Opsonization of *Actinobacillus actinomycetemcomitans* by Immunoglobulin G Antibodies to the O Polysaccharide of Lipopolysaccharide

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Received 2 June 1997/Returned for modification 24 July 1997/Accepted 12 August 1997

Sera of patients with localized juvenile periodontitis (LJP) patients colonized by *Actinobacillus actinomycetemcomitans* serotype b often contain markedly elevated levels of immunoglobulin G (IgG) antibodies to serospecific determinants in the O polysaccharide of lipopolysaccharide (LPS), as well as to outer membrane proteins of this species. IgG antibodies in LJP sera are known to opsonize *A. actinomycetemcomitans* for subsequent phagocytosis and killing by human neutrophils. The objective of this study was to determine whether outer membrane proteins or serospecific determinants in LPS are the primary target for opsonic IgG antibodies in LJP sera. An *A. actinomycetemcomitans* serotype b O-polysaccharide affinity column was constructed and subsequently used to purify LPS-specific IgG antibodies from LJP serum. The affinity-purified anti-LPS IgG antibodies were enriched in content of IgG2 (66.2%, compared with 37.0% in the total IgG fraction) and were immunospecific for *A. actinomycetemcomitans* serotype b LPS. In an opsonophagocytic assay using neutrophils from donors who were homozygous for the H131 allotype of Fcy receptor IIa (CD32), it was found that LPS-specific IgG antibodies exhibited substantially greater opsonic activity toward *A. actinomycetemcomitans* serotype b than an LJP IgG fraction that was depleted of LPS-reactive antibodies but contained antibodies against outer membrane proteins of this species. The results of this study indicate that serospecific determinants in the O polysaccharide of *A. actinomycetemcomitans* serotype b are a principal target for opsonic antibodies in sera of LJP subjects.

Sera of patients with localized juvenile periodontitis (LJP) often contain elevated levels of immunoglobulin G (IgG) antibodies reactive toward *Actinobacillus actinomycetemcomitans* serotype b (7, 12). These IgG antibodies recognize a diverse array of *A. actinomycetemcomitans* antigens including the RTX leukotoxin (25), as well as lipopolysaccharide (LPS) (8, 23, 30) and outer membrane proteins (28, 29, 32) present in the cell envelope. However, the serotype b antigen is considered the immunodominant antigen of *A. actinomycetemcomitans*. First described as a heat-stable, papain-resistant molecule with carbohydrate-like features (4), the serotype b antigen was reported to be a linear polymer composed of disaccharide units containing L-rhamnose and D-fucose (2). We and others provided evidence that the serotype b antigen is associated with the polysaccharide moiety of LPS (17, 30). More recently, we reported that the serotype b antigen is defined by the O polysaccharide (O-PS) of LPS and is a trisaccharide repeating unit consisting of D-fucose, L-rhamnose, and N-acetyl-D-galactosamine (18).

Given the identity of the LPS O-PS as the serotype-specific antigen of *A. actinomycetemcomitans* and its strong reactivity with IgG antibodies in LJP sera, we sought to define the functional properties of IgG antibodies directed to this surface-accessible structure. The polymorphic nucleolar neutrophil is considered to play a key role in host defense against periodontopathic bacteria (9, 15, 26). Efficient phagocytosis and subsequent intracellular killing of bacteria often require opsonization by specific antibody and/or complement. LJP sera have been found to contain IgG antibodies capable of supporting phagocytosis and killing of *A. actinomycetemcomitans* serotype b in the presence of complement (3). However, the antigenic specificity of these opsonic IgG antibodies has not heretofore been defined. Accordingly, the objective of this study was to examine the role of O-PS-directed IgG antibodies in promoting phagocytosis and killing of *A. actinomycetemcomitans* by human neutrophils. O-PS-specific IgG antibodies were prepared from LJP serum by affinity chromatography. The opsonic activity of these antibodies was compared with that of an IgG fraction that was depleted of O-PS-specific antibody but contained antibodies to *A. actinomycetemcomitans* outer membrane proteins. The results of this study indicate that IgG antibodies which recognize serospecific determinants in the O-PS of *A. actinomycetemcomitans* serotype b play a major role in opsonization of this species.

**MATERIALS AND METHODS**

**Patient serum.** Serum containing elevated levels of IgG antibodies to *A. actinomycetemcomitans* serotype b LPS, as well as to several outer membrane proteins, was obtained from a patient with LJP after informed consent was obtained. The IgG2 fraction of this patient’s serum had previously been found to exhibit significant opsonic antibody activity (33). Serum was also obtained from a patient with common variable hypogammaglobulinemia (CVH) and was used as a complement source in bactericidal assays. The CVH serum contained markedly depressed concentrations of IgG, IgA, and IgM but normal levels of complement proteins C3 and C4 and a normal 50% hemolytic complement value.

**Isolation of LPS and O-PS.** *A. actinomycetemcomitans* ATCC 43718 (strain Y4) was maintained on 5% sheep blood agar as described previously (3). Broth cultures (26 liters) were prepared in NIH thioglycolate broth (Difco, Detroit, Mich.) supplemented with 5 g of yeast extract and 1 g of sodium bicarbonate per liter and incubated at 37°C in humidified 5% CO2. The organisms were harvested by centrifugation, washed in phosphate-buffered saline (PBS; pH 7.2), and mixed with 1% (wt/vol) formalin. LPS was extracted into hot aqueous phenol as described previously (30), dialyzed against distilled H2O to remove phenol, and lyophilized. A 1-mg/ml (wt/vol) solution of *A. actinomycetemcomitans* Y4 LPS was prepared in 1.5% acetic acid (vol/vol) and incubated for 2 h at 100°C to liberate polysaccharide. Insoluble lipid A was removed by centrifugation and filtration (0.45-μm-pore-size filter). Following dialysis against distilled H2O by...
Polysaccharide-containing fractions were pooled, dialyzed against distilled H2O, dropwise manner with constant stirring. The reaction mixture was incubated for 1,3-diaminopropane dihydrochloride and 3 mg of sodium cyanoborohydride (0.15 M borate (pH 8.5)). Two milliliters of the same buffer, containing 58 mg of above), and lyophilized.

The included volume peak was retained, dialyzed against distilled H2O, and lyophilized. The O-PS was further resolved on a Sephadex G-50 column (1.5 by 62 cm) equilibrated with 50 mM pyridylacetic acid (pH 4.5). Collected fractions (1 ml) were equilibrated with PBS (pH 7.2) and stored at 4°C.

Preparation of O-PS affinity column. O-PS (27 mg) was suspended in 4 ml of 0.15 M NaCl (pH 8.5). Two milliliters of the same buffer, containing 58 mg of 1,3-diaminopropane dihydrochloride and 3 mg of sodium cyanoborohydride (both from Sigma Chemical Co., St. Louis, Mo.), was then added to the O-PS in dropwise manner with constant stirring. The reaction mixture was incubated for 6 h at 4°C. The reaction product was applied to a Bio-Gel P-60 column (1.5 by 62 cm) and eluted in 20 ml of 0.15 M NaCl (pH 5.4). Fractions (4 ml) were monitored for neutral sugar by the phenol-sulfuric acid method (see above). Polysaccharide-containing fractions were pooled, dialyzed against distilled H2O, and lyophilized.

CH-Sepharose (1.2 g [dry weight]; Sigma) was sequentially washed in 1 M HCl, distilled H2O, and then coupling buffer (0.1 M sodium bicarbonate [pH 7.8], 150 mM NaCl, and 5% CO2 with continuous end-over-end rotation (approximately 10 to 15 rpm)). At time zero and 90 min after addition of the bacterial inoculum, 50 ml samples were withdrawn from the reaction tubes and transferred to tubes containing 4.95 ml of sterile H2O, with 0.2% BSA filter sterilized. Following 1 h of incubation at ambient temperature (in order to lyse neutrophils), 50 µl of the lysate was removed and diluted into 3 ml of sterile half-strength brain heart infusion broth (Difco). Ten-microliter aliquots of this dilution were plated in triplicate onto Trypticase soy agar (Northeast Laboratory, Waterville, Maine). The plates were incubated at 37°C in humidified 5% CO2 for approximately 72 h, after which CFU per plate was enumerated. Controls included A. actinomycetemcomitans Y4 incubated without neutrophils in the presence of (i) heat-inactivated 5% CVH serum (50 ml; 30 min), (ii) native CVH/3% H2O, and (iii) heat-inactivated CVH serum. Additional controls included bacteria incubated with immunoglobulin and native or heat-inactivated CVH serum in the absence of neutrophils. The results are expressed as a percentage of the starting (time zero) inoculum.

RESULTS

Affinity purification of anti-serotype b LPS. The serotype b antigen of A. actinomycetemcomitans has recently been identified as the O-PS of LPS (18). To evaluate the biologic properties of IgG antibodies to this key surface antigen, we isolated the O-PS from A. actinomycetemcomitans serotype b LPS by acetic acid hydrolysis and subsequent chromatography on Sephadex G-50. As described previously in detail (18), this fraction contains the neutral sugars D-fucose and L-rhamnose, as well as N-acetyl-d-galactosamine, in a ratio of 1:1:1. The O-PS was derivatized with a diaminopropane spacer and subsequently coupled to CH-Sepharose. This affinity absorbent was used to isolate IgG antibodies from LJP serum which are specific for the A. actinomycetemcomitans serotype b antigen. Serologic specificity of IgG antibodies bound to the O-PS affinity column was confirmed by double-immunodiffusion analysis. As shown in Fig. 1, affinity-purified IgG specific for the O-PS of serotype b LPS exhibited immunoreactivity toward LPS from serotype b but not toward LPS from representative serotypes a or c strains. The affinity-purified IgG was similarly unreactive toward LPS derived from strains representing serotypes d and e (not shown). These results are consistent with evidence indicating that the O-PSs of A. actinomycetemcomitans serotypes a to e are structurally distinct (18, 19).

Immunoblot analysis. Antigenic specificity of the anti-LPS IgG was further characterized by immunoblot analysis using high-molecular-mass serotype b LPS and Sarkosyl-insoluble outer membrane complexes (containing LPS and outer membrane proteins) as test antigens. High-molecular-weight serotype b LPS electroblotted onto PVDF membranes produced a diffuse smear pattern when probed with the DEAE IgG fraction of high-titer LJP serum (Fig. 2). This smearing is characteristic of the serotype b LPS of A. actinomycetemcomitans (4, 17, 30). O-PS-specific anti-LPS IgG prepared by affinity chromatography produced a similar smear pattern, while IgG depleted of O-PS-specific IgG showed minimal immunoreactivity with high-molecular-mass LPS.

Immunoblot analysis performed using outer membrane
complexes of *A. actinomycetemcomitans* demonstrated that the DEAE fraction of LJP IgG contained antibodies to a number of outer membrane proteins of this species, as well as to LPS. Depletion of O-PS-specific antibodies did not alter immunoreactivity toward *A. actinomycetemcomitans* outer membrane proteins. However, the anti-LPS-depleted IgG no longer produced a smear pattern when reacted with electrophobbed outer membrane complexes.

**IgG1 and IgG2 antibodies in anti-LPS IgG.** High-titer LJP sera contain significantly elevated levels of IgG1 and IgG2 antibodies to *A. actinomycetemcomitans* serotype b LPS (31). Affinity purification of O-PS-specific IgG from LJP serum should, therefore, result in an enrichment of IgG1 and IgG2 antibodies to *A. actinomycetemcomitans* serotype b LPS. Using an IgG subclass-specific ELISA, we assessed the content of LPS-reactive IgG1 and IgG2 antibodies in the affinity-purified anti-LPS IgG. As shown in Fig. 3A, IgG1 antibody activity toward serotype b LPS was greater in the anti-LPS IgG fraction than in the DEAE IgG fraction, while IgG depleted of LPS-reactive antibody contained minimal IgG1 antibody activity toward LPS. Similarly, affinity-purified anti-LPS IgG showed enrichment of IgG2 antibody activity toward serotype b LPS (Fig. 3B).

**IgG subclass distribution of anti-LPS antibodies.** Polysaccharide antigens typically induce the production of IgG antibodies which are principally of the IgG2 subclass, although IgG1 antibodies are often present. Using a human IgG subclass-specific ELISA, we previously observed that sera of LJP patients contain substantial concentrations of IgG2 antibodies to *A. actinomycetemcomitans* serotype b LPS (31). We sought independent confirmation of this finding by examining the subclass distribution of affinity-purified IgG antibodies to the O-PS of *A. actinomycetemcomitans* serotype b. As shown in
In particular, structural analysis has revealed that the polysaccharide region of LPS was found to be associated with the serotype b-specific antigen (4, 23). This antigen has been identified as an important structure due to its surface accessibility (8, 14, 21, 22, 28). Monoclonal and polyclonal antibodies directed to LPS have been shown to promote killing of this organism by human neutrophils and to protect against experimental infections caused by certain species of gram-negative bacteria (24). Monoclonal antibodies to the serotype b antigen of *A. actinomycetemcomitans* have been shown to promote killing of this organism by human neutrophils in the presence of complement (35). IgG antibodies present in sera of LJP patients also promote phagocytosis and killing of *A. actinomycetemcomitans*.

Information pertaining to the influence of humoral immunity upon the dynamics of periodontal infection caused by *A. actinomycetemcomitans* serotype b is limited. Previous studies have suggested that fewer teeth are involved in LJP patients with high titers of IgG antibody to *A. actinomycetemcomitans* serotype b compared with patients with low to moderate anti-LPS antibody levels (5). Hence, antibodies directed to LPS-associated determinants may contribute to host defense against *A. actinomycetemcomitans* infection.

The O-PSs of gram-negative bacteria are immunologically important structures due to their surface accessibility (8, 14, 21, 22, 28). Monoclonal and polyclonal antibodies directed to LPS O-PSs have been demonstrated to protect against experimental infections caused by certain species of gram-negative bacteria and to promote their opsonization in vitro (16, 24). Monoclonal antibodies to the serotype b antigen of *A. actinomycetemcomitans* have been shown to promote killing of this organism by human neutrophils in the presence of complement (35). IgG antibodies present in sera of LJP patients also promote phagocytosis and killing of *A. actinomycetemcomitans*.

### DISCUSSION

LJP patients are frequently colonized by serotype b strains of *A. actinomycetemcomitans* (30). Moreover, LJP sera contain opsonic IgG antibodies capable of facilitating phagocytosis and killing of *A. actinomycetemcomitans* by human neutrophils (3). We sought to determine whether these opsonic IgG antibodies recognize LPS or outer membrane proteins of *A. actinomycetemcomitans*. Accordingly, we compared the abilities of affinity-purified anti-LPS IgG and IgG depleted of anti-LPS antibody to promote phagocytosis and killing of *A. actinomycetemcomitans* serotype b by neutrophils. The anti-LPS-depleted IgG contained antibodies reactive toward a number of outer membrane proteins of this strain (Fig. 2). Given that the affinity-purified anti-LPS IgG was highly enriched in content of IgG2, we used neutrophils from donors who were homozygous for the H131 allele of FcyRIIa (CD32), the only FcYR known to bind human IgG2 efficiently (27, 34). As depicted in Fig. 4, anti-LPS IgG exhibited substantially greater opsonic activity than anti-LPS-depleted IgG during a 90-min incubation period. In the presence of complement, anti-LPS IgG exhibited significantly greater opsonic activity (>90% loss of bacterial viability) at concentrations of <1 μg/ml. In contrast, opsonic activity of the anti-LPS-depleted IgG fraction was manifest only at concentrations exceeding 25 μg/ml in the presence of complement. Neither anti-LPS IgG nor anti-LPS-depleted IgG expressed opsonic activity in the presence of heat-inactivated complement, nor was opsonic activity observed in the presence of complement alone. Moreover, incubation of *A. actinomycetemcomitans* Y4 with anti-LPS IgG (50 μg/ml) in the presence of native 5% CSV serum yielded no loss of bacterial viability after 90 min (188.8% of the time zero inoculum), indicating that the anti-LPS IgG lacked bactericidal antibody activity.

### TABLE 1. Subclass distribution of total IgG, affinity-purified anti-LPS IgG, and anti-LPS-depleted IgG prepared from LJP serum

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (DEAE) IgG</td>
<td>50.6</td>
<td>37.0</td>
<td>6.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Affinity-purified anti-LPS IgG</td>
<td>26.5</td>
<td>66.2</td>
<td>0.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Anti-LPS-depleted IgG</td>
<td>58.0</td>
<td>33.8</td>
<td>5.9</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Subclass concentrations were determined by enzyme immunoassay (31). Total IgG was calculated by summation of the IgG1 through IgG4 subclass concentrations. Subclass distribution is expressed as a percentage of the total IgG concentration in each fraction.

* Prepared from high-titer LJP serum by ammonium sulfate precipitation and subsequent ion-exchange chromatography on DEAE-Sepharose.
serotype b by human neutrophils (3), although the antigenic specificity of these opsonic antibodies was not defined.

High-titer LJP sera contain IgG antibodies directed to serospecific determinants in LPS, as well as to outer membrane proteins of A. actinomycetemcomitans. The focus of the current study was to determine the role of IgG antibodies directed to serospecific determinants in the O-PS moiety of LPS in facilitating phagocytosis and killing of A. actinomycetemcomitans serotype b by human neutrophils. Accordingly, anti-LPS IgG antibodies, specific for the O-PS of serotype b LPS, were isolated from high-titer LJP serum by means of affinity chromatography. IgG antibodies eluted from the O-PS affinity column exhibited serotype specificity, reacting with LPS from serotype b but not from serotype a or c (Fig. 1). Moreover, these antibodies, when reacted with electoblotted serotype b LPS, produced a smear pattern characteristic of the serotype b antigen.

IgG antibodies depleted of O-PS-reactive antibody failed to produce this smear but were reactive toward a number of A. actinomycetemcomitans outer membrane proteins (Fig. 2).

IgG antibodies specific for the O-PS were found to be enriched in content of IgG1 and IgG2 antibodies to serotype b LPS (Fig. 3). As determined by an IgG subclass-specific immunoenzymetic assay, the affinity-purified antibodies contained 66.2% IgG2 and 26.5% IgG1. In contrast, the DEAE munoenzymetric assay, the affinity-purified antibodies contained 37.0% IgG2 and 50.6% IgG1. These results provide further evidence that IgG antibodies directed to serospecific determinants in A. actinomycetemcomitans LPS are primarily of the IgG2 subclass (13, 31).

Given that sera of high-titer LJP patients contain substantial amounts of IgG2 antibodies directly not only to LPS-associated determinants but also to at least one major outer membrane protein (32), we previously examined the opsonic activity of an IgG2 subclass-restricted fraction of LJP serum. When used in conjunction with complement and neutrophils expressing the H131 allotype of FcγRIIa, which binds human IgG2 efficiently, LJP IgG2 was highly opsonic for A. actinomycetemcomitans serotype b, whereas nonimmune IgG2 lacked such activity (33). Affinity-purified IgG1 from this same serum was also opsonic and synergized with the IgG2 fraction in supporting killing of A. actinomycetemcomitans. These results indicated that both IgG1 and IgG2 antibodies in LJP serum express opsonic activity toward this organism, although the antigenic specificity of these antibodies was not examined.

The results of the present study indicate that IgG antibodies to serospecific determinants in LPS play a greater role in supporting opsonophagocytosis of A. actinomycetemcomitans than do IgG antibodies which recognize outer membrane proteins of this species. Given that (i) IgG2 antibodies are opsonic for A. actinomycetemcomitans, (ii) LPS-specific IgG antibodies belong chiefly to this subclass, and (iii) affinity-purified anti-O-PS IgG contains 2.5 times as much IgG2 as IgG1, it is tempting to hypothesize that the opsonic activity of IgG specific for the O-PS of serotype b LPS is largely attributable to antibodies of the IgG2 subclass. To test this hypothesis, we are currently preparing IgG1 and IgG2 subclass-restricted fractions of anti-O-PS IgG and will assess the opsonic activity of each. Moreover, we will examine the influence of the H131/R131 polymorphism of FcγRIIa upon the expression of opsonic activity by IgG2 antibodies specific for the O-PS of A. actinomycetemcomitans.

ACKNOWLEDGMENTS

We thank R. Hamilton (Johns Hopkins University) for performing quantitative IgG subclass analyses and Elenora Altman (National Research Council, Ottawa, Canada) for helpful advice regarding preparation of the O-PS affinity column.

This work was supported by Public Health Service grant DE10041 from the National Institute of Dental Research.

REFERENCES


22. Saito, S., N. Takamatsu, N. Okahashi, N. Matsunoshita, M. Inoue, T. Take-


