Mechanism of Bacterial Resistance to Complement-Mediated Killing: Inserted C5b-9 Correlates with Killing for *Escherichia coli* O111B4 Varying in O-Antigen Capsule and O-Poly saccharide Coverage of Lipid A Core Oligosaccharide

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The interaction of C3 and terminal complement components with three isogenic strains of *Escherichia coli* O111B4 varying in outer membrane and capsule composition was examined. Strains CL99 and 1-1, which possess O-antigen capsule and 74 to 77% coverage of lipid A core oligosaccharide, were sensitive to killing in pooled normal human serum (PNHS) or magnesium ethylene glycoltetraacetic acid PNHS in the presence but not the absence of antibody, unthough 1-1 contained 35% more lipopolysaccharide than CL99 and was slightly less sensitive to alternative pathway killing. In contrast, strain 1-2 lacks O-antigen capsule but contains 84% coverage and resists serum killing in the presence and absence of antibody in both PNHS and magnesium ethylene glycoltetraacetic acid PNHS. All three strains consumed C3 and C9 when incubated in PNHS, but consumption was most rapid with 1-2, which also bound the largest number of C3 molecules per CFU. Between $15 \times 10^3$ and $24 \times 10^3$ molecules of C9 per CFU bound to CL99 and 1-1 during incubation in 10% PNHS or 10% magnesium ethylene glycoltetraacetic acid PNHS, and binding was relatively stable. Binding and release of $3 \times 10^4$ to $8 \times 10^4$ molecules of C9 per CFU was observed for strain 1-2. The majority of C9 bound to CL99 and 1-1 in the presence of antibody distributed with the outer membrane after lysis of the organisms in a French press, whereas only 16.1 to 20.1% of C9 was deposited on these organisms in the absence of antibody, and 31.5 to 39.8% of C9 on strain 1-2 with or without antibody sedimented with the outer membrane. Between $4.6 \times 10^4$ and $5.5 \times 10^4$ molecules of C9 per CFU remained bound in a salt- and trypsin-resistant form to the outer membrane of organisms that were killed, whereas fewer than $1.4 \times 10^3$ molecules of C9 per CFU were bound to the outer membrane of organisms not killed by serum. These results indicate that C5b-9 that is bound to the outer membrane of *E. coli* O111B4 in a form resistant to salt or protease elution correlates with bacterial killing.

Resistance to complement-mediated killing is an attribute of many gram-negative bacteria. The presence of the smooth phenotype, that is, lipopolysaccharide (LPS) molecules containing long polysaccharide side chains (O-antigen), is the characteristic of the gram-negative cell envelope most clearly correlated with serum resistance. This conclusion is based most firmly on studies done comparing rough isogenic mutants with smooth parental strains of *Escherichia coli* and *Salmonella* spp. (13, 14, 17). Cells lacking O-antigen are generally killed directly by complement in the absence of specific antibody but become resistant to serum when O-antigen is synthesized (3). In contrast, the role of capsular polysaccharides and outer membrane proteins in conferring serum resistance is less completely defined (12, 15, 16, 18).

We previously reported on the mechanism of serum resistance for a smooth, unencapsulated strain of *Salmonella minnesota* (9, 10). This organism activated complement efficiently, and a complement membrane attack complex, C5b-9, formed on the bacterial surface. The complex was not bactericidal and was released from the surface of the organism. Apparently, the complex did not insert into hydrophobic outer membrane domains. In those initial studies, we examined neither the amount of LPS per organism nor the distribution of O-antigen. Furthermore, attachment of C5b-9 to whole organisms was not compared with the C5b-9 binding to the bacterial outer membrane.

In this report, we examined in quantitative fashion the interactions of C3 and terminal complement components with the three isogenic strains of *E. coli* O111B4 that vary in serum sensitivity and outer membrane composition. The strains were characterized with regard to the amount of LPS per organism, the presence of a surface polysaccharide capsule (O-antigen capsule), and the distribution of O-antigen (R. C. Goldman, K. A. Joiner, and L. Leive, submitted for publication). The extent of coverage of lipid A core units of LPS with O-antigen was determined. The use of isogenic strains permits a more definitive comparison of C5b-9 attachment to outer membranes of varying composition than would be possible if nonsisogenic strains were studied. Our results indicate that binding of C5b-9 to the bacterial outer membrane in a form that is resistant to salt or protease elution is associated with bacterial killing.

MATERIALS AND METHODS

The following buffers were used (9): Hanks balanced salt solution (HBSS) containing 0.15 mM CaCl$_2$ and 1.0 mM MgCl$_2$ (HBSS$^+$); isotonic Veronal-buffered saline containing 0.1% gelatin, 0.15 mM CaCl$_2$, and 1 mM MgCl$_2$ (VBSS$^+$); and VBSS$^{++}$ diluted with 5% glucose to 0.060 μm.

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**Bacteria.** *E. coli* CL99 is a strain of *E. coli* O111B4 lacking the enzyme uridine diphosphate galactose epimerase. Characteristics of this strain have been described in detail previously (4). Briefly, the strain was derived from an isolate originally reported by Heath by curing the isolate of a lysogenic phase (2). The strain produces only rough LPS when grown in the absence of galactose but produces both an O-antigen capsule and smooth LPS when grown in the presence of galactose.

The O-antigen capsule is biochemically and immunologically similar to the polysaccharide portion of LPS from the same organism (4). This O-antigen capsule inhibits the agglutination of unheated cultures of CL99 by O-serum, whereas heat treatment of CL99 releases O-antigen capsule and renders bacterial cells agglutinable in O-serum. Thus, although the O-antigen capsule is not biochemically analogous to the acidic exopolysaccharide capsules of *E. coli* (K antigens), it apparently serves the same role in masking outer membrane constituents. Furthermore, the O-antigen capsule is a surface polysaccharide, which behaves like many *E. coli* capsules in that it remains associated with outer membranes prepared by relatively gentle procedures (EDTA plus lysozyme) but separates from outer membranes prepared with the use of more vigorous procedures (French press lysis).

Strain CL99 has 77% coverage of lipid A core oligosaccharide with polysaccharide and an average of 9.6 O-units per molecule of lipid A core oligosaccharide (Goldman et al., submitted for publication). Strains 1-1 and 1-2 were derived from CL99 by serial passage in serum after presensitization with bactericidal rabbit antibody to *E. coli* O111B4. Both 1-1 and 1-2 contain the uridine diphosphate galactose epimerase enzyme mutation. Strain 1-1, when grown in the presence of galactose, contains an O-antigen capsule, has 74% coverage of lipid A core oligosaccharide with polysaccharide, has an average of 9.0 O-units per molecule of lipid A core oligosaccharide, and contains 40% more LPS per organism than does CL99. Strain 1-2, grown with galactose, lacks O-antigen capsule, has 86% coverage of lipid A core oligosaccharide with polysaccharide, an average of 11.7 O-units per molecule, and contains 50% more LPS per organism than does CL99. Isolates CL99 and 1-1 are killed by serum in the presence of agglutinating antibody to whole cells of *E. coli* O111B4, but not in its absence. In contrast, strain 1-2 resists serum killing in both the presence and the absence of agglutinating antibody.

Bacteria were grown to an optical density at 600 nm of 0.5 to 0.7 in defined media containing galactose, as previously reported (4). The organisms were washed three times in HBSS ++ at 4°C and suspended to an optical density at 600 nm of 1.500 for use in experiments.

**Antibody preparation.** Antiserum to *E. coli* O111B4 (ATCC 12015) was raised in rabbits as previously described (8). *E. coli* 12015 is similar to strain CL99 (grown with galactose) in that it possesses both smooth LPS and O-antigen capsule. The immunoglobulin G (IgG) fraction from this antiserum was prepared by octanoic acid precipitation and batch absorption on Whatman DE52 in acetate buffer (pH 5.6) (9). The material contained more than 95% IgG as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For some experiments, IgG was radiolabeled with 125I Na by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) to a specific activity of 6 x 105 cpm/μg.

**Purification and radiolabeling of complement components.** C3 and C9 were purified from fresh human plasma by using modifications of the procedure published by Hammer (6, 9). Both preparations gave single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a single line on double diffusion when tested with antisera to whole human serum. The specific hemolytic activities of the purified preparations were as follows: C3, 51 U/μg (serum, 5 x 106 U/ml); and C9, 890 U/μg (serum, 4 x 106 U/ml).

C3 and C9 were radiolabeled with 125I Na by using Iodobeads to specific activities of 2.5 x 105 cpm/μg for C3 and 6.2 x 105 cpm/μg for C9.

**Serum bactericidal assays.** Serum bactericidal assays were performed as previously described (8). Briefly, organisms at an optical density at 600 nm of 1.500 in HBSS ++ were presensitized with 50 μg of preimmune or immune IgG per ml (final concentration). After presensitization for 20 min at room temperature with periodic agitation, bacterial suspensions were mixed (vol/vol) with dilutions of absorbed pooled normal human serum (PNHS) to yield final serum concentrations of 10, 20, or 40%. Serum had been previously absorbed with strain 12015 to remove specific antibody (8). The mixtures were incubated for 60 min at 37°C; then samples were removed for quantitation of viable bacteria. Killing was expressed relative to bacterial colony counts in control tubes containing equivalent percentages of PNHS which had been heated for 30 min at 56°C to inactivate complement. For some experiments, serum was chelated with 10 mM ethylene glycoltetraacetic acid containing 2 mM MgCl2 (Mg EGTA) to block classical pathway activation.

**Consumption of C3 and C9.** Consumption of hemolytic C3 and C9 from mixtures of bacteria and serum was measured by using published procedures (9).

**Binding of C3 and C9.** Binding of C3 and C9 to the bacterial surface was measured as described previously (9). Briefly, mixtures of serum and bacteria were prepared as described above for the serum bactericidal assay, except that either 125I-C3 or 125I-C9 was added to the mixture. Samples were incubated at 37°C. At designated intervals, samples of 150 μl were removed and added to microcentrifuge tubes containing 1 ml of cold HBSS ++, and the tubes were centrifuged for 5 min at 12,500 x g. The supernatants were aspirated, and the pellets were counted in a gamma counter. Controls for nonspecific binding were bacteria that were incubated in serum previously heated for 30 min at 56°C to block complement activation. Molecules of C3 and C9 bound to the bacterial surface were calculated as described previously.

**Separation of O-antigen capsule and outer membrane.** Lysis of strains CL99, 1-1, and 1-2 in a French pressure cell, followed by ultracentrifugation, provides a method for separating outer membrane from cytoplasmic constituents and O-antigen capsule (4). Lysis in a French pressure cell released >99% of O-antigen capsule from intact organisms. The procedure of French press lysis disrupts the cytoplasmic membrane into small fragments that do not sediment under the conditions used for pelleting outer membranes in this study (R. C. Goldman, unpublished observations). Mixtures of bacteria and serum prepared as described for C3 and C9 binding assays were incubated for 60 min at 37°C, washed three times in HBSS ++, and then lysed by two passages in a French pressure cell at 16,000 lb/in². Outer membranes were pelleted by centrifugation in an air-driven ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) for 20 min at 100,000 x g. The outer membrane pellets and supernatants were counted in a gamma counter to determine the distribution of C3 and C9. In preliminary experiments, in which we used intrinsically labeled bacteria and differentially labeled C9, we showed that these conditions pelleted 90% of the...
outer membranes and less than 15% of the C5b-9 complexes that were not associated with the outer membrane. Control experiments also indicated that the presence of antibody did not lead to artifactual sedimentation of small complexes of C5b-9 and bacterial outer membranes by a process of aggregation.

**Elution of C9 from outer membranes with NaCl and trypsin.** Outer membranes containing 125I-C9 were prepared from strains CL99, 1-1, and 1-2 as described above. These membrane preparations were suspended in either 1 M NaCl or 0.1% trypsin tosylsulfonyl phenylalanyl chloromethyl ketone and incubated for 30 min at 37°C. Susensions were centrifuged for 20 min at 100,000 x g in an air-driven ultracentrifuge. The supernatant and outer membrane pellet were counted in a gamma counter to determine the percentage of 125I-C9 released from the membranes, and the total number of molecules of C9 remaining associated with the membrane was calculated.

**RESULTS**

**Serum bactericidal assays.** Strains CL99 and 1-1 were resistant to killing by 10% PNHS and 10% Mg EGTA PNHS in the absence of immune IgG (Table 1). In contrast, these same strains were killed by 0.44 to 1.49 log_{10} decrease presensitization with immune IgG. The killing of strain 1-1 was significantly less than the killing of strain CL99 in 10% Mg EGTA PNHS. The killing increased in a dose-related fashion as the serum concentration was raised to 20 or 40% for immune IgG-presensitized organisms, compared with minimal killing of nonpresensitized bacteria under the same conditions (data not shown). Strain 1-2 was not killed in 10% PNHS or in 10% Mg EGTA PNHS, in either the presence or absence of immune IgG. These results confirm and extend our previous observations with the three isogenic strains of *E. coli* 0111B4.

**Consumption of C3 and C9.** Substantial consumption of C3 and C9 by strains CL99, 1-1, and 1-2 was observed in 10% PNHS and 10% Mg EGTA PNHS after a 60-min incubation at 37°C. Consumption of C3 exceeded 72% and consumption of C9 exceeded 85% for all three strains in the presence or absence of immune IgG. This result indicates that differences in killing between strains and differences in killing induced by antibody are not a consequence of inefficient complement activation. Rather, the extent and stability of C3 and C5b-9 deposition on the bacterial surface must vary. This possibility was examined in the next series of experiments.

**Binding of C3 and C9 to CL99, 1-1, and 1-2.** More molecules of C3 bound to strain 1-2 than to strains CL99 or 1-1 during incubation for 60 min in 10% PNHS or 10% Mg EGTA PNHS. Presensitization with immune IgG increased C3 binding on all three strains in comparison with incubation with nonimmune IgG (Table 2).

**French press lysis.** The distribution of C9 deposited on strains CL99, 1-1, and 1-2 was examined after separation of outer membrane from O-antigen capsule (Table 3). The majority of C9 bound to CL99 and 1-1 in the presence of antibody distributed with the outer membrane, whereas only 16.1 to 20.1% of C9 deposited on these strains in the absence of antibody was associated with that fraction. From 31.5 to 39.8% of C9 deposited on 1-2 with or without antibody sedimented with the outer membrane. These results suggested that C5b-9 deposited on CL99 and 1-1 in the absence of antibody and therefore in the process of killing were absorbed within or underneath the capsule. This was in accord with our previous results on C5b-9 attachment to the O-antigen capsule bearing *E. coli* 0111B4 strain 12015 (11). Addition of antibody resulted in a more firm association of C5b-9 with the outer membrane in the presence of bacterial killing (strains CL99 and 1-1), but not in the absence of killing (strain 1-2).

**Elution of C5b-9 with NaCl and trypsin.** The nature of attachment of C5b-9 to the outer membrane of the three strains of *E. coli* 0111B4 was investigated next (Table 4). Outer membranes bearing 125I-C9 were separated as described above and then suspended in either 1 M NaCl or 0.1% trypsin for 30 min at 37°C. The C9 that remained associated with the outer membrane was then quantitated after ultracentrifugation to repellet the outer membrane fraction. The total number of molecules of C9 per CFU were then calculated for the NaCl- and trypsin-resistant molecules by assuming 100% recovery of outer membranes during French press lysis and elution steps. The results indicated that 4.2 x 10^3 to 5.4 x 10^3 molecules of C9 per CFU remained bound to the outer membrane of the organisms that were killed (e.g., CL99 and 1-1 with antibody), whereas...
fewer than $1.4 \times 10^3$ molecules of C9 per CFU were bound to the organisms not killed by serum (CL99 and 1-1 without antibody and 1-2 with and without antibody).

**DISCUSSION**

We studied the interaction of C3 and terminal complement components with three isogenic strains of *E. coli* O111:B4 that vary in the presence of O-antigen capsule, the amount of LPS, and the percentage of coverage of lipid A core oligosaccharide with O-polysaccharide. Our results indicate that bacterial killing is associated with increased binding of C5b-9 to the outer membrane in a form resistant to salt and protease release. Although the presence of more than one variable or of unidentified variables in the outer membrane composition of the three strains that were used obviates drawing absolute conclusions about the bacterial factor mediating serum resistance, firm conclusions can be drawn about the form of C5b-9 associated with bacterial killing. In particular, resistance to elution with 1 M NaCl suggests that the C5b-9 complex is associated with the outer membrane by hydrophobic bonds. Resistance to protease release has multiple possible interpretations, including insertion of C5b-9 into protected hydrophobic domains (5), intrinsic resistance of the complex to proteolysis (1, 19), or failure of the proteolyzed complex to be released because of protease-resistant bonds with the outer membrane. Nonetheless, the most reasonable supposition from the data presented here is that the C9 that is resistant to salt and trypsin release is inserted into hydrophobic domains of the outer membrane.

The minimum number of C5b-9 complexes per organism required for bacterial killing is unknown. Inoue et al. (7) have estimated that as many as 1,450 C5b-9 complexes may be required for the killing of a rough strain of *E. coli*. Direct measurements of C5b-9 complex deposition on a deep rough isolate of *S. minnesota* (Re 595) suggested that as few as 45 complexes per organism were capable of killing 90% of 5 x $10^8$ organisms (unpublished observations). In the present study, 5 × 10^3 molecules of inserted C9 or ca. 800 to 1,000 C5b-9 complexes (assuming a C9:C5b-8 ratio of 5:1 to 6:1 on

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**TABLE 3. Distribution of C9 on strains CL99, 1-1, and 1-2 after lysis by French press**

<table>
<thead>
<tr>
<th>Organism (no. of exps)</th>
<th>+ Antibody</th>
<th>- Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>CL99 (n = 3)</td>
<td>25.4 ± 7.1</td>
<td>79.9 ± 15.5</td>
</tr>
<tr>
<td>1-1 (n = 2)</td>
<td>18.0 ± 8.5</td>
<td>82.0 ± 8.5</td>
</tr>
<tr>
<td>1-2 (n = 3)</td>
<td>68.5 ± 10.3</td>
<td>31.5 ± 10.3</td>
</tr>
</tbody>
</table>

* Presensitized and nonpresensitized bacteria at 10⁶ CFU/ml were mixed (vol/vol) with absorbed PNHS containing [125I]C9 to achieve a final serum concentration of 10%. The mixtures were incubated for 60 min at 37°C, washed three times, and then passed twice through a French press cell at 16,000 lb/in². Outer membranes were pelleted by centrifugation in an air-driven ultracentrifuge for 20 min at 20 lb/in², and the distribution of [125I]C9 was determined. Values represent the mean ± standard deviation for n experiments done in duplicate, with averages of the duplicates for statistical evaluation.

**TABLE 4. Total number of molecules of C9 bound to the outer membrane of strains CL99, 1-1, and 1-2 in NaCl- and trypsin-resistant form**

<table>
<thead>
<tr>
<th>Organism (no. of exps)</th>
<th>+ Antibody</th>
<th>- Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
<td>Trypsin</td>
</tr>
<tr>
<td>CL99 (n = 3)</td>
<td>5,462 ± 1,729</td>
<td>4,589 ± 1,131</td>
</tr>
<tr>
<td>1-1 (n = 1 or 2)</td>
<td>5,510 ± 1,589</td>
<td>4,836 ± 1,534</td>
</tr>
<tr>
<td>1-2 (n = 3)</td>
<td>465 ± 139</td>
<td>398 ± 204</td>
</tr>
</tbody>
</table>

* Experimental conditions were as outlined in Table 3, footnote a. The outer membrane pellet obtained by ultracentrifugation was suspended in either 1 M NaCl or 0.1% trypsin tosylsulfonyl phenylalanyl chloromethyl ketone for 30 min at 37°C. The outer membranes still bearing [125I]C9 were then collected by centrifugation at 20 lb/in² for 20 min, and bound counts were determined. The number of molecules of C9 per CFU were then calculated by assuming 100% recovery of outer membranes during French press lysis and elution steps. Values represent the mean ± standard deviation for n experiments done in duplicate, with averages of the duplicates for statistical evaluation.
E. coli O111B4 under these conditions [8] were sufficient for killing of 90% of strain CL99 and 80% killing of strain 1-1 in the presence of antibody.

The observation that rapid initial binding of C9 to strain 1-2 was followed by release of C9 from the bacterial surface was consistent with our previous observations on C5b-9 binding to unencapsulated, serum-resistant E. coli and Salmonella strains. Stable binding of C9 to the encapsulated E. coli strains, CL99 and 1-1, in the absence of antibody, and therefore in the absence of killing, was similar to our previous results with E. coli 12015. The demonstration that 80 to 84% of the C5b-9 that was bound to CL99 and 1-1 in the absence of antibody was released with French press lysis suggested that nonbactericidal C5b-9 was trapped either within or beneath the O-antigen capsule, as had been demonstrated for strain 12015. In studies with 12015, however, the nature of attachment of C5b-9 to the outer membrane was not studied. Furthermore, comparisons were not made between strains varying in outer membrane composition and serum sensitivity. Therefore, the attachment of C5b-9 by hydrophobic bonds to the outer membrane in the presence, but not the absence, of killing was inferred but not proven. In the experiments reported here, this postulate was examined by direct testing. We found that the increased LPS content and coverage of lipid A core oligosaccharide with polysaccharide on serum-resistant strain 1-2 is associated with release of bound C5b-9 from the bacterial surface and outer membrane. It is our contention that C5b-9 release from strain 1-2 results because stearic hindrance by LPS prevents the complex from inserting into hydrophobic membrane domains. Neither the presence of O-antigen capsule (CL99, 1-1) nor the presence of increased amounts of LPS (1-1) is able to mediate resistance to killing in the presence of antibody. Killing is associated with binding of C5b-9 to the bacterial outer membrane in a NaCl- and protease-resistant form.

LITERATURE CITED