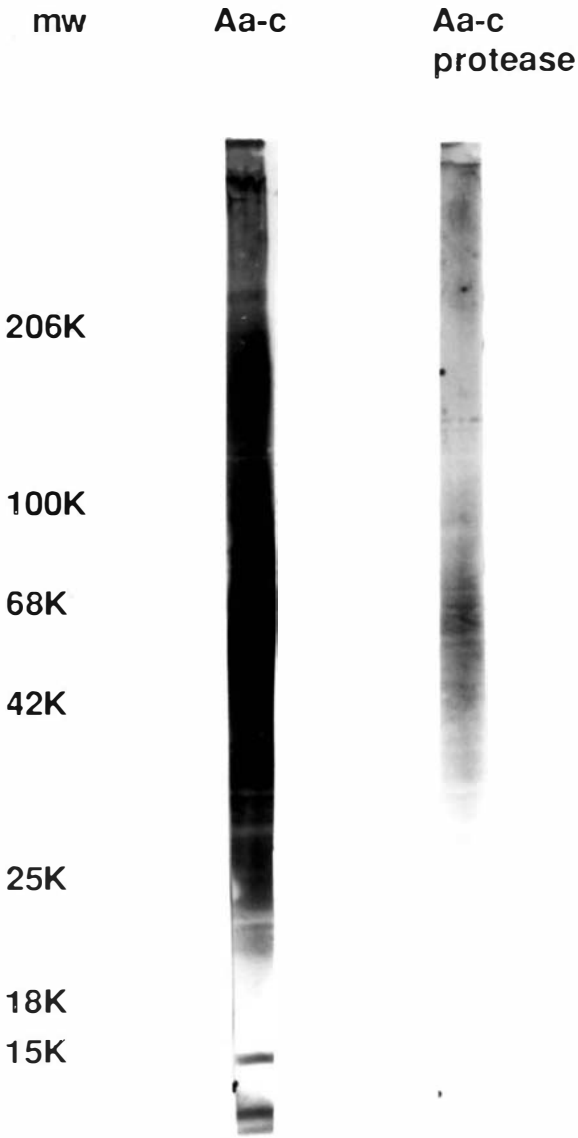
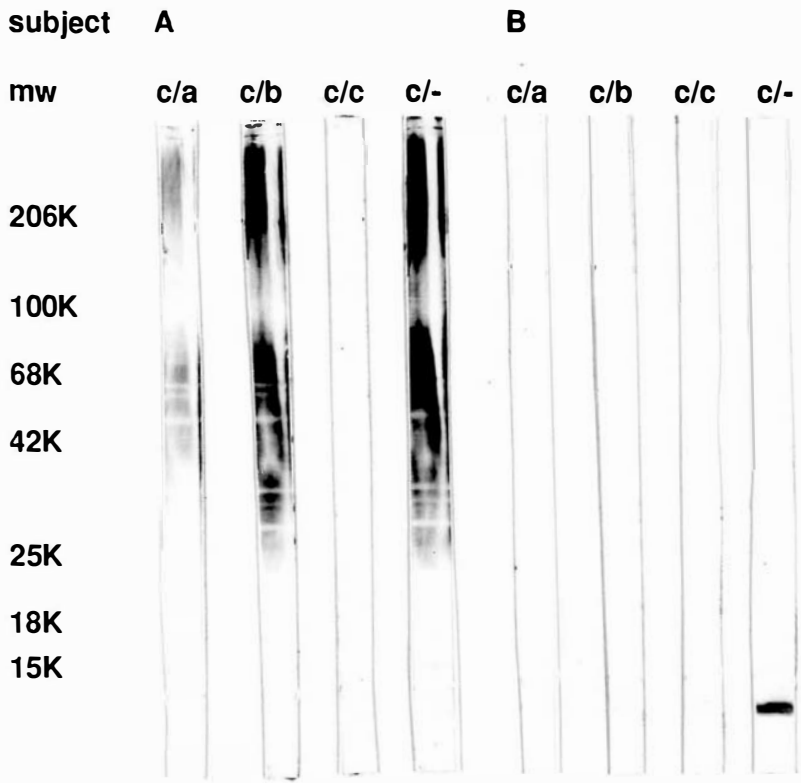

mw
Aa-c
Aa-c protease
206K
100K
68K
42K
25K
18K
15K

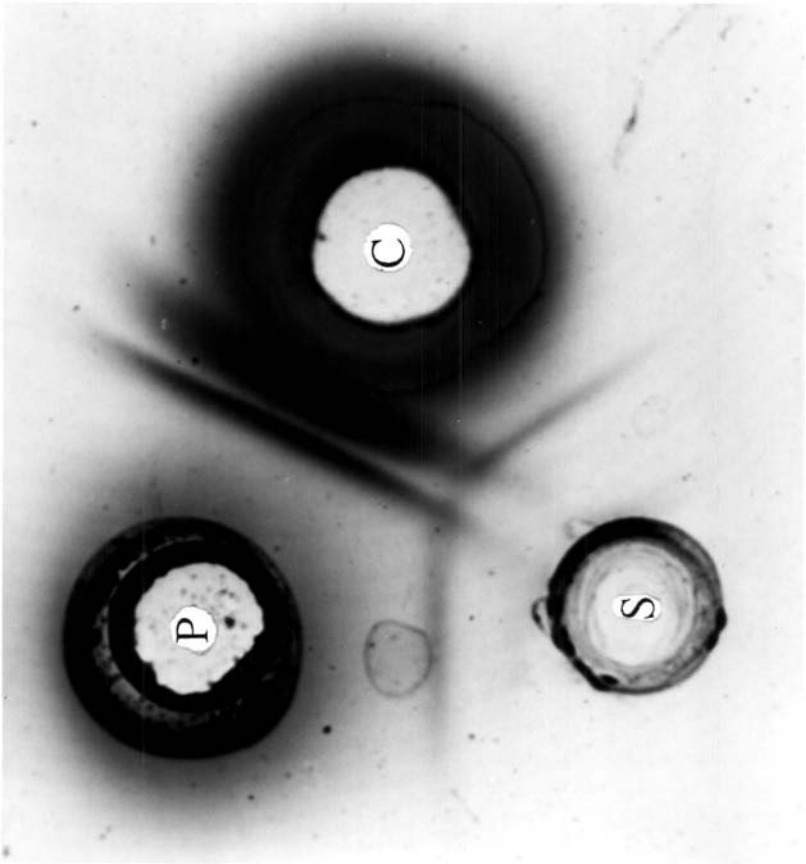
FIG. 14. Competition assay using the western blot technique showing that the 15Kd antigen is an Aa common antigen and the smeared antigen is a serotype specific antigen. Patient A: a specific responder to serotype-c, Patient B: a patient that responded nonspecifically to serotype-c, c/a: blot of serotype-c stained with serum preincubated with serotype-a antigen, c/b: blot of serotype-c stained with serum preincubated with serotype-b antigen, c/c: blot of serotype-c stained with serum preincubated with serotype-c antigen, c/-: blot of serotype-c stained with untreated serum.


subject
A
B
mw
c/a
c/b
c/c
c/-
c/a
c/b
c/c
c/-
206K
100K
68K
42K
25K
18K
15K

c/c patient A) and not with the other two antigens (lane c/a, c,b patient A). Serum from the patient with the nonspecific response lacked reactivity with the smeared antigen but did recognize the 15Kd antigen (lane c/- patient B). The 15Kd antigen was again eliminated with antigen from any of the three serotypes (lanes c/a, c/b, c/c patient B) confirming the lack of serotype specificity.

The appearance of the smeared immunodominant antigen on western blot and its resistance to protease was consistent with either LPS or a carbohydrate antigen. To further characterize the antigen, double immunodiffusion analysis was performed. This analysis utilized serotype-c specific rabbit antisera which has been shown to have reactivity with mannan from serotype-c and no reactivity with LPS (31). Antigen from serotype-c was treated with protease K to remove protein antigens and then added to the center well. Serum from a patient known to be positive for the smeared antigen was placed in one peripheral well and the serotype specific rabbit antisera was placed in an adjacent peripheral well. This analysis resulted in a line of identity indicating that the non-protein antigen recognized by the rabbit antiserum was the same antigen as the immunodominant antigen recognized by the patient's serum (Fig. 15). This result was found for 9 of 11 of the serotype-c specific high responders that had the smeared antigen as a dominant antigen. The remaining 2 subjects showed a reaction of non-identity with the serotype specific antigen recognized by the rabbit antiserum. The immunodominant antigen in man is apparently the mannan described by Zambon (31). When antigen not subjected to proteolysis was used other precipitin lines were observed that were eliminated by the enzyme digestion indicating that the proteolytic digestion was effective.

FIG. 15. Double-immunodiffusion analysis (Ouchterlony technique) showing that rabbit serotype-c specific antiserum and patient serum recognize the same antigen in serotype-c sonicate that has been treated with protease K. Peripheral wells P and C contained patient serum and rabbit serotype-c specific antiserum respectively (20ul of undiluted serum for each). The center well S contained 5ul of serotype-c sonicate (originally having a protein concentration of 7ug/ml) that was treated with protease K. Note precipitin lines between P and C. These are absorbtion lines which derive from the fact that the serotype specific rabbit antiserum was absorbed with antigen preparations from the other two serotypes. Therefore, the rabbit antiserum contains antigens form serotypes -a and -b. Several different concentrations of antigen and sera were used and only one precipitin line was found in any case.


C
P
S

DISCUSSION

Analyses of the antibody reactive with AaY4 sonicate by RIA and western blot analysis strongly suggest that the immunodominant antigen for AaY4 is a carbohydrate. The immunodominant antigen is 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 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, resistant to proteolytic digestion, purified with carbohydrate, and appeared like carbohydrate on western blot (i.e., carbohydrate does not focus into a discrete band on western blot). 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 AaY4 may represent capsular carbohydrate as it is a surface antigen readily detectable on whole cells using immunofluorescence (30). A lipid A binding affinity column which bound AaY4LPS failed to retain the "smear antigen" found on western blots of AaY4 carbohydrate extract. This further supports the idea that the immunodominant antigen may be a capsular polysaccharide.

The next set of experiments examined the immunodominant antigen in AaY4 for antibody positive subjects that had a range of titers. This would determine if an immunodominant response to the serotype specific carbohydrate antigen was restricted to the high responding patients. To

determine if the immunodominant antigen for antibody positive subjects with a range of antibody titers would be the same immunodominant antigen found in the high responding subjects, patients were selected to include subjects of both races with a range of antibody titers to AaY4. The results showed that the immunodominant antigen of AaY4 in antibody positive, serotype specific, early onset periodontitis patients was the serotype-b antigen as was previously found in earlier work limited to high responding patients. This was the case for the entire range of antibody specific responses although most of the very low titers were not AaY4 specific. The competition (inhibition) assay showed that these low responding subjects generally had a specific response to serotype-a or-c. Thus, the assay probably measured low titer antibody directed towards cross reactive antigens when AaY4 was used as the antigen for RIAs and western blots.

The relationship between antibody response to the "smeared" antigen and race was also examined as initial work showed that most high responders to the smeared antigen were black. The second set of experiments indicate that both black and white subjects with specific antibody responses to AaY4 were responding to the serotype specific antigen. While this seems to suggest the absence of a race affect on the antibody response, it should be understood that only those white subjects that had a specific response to AaY4 were selected for the study. Gunsolley *et al* have shown that the majority of white subjects were not seropositive for AaY4, while the vast majority of black subjects were seropositive (in press Gunsolley *et al*, 7). It was also noted that white subjects had lower antibody titers

than black subjects. Either white subjects do not respond to AaY4 as frequently or with as high an antibody titer as black subjects, or they are not as frequently infected with Aa as blacks.

A similar race effect has been shown for the antibody response to carbohydrate antigens of *Haemophilus influenzae,* which has been attributed to an immune response gene associated with Km1 immunoglobulin light chain allotype (5). It has also been suggested that major histocompatibility complex (specifically HLA-DQ molecules) may be responsible for the ability to respond to microbial mannans found in Aa (32).

Examination of the data revealed that, with two exceptions, AaY4 specific sera had an antibody titer of 43,000 units of antibody or greater. I examined the dominant antigens of the non-specific group and found that 4 out of 9 had a response to a smeared antigen. The response to the smeared antigen in these subjects was not serotype specific indicating that having antibody reactive with a smear is not sufficient to verify a response to the serotype specific antigen.

Based on these data, it is suggested that any study of antibody responses to antigens of Aa should include assays of specificity to try to assure that the patients have been exposed to the antigen of interest. Subjects with moderate to low antibody responses would include many individuals responding non-specifically to the serotype of interest. The result would be highly variable data with apparent immunodominant responses to different cross reactive antigens.

Analysis of antibody reactive with *A. actinomycetemcomitans* serotype-a by limiting dilution analysis of western blots indicated that the immunodominant antigen for serotype-a was highly variable from patient to

patient. In contrast, most patients with high antibody titers to serotype-c had a 15Kd protein antigen and/or a diffuse band as their dominant antigens. Results of double immunodiffusion assays indicated that the smeared antigen is the serotype-c specific antigen. This antigen has been shown to be mannan and not LPS and appears to be part of the bacterial capsule (31). I particularly wanted to focus attention on dominant antigens that elicited a response in a majority of the high responder patients as it is the response to antigens that might provide information of prognostic value for the patient and clinician. These dominant antigens might also make good candidates for vaccines that could elicit protective antibody responses. For serotype-a, no particular dominant antigen was detectable in the majority of patients. Several dominant antigens were detected for serotype-c but only two were found in the majority of the serotype-c high responders. The 15Kd antigen was sensitive to protease K digestion and therefore appeared to be a protein. This antigen also appeared to be common to all three serotypes and was often present in western blots. This band could be eliminated in competition assays where serum was incubated with any of the three Aa serotype sonicates. This was not the case for the smeared serotype-c antigen. This antigen, which was recognized by the majority of serotype-c high responders, gave a reaction of identity with the serotype-c specific rabbit antisera in double immunodiffusion assays, indicating that this antigen was serotype-c specific. Furthermore, antigen could only be eliminated from western blots when serum was incubated with serotype-c sonicate but not-a or-b.

When these results are compared with previous data from serotype-b it is apparent that the dominant antigen for serotype-b is similar to the serotype-c smeared antigen. Ouchterlony tests indicate that these antigens are the serotype specific carbohydrate and may represent capsular

polysaccharides. LPS has not been ruled out as the dominant antigen for serotype-b. However, analysis of sugar residues from the serotype specific carbohydrate by Zambon *et al* suggest that this antigen is not LPS (32). For the 2 subjects (2 of 12 serotype-c specific high responders) having a reaction of non-identity with the serotype-c specific rabbit antiserum, LPS remains a possibility.

The serotype specific carbohydrate, which is known to be associated with the cell surface of Aa and may represent capsule, could be a virulence factor for Aa as it is for several other organisms. Purified serotype specific carbohydrate from Aa serotype-c is the serotype specific antigen and has been identified as mannan (30,31). Capsular polysaccharide is known to inhibit phagocytosis of many bacteria (e.g., *S. pneumoniae*). In part, this appears to be the result of inhibition of the alternative complement pathway and decreased opsonization (10,12). Mannans isolated from *S. cerevisiae* have been shown to inhibit the respiratory burst and release of myeloperoxidase in phagocytes (29). Therefore, the serotype specific carbohydrate may be a virulence factor for Aa.

Pneumococcal capsular polysaccharide has been shown to persist for long periods in mice; such polysaccharides have a half-life of up to 50 days (22). If the AaY4 carbohydrate is as resistant to degradation *in vivo* (by e.g., bacterial and PMN proteases) as it is *in vitro,* it could also persist for long periods of time within the periodontal tissues. Bacteria (14,21) and antigens of AaY4 (3,4) are observable within the periodontal tissues of juvenile periodontitis subjects. If the carbohydrate of AaY4 persists in the tissues, 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. For example, a T dependant (IgG) response is not typically seen with a carbohydrate antigens. 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, either 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 node. Future studies are planned to examine the behavior of the AaY4 carbohydrate antigen in different antigen presentation systems to determine the mechanism of production of the IgG response.

High antibody titers to AaY4 have been associated with localization and decreased severity of disease in juvenile periodontitis (6,20). Future studies will examine the relationship of antibody response to the immunodominant antigen as a function of disease severity and extent to see if 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, measuring antibody to the immunodominant serotype specific carbohydrate antigen may decrease false positive results in antibody data due to cross-reactive antigens shared by the three serotypes.

The immunodominant carbohydrate antigens for serotypes-b and-c appear to be potent antigens, at least for black subjects. The enormous antibody response (very high titer) observed for serotype-c and especially serotype-b may be due in part to the presence of highly immunogenic epitopes on these antigens or it may be due to long term persistence of these antigens in the periodontal tissues. Bacteria (14,21) and antigens (3,4) of Aa are observable

in the periodontal tissues. The serotype-c and-b specific carbohydrate antigens have been shown to be stable to protease and the serotype-b specific antigen will withstand boiling for 45 minutes. Therefore, both potent immunogenicity as well as stability may explain the extremely high antibody titers that have been observed.

I found that the immunodominant antigen was often not the most intensely staining antigen at low serum dilution. Some bands that stained quite intensely at low serum dilution did not titer out very far as blots were stained with increasing dilutions of serum. Therefore, I feel, it is important to dilute the serum with limiting dilution analysis as was done here. Staining intensity alone is not adequate to establish the immunodominance of particular antigens on western blots.

I my conclusion, the immunodominant antigen for Aa serotype-a is highly variable from patient to patient. Most patients with high antibody titers to serotype-c responded to a 15Kd protein antigen and/or a smeared antigen. The immunodominant antigen for AaY4 in both high responders and specific responders over a range of antibody titers was also a smeared antigen. This smeared antigen recognized by the patient's serum was also recognized by serotype specific rabbit antiserum. These data support the conclusion that the smeared immunodominant antigen was the serotype specific carbohydrate which appears to be part of the Aa capsule (30,31,32). The carbohydrate antigens therefore appear to be highly immunogenic and may be important antigens for Aa serotypes-b and-c. This potent immunogenicity may explain the enormous antibody titers that are found even when AaY4 is found in low numbers. The immune response to these antigens may relate to the severity and extent of disease. Future work will examine the relationship of these responses to clinical status.

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Literature Cited

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