Heterogeneity of Antibodies Reactive with the Dominant Antigen of *Actinobacillus actinomycetemcomitans*

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The serotype b-specific carbohydrate antigen (SbAg) of *Actinobacillus actinomycetemcomitans* Y4 is reported to be the O antigen of lipopolysaccharide, and the highest titers of serum antibody reactive with *A. actinomycetemcomitans* in early-onset periodontitis (EOP) patients bind SbAg. These high titers of serum antibody reactive with SbAg are associated with a lesser extent and severity of periodontal disease. The aim of this study was to determine if a limited number of genes code for anti-SbAg antibodies as has been shown for immunoglobulin G (IgG) reactive with the type b polysaccharide from *Haemophilus influenzae*. Serum IgG reactive with the SbAg was prepared from 20 high-titer EOP patients by affinity chromatography. The IgG subclass concentrations were determined, and heterogeneity was analyzed by isoelectric focusing (IEF). IgG2 was the dominant subclass (83% of total IgG) in the anti-SbAg IgG fraction and represented an average of 1.33% of total serum IgG2. The IgG2 reactive with SbAg was isolated from the affinity-purified IgG fraction by affinity chromatography with protein A and subclass-specific monoclonal antibodies. On IEF gels, only 4 to 20 bands were observed in the anti-SbAg IgG fractions, indicating limited heterogeneity. N-terminal amino acid sequence analysis of eight representative anti-SbAg IgG2 preparations indicated that variable heavy and light chains consisted largely of VHIII and V\(_{\text{II}}\), respectively. However, a significant fraction of anti-SbAg may use \(V_{\text{H}}\) and \(V_{\text{L}}\) genes with blocked N termini. In short, these findings indicate that IgG reactive with SbAg is very much like the antibody reactive with *H. influenzae* type b polysaccharide. Similarities include IgG2 dominance, limited bands on IEF gels, supporting an oligoclonal response, and use of genes from \(V_{\text{H}}\)III and \(V_{\text{L}}\)II regions.

*Actinobacillus actinomycetemcomitans* has been classified into five serotypes (a, b, c, d, and e) (20). Serotype b strains are recovered from patients with localized juvenile periodontitis (LJP) more frequently than are other serotypes, suggesting a high periodontopathic potential for serotype b strains (30). The serotype b-specific carbohydrate antigen (SbAg) is reported to be a polymer consisting of L-rhamnose and D-fucose, with a trace amount of fatty acid (3). Recent studies indicate that the SbAg is the O antigen of lipopolysaccharide (LPS) and consists of a repeating trisaccharide unit composed of L-rhamnose, D-fucose, and D-GalNAc residues (1:1:1) (16, 17, 29). In addition, it is known that the highest titers of antibody reactive with *A. actinomycetemcomitans* Y4 in patients with early-onset periodontitis (EOP) are reactive with the SbAg (5). Furthermore, it is known that the vast majority of anti-SbAg antibody in EOP patients is immunoglobulin G2 (IgG2) (14, 28). In previous work, we found that a high level of antibody reactive with *A. actinomycetemcomitans* is associated with lesser extent and severity of disease in EOP patients (7, 19), and in recent studies this apparent protection correlated with antibody reactive with the SbAg (4). These results suggest that a vaccine containing SbAg could protect against EOP and prompted this study to further characterize the antibody reactive with the SbAg.

*Haemophilus influenzae* causes bacterial meningitis and is a serious pathogen in infants and children. *H. influenzae* type b polysaccharide (Hib-PS), a linear polymer of ribose-(1-1)-ribitol-5-phosphate, is widely used clinically as a vaccine which protects against this disease. The human antibody response to Hib-PS is one of the best-characterized human antigen-specific antibody responses (22). Antibodies of the IgG2 subclass contribute substantially to the IgG antibody response of most adults to immunization with Hib-PS (24). Isoelectric focusing (IEF) profiles of anti-Hib-PS IgG antibodies are restricted and often shared by unrelated subjects (9). The anti-Hib-PS response appears to be oligoclonal, with frequent use of \(V_{\text{H}}\)III and \(V_{\text{L}}\)II genes (22).

The similarities between Hib-PS and SbAg prompted us to hypothesize that the immunoglobulin V gene usage for anti-SbAg IgG2 would be limited, as has recently been shown for Hib-PS (23). Twenty clonally purified anti-SbAg IgG2 fractions were prepared to analyze for N-terminal amino acid sequence. Our results confirm that the high levels of anti-SbAg IgG result mainly from the IgG2 subclass. The limited bands on IEF gels lend support to the hypothesis that the anti-SbAg response is oligoclonal like the Hib-PS response, and the high use of genes from \(V_{\text{H}}\)III and \(V_{\text{L}}\)II regions suggested that the number of genes coding for the anti-SbAg IgG2 is limited as it is for anti-Hib-PS.

**MATERIALS AND METHODS**

**Human subjects.** Twenty EOP patients who had elevated IgG titers to the serotype b-specific carbohydrate were selected from patients seen at the Clinical Research Center for Periodontal Diseases. Fourteen of the patients had LJP (such patients had to be less than 30 years of age and have a localized pattern of periodontal destruction limited to first molars and incisor teeth and up to two additional teeth), and the other six patients had the more generalized or severe form of EOP (these patients were less than 35 years of age and had 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). The majority of the subjects were black (18 of 20) and had LJP (14 of 20). Sera were obtained...
by venipuncture at the time of diagnosis and before any periodontal therapy. Sera were stored at −70°C until the day before the assay.

**Preparation of the SbAg.** *A. actinomycetemcomitans* ATCC 43518 (previously designated Y4; serotype b) was obtained from the American Type Culture Collection. The organism was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% yeast extract (Difco) at 37°C for 3 days in a 5% CO2 atmosphere. A phenol-water extract was prepared by lyophilized whole cells by the method of Westphal and Jann (27), followed by treatment with nuclease P1 (Worthington Biochemicals, Lakewood, N.J.). The phenol-water extract was further purified by gel filtration on Sephadex G-200 (Pharmacia Biotech, Piscataway, N.J.) in the presence of deoxycholate (15). The high- and the low-molecular-weight carbohydrate peaks were eluted as described previously (12). The high-molecular-weight carbohydrate peak was then applied to a column (1.5 by 20 cm) containing polymyxin B beads (Affi-Prep polymyxin matrix; Bio-Rad Laboratories, Hercules, Calif.), which removes residual low-molecular-weight lipids but not the O antigen of LPS (10, 25). Pass-through fractions were collected, lyophilized, and used as SbAg.

A portion of SbAg was coupled to α-aminohexyl agarose (A6017; Sigma Chemical Co., St. Louis, Mo.) with cyanogen bromide. It was confirmed by monitoring for carbohydrate that over 93% of SbAg was coupled to the agarose. Unoccupied active sites of the agarose were blocked by incubation with 1 M monothanolamine for 2 h at room temperature. The SbAg coupled to the agarose was used in the isolation of anti-SbAg antibodies.

**Isolation of anti-SbAg antibodies.** A crude IgG fraction was prepared from 5 ml of the patient’s serum by ammonium sulfate precipitation. The crude IgG fraction was applied to a small column (Poly-Prep chromatography column; Bio-Rad) containing polymyxin B agarose. The mixture was incubated for 2 h at room temperature and then washed thoroughly with phosphate-buffered saline (PBS) and PBS buffer containing 1 M NaCl. After another wash with PBS, an antibody fraction was eluted with 8 ml of 3.5 M MgCl2 (26). The fraction was immediately dialyzed against PBS and concentrated by ultrafiltration with a Centricon-30 concentrator (30,000-molecular-weight cutoff; Amicon Inc., Beverly, Mass.) and used as the anti-SbAg IgG. The anti-SbAg IgG was further purified by using a protein A column (ImmuPure Plus immobilized protein A; Pierce Chemical Co., Rockford, Ill.), followed by affinity chromatography with an exclusion monoclonal antibody (mouse anti-human IgG1, IgG3, and IgG4 monoclonal antibody 3H9 [Calbiochem Co., La Jolla, Calif.]) coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech). The obtained fraction was used as the anti-SbAg IgG2.

**IEF.** An IgG gel (0.4 mm thick, pH 3 to 10) containing 5% polyacrylamide was prepared on a thin film (gel support film for polyacrylamide; Bio-Rad). An IEF gel (0.4 mm thick, pH 3 to 10) containing 5% polyacrylamide was prepared on a thin film (gel support film for polyacrylamide; Bio-Rad). The anti-SbAg IgG2 was applied to the surface of the gel and allowed to diffuse for 15 min. The gel was turned upside down and placed on model 111 mini-IEF cell (Bio-Rad). Focusing was carried out in a stepwise fashion (100 V for 15 min, 200 V for 15 min, and 450 V for 60 min) as described in the manual supplied with the instrument. After electrofocusing was completed, the gel was removed and stained with Coomassie brilliant blue R-250.

**Serum IgG2 avidity to SbAg.** Serum IgG2 avidity to SbAg was determined by measuring the degree of ammonium thiocyanate-induced dissociation of antibody-antigen binding by the method of Pullen et al. (18). Microtiter plates (Immulon 4; Dynatech, McLean, Va.) were coated with SbAg suspended in carbonate buffer (1 mg/ml) overnight at 4°C. After blocking unoccupied sites with PBS containing 1% dry milk, a 1:1 dilution of serum, which had been determined from a linear portion of each serum dilution curve in preliminary experiments, was added to all wells of microtiter plates, and plates were incubated for 2 h. Graded concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mg/ml) of ammonium thiocyanate were added to different wells of each plate, and the plates were incubated for 1 h. The wells containing PBS without thiocyanate were used as controls. A 1:1,000 dilution of mouse anti-human IgG2 monoclonal antibody (HP6062 for kappa chain and HP6065 for lambda chain; Calbiochem) monoclonal antibodies and a known standard serum (a control human serum included in the human kappa total and lambda total RID kit; The Binding Site) were used.

**RESULTS**

**IEF profile of the anti-SbAg IgG.** The complexity of IEF profiles is an indicator of antibody heterogeneity. For example, purified human IgG1, -2, -3, and -4 myeloma proteins (Calbiochem) gave four to six bands on IEF gels with pl values between 7.6 and 8.0 for IgG1, 7.8 and 8.2 for IgG2, 6.9 and 7.2 for IgG3, and 7.3 and 7.7 for IgG4 (Fig. 1). We also examined the mouse anti-human IgG subclass monoclonal antibody used in ELISA and saw similar banding and limited complexity (data not shown). We reasoned that if antibody reactive with SbAg is monoclonal or oligoclonal, like that for anti-Hib-PS, then the antibody should be resolvable into discrete bands as it is for anti-Hib-PS (9, 23). The analysis revealed 10 to 20 bands in most patients (patients 1, 2, 4, 7, 8, 12, 14, and 19), but simpler patterns with only three to five bands were also seen (patient 19) (Fig. 1). The pI values of these anti-SbAg IgGs ranged from 6.7 to 8.2, and some patients appeared to have bands in common (for example, bands at around pH 7.1 to 7.2 for}
patients 8, 12, and 14). IEF gels from 17 patients were examined, and distinct banding patterns were consistently apparent, although three of the patterns were weak and difficult to photograph. The eight patients represented in Fig. 1 were typical of the group and were used to study the N-terminal amino acid sequences (see below). We also found very similar avidities for 19 of the 20 samples, between 2.2 and 3.5 M; the single outlier was at 1.51 M.

Concentrations of IgG subclasses and kappa and lambda chains. To help define the nature of the preferential use of IgG2 heavy chains and to determine if there is a preferential use of light chains, the anti-SbAg antibodies were analyzed (Table 1). In sera of these patients, IgG1 was the highest in concentration of the IgG subclasses (91.64 mg/ml), IgG2 (6.91 mg/ml), IgG3 (1.12 mg/ml), and IgG4 (0.32 mg/ml). In contrast, IgG4 was at 1.51 M.

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Serum (mg/ml)</th>
<th>Anti-SbAg IgG (µg/ml)</th>
<th>Anti-SbAg IgG2 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>11.53 ± 1.19</td>
<td>10.16 ± 2.03</td>
<td>ND</td>
</tr>
<tr>
<td>IgG2</td>
<td>6.91 ± 0.64</td>
<td>91.64 ± 20.03</td>
<td>ND</td>
</tr>
<tr>
<td>IgG3</td>
<td>1.12 ± 0.13</td>
<td>1.74 ± 0.34</td>
<td>ND</td>
</tr>
<tr>
<td>IgG4</td>
<td>0.32 ± 0.06</td>
<td>0.58 ± 0.11</td>
<td>ND</td>
</tr>
<tr>
<td>Kappa</td>
<td>ND</td>
<td>ND</td>
<td>26.78 ± 3.77</td>
</tr>
<tr>
<td>Lambda</td>
<td>ND</td>
<td>19.58 ± 3.90</td>
<td></td>
</tr>
</tbody>
</table>

*All values are given as means ± standard errors (n = 20). Concentrations are expressed as amounts prepared from 1 ml of serum. ND, not determined.

†IgG2 in the anti-SbAg IgG is significantly higher in concentration than other subclasses (P < 0.01).

The results reported here lend support to the hypothesis that serum immunoglobulins reactive with the SbAg from *A. actinomycetemcomitans* Y4 are limited in heterogeneity like those reactive to the Hib-PS antigen from *H. influenzae*. The anti-SbAg IgG fractions obtained by affinity chromatography showed a limited number of bands on IEF gels (4 to 20 bands). These results indicate that the antibody responses to SbAg were oligoclonal and may even represent monoclonal responses in some patients, since a single clonal antibody usually exhibits three to five spectrotypic bands, as indicated in Fig. 1. Amino acid sequencing revealed a frequent use of genes from VHIII and Vll regions, suggesting that the number of variable-region genes coding for the anti-SbAg IgG2 is limited as it is for anti-Hib-PS (23). Furthermore, the heavy-chain gene used overwhelmingly codes for IgG2. Wilson and Hamilton first determined the subclass distribution of IgG antibody reactive with the SbAg and reported that high-titer LJP sera contained 7.78 µg of IgG1 and 136.54 µg of IgG2 per ml (28). We found a similar distribution (8.8 µg/ml for IgG1 and 65.7 µg/ml for IgG2) (14), and others also found that IgG2 accounted for the major quantitative response to the carbohydrate in both patients and control subjects (13). In the present study, the anti-SbAg IgG fraction purified from each patient’s samples (patients 1, 4, 8, and 19). Only primary residues were detected at most positions, although secondary residues were also detected in two samples (positions 3 and 9 of patient 2 and position 1 of patient 14). The primary residues of the four samples were similar to those of VHIII. Interestingly, the primary residues at position 10 were aspartate for two samples (patients 2 and 12) and alanine for one sample (patient 7), instead of glycine of VHIII.

Nine to twenty-one residues of the kappa light chains were determined for all eight of the samples tested (Table 3). The primary residues of the eight samples were consistent with the Vkl subgroup, and the sequence matched the sequence for the A3/A19 genes (21), suggesting that anti-SbAg likely uses the A3/A19 gene products. However, secondary residues were detected at several positions in patients 2, 7, and 8. Glutamate at position 1, which is the invariant residue of VHIII, was observed in patients 7 and 8. Glutamine at position 3, which is the invariant residue of Vkl, was observed in patients 2, 7, and 8. Alanine and threonine were observed at position 10 in patients 7, 8, 12, and 19. Alanine was observed at position 10 as a primary residue in patients 7 and 19, and the alanine was confirmed as a primary residue in a repeat of the sequence analysis.

### DISCUSSION

The N-terminal sequences of heavy and light chains in the anti-SbAg IgG2. The IEF profiles were resolvable into distinct bands similar to those seen with anti-Hib-PS, and we reasoned that the gene usage might be limited in anti-SbAg antibodies as it is for anti-Hib-PS. Fifteen to eighteen residues of heavy chains could be determined for four samples (patients 2, 7, 12, and 14) (Table 2). No sequence was obtained for the other four samples (patients 1, 4, 8, and 19). Only primary residues were detected at most positions, although secondary residues were also detected in two samples (positions 3 and 9 of patient 2 and position 1 of patient 14). The primary residues of the four samples were similar to those of VHIII. Interestingly, the primary residues at position 10 were aspartate for two samples (patients 2 and 12) and alanine for one sample (patient 7), instead of glycine of VHIII.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Position of amino acids relative to VHIII sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E V Q L V S G G L V Q P G G S L R L</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>V</td>
</tr>
<tr>
<td>12</td>
<td>A</td>
</tr>
<tr>
<td>14</td>
<td>D</td>
</tr>
<tr>
<td>T</td>
<td>V</td>
</tr>
</tbody>
</table>

*Boldface letters in the VhIII sequence (11) indicate invariant residues. A dash indicates the same residue as that of VhIII; an X indicates no detectable residue at the position. Primary (above) and secondary (below) residues were detected in some patients. No sequence was obtained for patients 1, 4, 8, and 19. All sequences were similar to that of VhIII.*
serum was nine times higher in mean IgG2 than the next-highest subclass (91.64 µg/ml, versus 10.16 µg/ml for IgG1). The anti-SbAg IgG2 represented 1.33% of the total serum IgG2, and this was also about 10 times higher for the other subclasses. This high usage of the constant-region gene coding for IgG2 has been observed with other bacterial polysaccharide antigens like Hib-PS, but the IgG2 is even more dominant for SbAg than for Hib-PS, which elicited more IgG1 (24).

We isolated IgG2 from anti-SbAg IgG fractions and found that the lambda chain was used almost as frequently as the kappa chain in the anti-SbAg IgG2 fractions. Only kappa chain-like amino acid residues were detected by amino acid sequencing, and this is likely attributable to a technical difficulty in sequencing the lambda chain. A previous study indicated that the Edman method of N-terminal analysis failed to demonstrate any PTH derivative in most of the lambda chains, suggesting that these light chains had a blocked κ-amino group (8). The amino acid residue most commonly observed at position 1 is pyrrolidone carboxylic acid for VκI, VκII, and VκV (11). Taken together, the data indicate that lambda chains in the anti-SbAg IgG2 likely have a blocked N-terminal amino acid residue. Similarly, we were unable to get sequence data for a number of heavy chains, suggesting that many of our Vκ sequences were blocked (Table 2). The κ-amino groups of certain heavy chains are known to be blocked, and this may explain why we were unable to get sequences for some heavy chains in the anti-SbAg IgG2 fractions. Peptide fragments can be obtained from proteins with blocked N-terminal residues by digesting heat-denatured protein with an enzyme like pepsin. However, we could not follow these procedures in the present study because of the small amounts of the affinity-purified anti-SbAg IgG2 fraction available.

We found that VκIII and VκII are commonly expressed heavy and light chains among purified serum anti-SbAg IgG2 antibodies. The primary residues at position 10 of heavy chains were aspartate or alanine for three of four samples, instead of glycine of VκIII. Glycine, aspartate, and alanine are coded by GGX, GX, and GCX, respectively. Similarly, the primary residues at position 10 of light chains were threonine or alanine for four of eight samples, instead of serine of VκII. Serine, threonine, and alanine are encoded by TCX, ACX, and GCX, respectively. These changes of the amino acid residue may result from a point mutation of the first or second base of the codon. Glutamine at position 1 found in light chains of patients 7 and 8 may be due to VκIII instead of a point mutation, since leucines at position 4, glycines at position 9, and serines at positions 12 and 14 are the most commonly observed amino acid residues of VκIII. Glutamines at position 3 (patients 2, 7, and 8) also may be due to VκI. Scott et al. characterized human IgG antibodies to Hib-PS in a series of studies (reviewed in reference 23). They immunized 14 adult volunteers with Hib-PS vaccine and isolated anti-Hib-PS IgG antibody from each volunteer’s serum, showing that VκIII and VκII are the most commonly expressed heavy and light chains among clonally purified serum anti-Hib-PS antibodies. They also described that a number of these anti-Hib-PS antibodies show complete amino acid homology with the translated amino acid sequence of the germ line VκII gene A2. We could not determine the genes coding the anti-SbAg IgG2. However, because of the S instead of T at position 7, P instead of V at position 12, and E instead of Q at position 17, the VκII gene(s) used in the anti-SbAg response is probably not A2 but A3/A19 (21). To determine the genes, a human heterohybridoma or much larger amount of clonally purified human anti-SbAg IgG2 would be needed as described in other studies (1, 2, 23). Recently, Feeney et al. investigated Navajos and controls for the A2 gene (6). Over half of the Navajos studied, but only one control individual, had a new allele of A2, termed A2b, with three changes from the published A2 germ line sequence, indicating that a defective Vκ-A2 allele in Navajos may play a role in increased susceptibility to meningitis caused by H. influenzae.

In summary, our data indicate important similarities in human antibody responses to the SbAg from A. actinomyctecomitans and Hib-PS, and it appears that the number of genes used to make the IgG2 antibodies is limited in both responses. The human antibody response to Hib-PS is one of the best-characterized human antigen-specific antibody responses. The vaccine made using Hib-PS is widely used clinically and protects against bacterial meningitis caused by H. influenzae. In previous work, we found that antibody reactive with A. actinomyctecomitans is associated with better periodontal health in patients with EOP (7, 19), and in recent studies this apparent protection correlated with antibody reactive with the SbAg (4). These results suggest that a vaccine containing SbAg could
protect against EOP, and we look forward to developments in this area.

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