JOURNAL OF BACTERIOLOGY, Sept. 1984, p. 877–882
0021-9193/84/090877-06$02.00/0
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Vol. 159, No. 3

Serum-Resistant Mutants of *Escherichia coli* O111 Contain Increased Lipopolysaccharide, Lack an O Antigen-Containing Capsule, and Cover More of Their Lipid A Core with O Antigen

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Received 13 February 1984/Accepted 4 June 1984

***Escherichia coli* strains of group O111 were characterized with respect to sensitivity to complement killing, amount of lipopolysaccharide and O antigen-containing capsule, and distribution of O antigen. All wild-type *E. coli* O111 strains were resistant to complement killing in the absence of specific antibody. Presensitization of strains with antibody to whole cells (OK antibody), followed by incubation in 50% pooled normal human serum as a source of complement, subdivided wild-type strains into three types: completely resistant, partially resistant, and sensitive. Completely and partially resistant mutants were isolated by cycles of serum killing, starting with one sensitive strain. Completely resistant mutants had no O antigen-containing capsule, but had 50% more lipopolysaccharide than did the parent, and this lipopolysaccharide had 30% fewer lipid A core molecules devoid of O antigen. Partially resistant mutants still had O antigen-containing capsule, but contained 40% more lipopolysaccharide than did the parent; the extent of coverage of lipid A core with O antigen remained unchanged, No correlations were found between outer membrane protein composition and the degree of serum resistance. Since the terminal membrane attack complex (C5b-9) must stably insert into a hydrophobic membrane site to effect killing, we conclude that both increased lipid A core and increased coverage of lipid A core with O antigen preclude access of C5b-9 to lethal sites on the cell surface.**

The cell surface of pathogenic bacteria is a primary factor determining the outcome of contact with a potential host. Once access to a given tissue is gained, a process which may in itself be influenced by specific factors on the bacterial surface (20, 22), dissemination by way of the bloodstream occurs when the pathogen escapes serum bactericidal reactions (1, 29, 30). Although several systems exist for maintaining sterility of serum, complement-mediated killing is of clear importance with respect to gram-negative bacteria (24, 25, 33, 34).

Many of the unique properties of gram-negative bacteria are due to the outer membrane and its associated surface antigens, polysaccharides, lipopolysaccharides (LPS), and proteins. LPS is a major structural component of the outer membrane and is a central factor mediating impermeability to hydrophobic compounds (7, 12), and an important virulence factor (28). LPS consists of a lipid, lipid A, linked to a core oligosaccharide, which is in turn linked, in many naturally occurring strains, to an O-antigen polysaccharide. This polysaccharide is of variable length and is constructed of a repeating unit of four to seven monosaccharides (15). The specific carbohydrate composition of the lipid A-linked O antigen is now known to influence alternative pathway activation of complement and subsequent deposition of ligands for receptor-mediated phagocytosis by macrophages (13, 14). In addition, although cells lacking O antigen are in many cases killed directly by complement in the absence of specific antibody, they can become resistant by making O antigen (30) or more lipid A core (30, 31).

Surface polysaccharides that are not linked to lipid A (capsules) and outer membrane proteins also may influence the outcome of serum bactericidal reactions. Thus, even cells that lack O antigen can become resistant to complement killing when they are able to synthesize the K1 capsule (3, 21); this same surface antigen is a virulence determinant of *Neisseria meningitidis* type B (26). Similarly, a polyribophosphate capsule is a virulence determinant in *Haemophilus influenzae* type B (17, 27). Finally, the outer membrane protein coded by the plasmid-borne *traT* gene also increases bacterial resistance to complement-mediated killing (16), although the degree of resistance is apparently less than that caused by K1 capsules.

The precise mechanisms by which these various surface antigens render cells resistant to complement killing is currently being investigated (8, 9, 9a, 9b, 9c, 11a, 23). We now know that cells that contain O antigen are resistant, in the absence of antibody, due to failure of the complement C5b-9 complex to insert into the cell membrane (8, 9), rather than due to a lack of complement activation or initial surface attachment of terminal complement components. In contrast, sialic acid, the monomeric unit of the K1 capsule, is known to inhibit alternative pathway activation (19).

We have previously shown that antibody is required for complement killing of *E. coli* O111 and that antibody functions not primarily to increase deposition of terminal components, but rather to increase the bactericidal efficiency of C5b-9, possibly by allowing access of terminal components to a lethal membrane site (9a, 9b, 9c). We have now isolated variants that are resistant to antibody-mediated complement killing and herein report the changes that have occurred on the bacterial cell surface.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Four strains of *E. coli* group O111 were studied: CL99, derived from a *galE* strain lacking UDP-glucose epimerase (4) originally obtained from E. Heath (Department of Biochemistry, University of Iowa College of Medicine, Iowa City); Stoke W (obtained

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from the Centers for Disease Control, Atlanta, Ga.); and 12015 and 29552 (obtained from the American Type Culture Collection, Rockville, Md.). Strain CL99, when grown in medium containing galactose, and strains Stoke W and 12015 all contain two fractions of O antigen with the same carbohydrate composition and antigenic determinants (6). Each fraction represents 50% of the total O antigen; fraction I is not linked to lipid A core, whereas fraction II is so linked. Fraction II thus represents the LPS of the cell, whereas fraction I is a very large polysaccharide, apparently unlinked to lipid or protein, which from its location and properties is a capsule for the cell. It differs from other, more well-known capsules in having the same composition as the O antigen of the cell's LPS, and we will refer to it herein as O antigen capsule.

Strains CL99, 12015, and Stoke W are inagglutinable in O serum before heating at 100°C for 1 h, whereas strain 29552 is fully agglutinable, with or without heating (6). These observations suggested that O-antigen capsule inhibits agglutination of cells by O serum. We recently were informed that strain 29552 was inagglutinable in O serum before heating at 100°C when originally deposited with the Centers for Disease Control (B. Davis, personal communication) and the American Type Culture Collection (R. L. Gherna, personal communication). Thus it appears possible that, upon storage, strain 29552 lost the capacity to synthesize O-antigen capsule, simultaneously becoming agglutinable without heating.

Cells were grown in defined medium (WMS) at 37°C as described previously (4). Cells of CL99 were grown with added galactose (1×10^{-4} M final concentration) to permit production of O antigen, unless otherwise indicated. MacConkey agar base containing 0.1% galactose and agar plates containing M9 salts (2) plus 0.1% galactose as the sole carbon source were used to test for galactose fermentation. Cells were grown in low-phosphate medium (4) for labeling with [^{33}P]phosphate and subsequent analysis of LPS (see below).

Serum bactericidal assay. The extent of killing by pooled normal human serum (PNHS) was determined as previously described (10) with cells grown in WMS to midexponential growth ($\sim 3 \times 10^8$ cells per ml). Cells were presensitized with specific antibody (1:64 final dilution) by incubation at room temperature for 20 min and then mixed with an equal volume of PNHS. The mixture was incubated at 37°C for 30 to 40 min, at which time viable counts were taken and compared with control values (cells not exposed to presensitizing antibody or PNHS).

Selection of serum-resistant mutants. Mutants resistant to serum killing were selected as follows. Cells (5×10^8 to 1×10^9) were presensitized with antibody and subjected to serum killing as given above. Survivors (5×10^5 to 1×10^7) were washed with phosphate-buffered saline by centrifugation, inoculated into fresh WMS medium (5 to 10 ml), and grown overnight at 37°C. The next morning the cells were diluted 50-fold into fresh WMS; when the cells were reaching midexponential growth ($\sim 3 \times 10^8$ cells per ml), serum killing was repeated. All mutants were derived from the starting strain, CL99, since they retained *galE*, as shown by the inability to ferment galactose (test described above).

Each selection for serum-resistant mutants started from single colony isolates of CL99 that were tested for serum sensitivity and agglutination with O serum before and after heating at 100°C in phosphate-buffered saline (see below). A fresh, control culture of CL99 was tested in parallel with each cycle of serum killing to monitor efficiency of killing and to verify selection of resistant variants. When cultures became resistant to serum killing, cells were plated to single colonies that were picked at random and tested for lack of ability to ferment galactose, agglutination with antibody specific for *E. coli* O111, and resistance to serum killing.

Agglutination reactions. Cells were tested for agglutination by O serum before and after heating at 100°C in phosphate-buffered saline for 1 h as previously described (6).

Antibodies to *E. coli* O111. OK serum raised in rabbits was prepared against strain 12015 as described previously (6). OK serum against strain Stoke W was purchased from Difco Laboratories (Detroit, Mich.). Both antisera were highly bactericidal for CL99 when used with PNHS as a complement source.

Analysis of outer membrane proteins. Outer membranes were prepared and protein was analyzed on 12.5% polyacrylamide gels containing sodium dodecyl sulfate as described previously (4).

Analysis of LPS. Cells were grown in low-phosphate medium and labeled with [^{33}P]phosphate (NEZ 080; New England Nuclear Corp., Boston, Mass.) as previously described (4). Total cellular LPS was prepared for gel electrophoresis as follows. Cells from 10 ml of culture labeled with 20 μCi of [^{33}P]phosphate per ml were washed twice with 0.01 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid–NaOH (pH 7.4) by centrifugation, and the final pellet was suspended in 0.125 ml of 0.06 M Tris-hydrochloride (pH 6.8) containing 1.0% sodium dodecyl sulfate and immediately heated at 100°C for 5 min. Samples were cooled, diluted to 2.5 ml with the same Tris buffer, but lacking sodium dodecyl sulfate, and digested with RNase A (Sigma Chemical Co., St. Louis, Mo.) (10 μg/ml, final concentration) at 37°C for 16 to 20 h. Proteinase K (Sigma) was added to 10 μg/ml, and incubation was continued at 37°C for 4 h. Tris buffer was removed by chromatography on Sephadex G-25 (PD-10 columns; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) in 0.01 M ammonium acetate (pH 8.1). LPS-containing fractions (the 3.5-ml void volume) were lyophilized twice. Samples were then prepared for gel electrophoresis in 7 to 20% linear gradients of acrylamide containing sodium dodecyl sulfate as previously described (4). Gels were dried and exposed to X-ray film (Kodak AR film) for various lengths of time. Data on X-ray films were photographed onto Ektapan 4162 (Eastman Kodak Co., Rochester, N.Y.), and each sample lane was optically scanned with a Perkin-Elmer 1010G microdensitometer (32). Data were digitized and quantitated by image analysis programs written for the PDP-11/70 computer as previously described (5, 32). The percentage of total label in each LPS band was then calculated for each gel lane. Data generated by the image analysis program are accurate to less than 0.1% error. Experimental variation due to sample preparation gave less than 10% error in all parameters of LPS analysis.

Analytical procedures. Colitose, a carbohydrate residue in O antigen of *E. coli* group O111, and 2-keto-3-deoxyoctonate, a component of the core region of LPS, were assayed colorimetrically as previously described (6). Protein was assayed by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard. The presence or absence of O-antigen capsule was determined by density gradient centrifugation of total cellular O antigen after extraction with phenol as described previously (6).

RESULTS

Serum killing of four strains of *E. coli* group O111. Since surface antigens can influence the outcome of serum bacteri-

TABLE 1. Serum sensitivity of *E. coli* O111 strains

Strain	No. of cells exposed to serum	No. of survivors after 40-min treatment[a]	
		Cycle 1	Cycle 2
CL99	5.0×10^8	2.4×10^6	$< 2.0 \times 10^4$
12015	3.1×10^8	4.1×10^6	$< 2.0 \times 10^4$
Stoke W	1.9×10^8	5.1×10^7	1.2×10^7
29552	5.6×10^8	5.5×10^8	5.6×10^8

[a] Cells were presensitized with antibody and exposed to 50% PNHS for 40 min at 37°C for cycle 1. Survivors were washed, presensitized with antibody, and treated again with 50% PNHS for cycle 2. Cell numbers were determined by plating for viable counts.

cidal reactions, we examined complement killing of *E. coli* group O111 strains CL99, 12015, and Stoke W, which contain O-antigen capsule, and strain 29552, which does not. All four strains were resistant to killing by PNHS in the absence of specific antibody (9a; data not shown). Strains CL99 (grown with galactose), 12015, and, to a lesser extent, Stoke W, were killed by PNHS after presensitization with OK serum raised in rabbits; however, strain 29552 was totally resistant (Table 1). These characteristics were maintained during two sequential cycles of incubation in serum (Table 1). Thus the absence of O-antigen capsule appeared to correlate with resistance to antibody-mediated killing by serum. We therefore reasoned that serum-resistant mutants selected from strain CL99 should have lost the ability to make capsule.

Selection of serum-resistant mutants. Serum-resistant mutants of strain CL99 (grown with galactose) were selected by repeated cycles of serum killing with presensitized cells and PNHS. Surviving cells from each cycle were grown as described above before the cycle of serum killing to allow proliferation of the surviving cells. After four to six cycles of killing, completely resistant cultures were obtained (Table 2). In one experiment, after four cycles, two independent cultures became fully resistant to killing. Twenty single colony isolates from each were tested and were found to be (i) completely resistant to serum killing and (ii) fully agglutinable by anti-*E. coli* O111 O serum before heating to 100°C. In a second experiment, six single colony isolates were cycled through serum killing. Four yielded colonies with the properties described above, but two yielded serum-resistant colonies of both the first type and of a second type that was (i) partially resistant to serum killing and (ii) inagglutinable in O serum before heating. This latter phenotype is similar to that strain Stoke W, except for galactose fermentation. All

TABLE 2. Development of serum resistance in serum-cycled cultures[a]

Serum cycle no.	No. of cells exposed to serum	No. of survivors after 40-min treatment	
		Serum cycled	Control[b]
1	4.0×10^8	3.0×10^5	4.0×10^5
2	1.4×10^9	7.0×10^6	1.3×10^6
3	6.0×10^8	2.6×10^8	2.6×10^6
4	2.9×10^8	3.3×10^8	9.4×10^5

[a] Cells were presensitized with antibody and exposed to 50% PNHS for 40 min at 37°C. A portion was removed for viable counts (number of survivors), and the remaining cells were grown in fresh medium and subjected to serum killing the following day. This was repeated daily until the culture became fully resistant.

[b] A fresh culture of serum-sensitive strain CL99 was grown each day and subjected to serum killing to verify development of resistance in cycled cultures.

isolated colonies retained the parental properties of being unable to ferment galactose or to make O antigen in the absence of galactose.

Two serum-resistant mutants of each type were selected for further study. Strains 1-2 and 1-4, which were type 1, were found to lack detectable O-antigen capsule, whereas strains 1-1 and 2-4, which were type 2, had normal amounts of O-antigen capsule (Table 3). Thus it appears that two types of serum-resistant mutants can be selected from strain CL99: those like strain 29552, which have lost the ability to make O-antigen capsule and have become fully resistant to serum killing, and those like strain Stoke W, which still make O-antigen capsule, but have become partially resistant to killing.

LPS analysis of strains CL99, 1-1, and 1-2. Previous results indicated that although strain 29552 no longer made O-antigen capsule, it still contained approximately the same amount of total O antigen as strains CL99, 12015, and Stoke W (6, unpublished results). This suggested that O antigen originally destined for O-antigen capsule was somehow diverted into LPS and that this resulted in resistance to antibody-mediated serum killing. However, as these four strains were independent isolates that have been passaged and stored for various times, and as the original 29552 isolate that showed inagglutinability in O serum before heating (see above) is not available for comparison, we felt that no firm conclusion could be drawn from the study of these strains.

Parental strain CL99 and its serum-resistant mutants 1-1 and 1-2 are by contrast nearly isogenic; no mutagens were used during selection, and they are likely separated by one mutation (see below). We therefore analyzed LPS from these strains by gel electrophoresis. Despite the lack of O-antigen capsule, these strains contained nearly the same amount of total O antigen as parental strain CL99 (Table 4). We therefore anticipated four possible conclusions: LPS from strain 1-2 would (i) be present in greater amounts, but otherwise be identical to that from strain CL99, (ii) have a greater average number of repeats of O antigen units per molecule of lipid A core, (iii) have greater coverage of lipid A cores with O antigen, or (iv) a combination of the above possibilities.

The average number of O antigen units per molecule of lipid A is 9.6 for strain CL99 (Table 4), and 23% of the lipid A core molecules are devoid of O antigen; the exact distribution of O antigen units is given in Fig. 1A. In contrast, in strain 1-2 the average number of O unit repeats per molecule of lipid A is increased to 11.7 (Table 4), and the number of lipid A core molecules devoid of O units decreased to 16%; the exact distribution of O units is given in Fig. 1C. However, these changes are only sufficient to account for 185 nmol of O-antigen colitose in LPS per mg of cell protein, and cells contained 286 nmol of O-antigen colitose per mg of protein (Table 4).

The remaining increase of colitose in LPS is accounted for by an increase in total LPS molecules, as reflected by an

TABLE 3. Characteristics of serum-resistant variants

Type	Strain	% Survival after serum treatment	Agglutination in O serum before heating	Presence of O antigen fraction I
1	1-2	>95	+	−
	1-4	>95	+	−
2	1-1	10 to 30	−	+
	2-4	10 to 30	−	+

TABLE 4. Characterization of LPS and O antigen from strain CL99 and serum-resistant variants 1-1 and 1-2

Strain	nmol/mg of protein[a]		% Total O antigen as[b]:		Average no. of O-units per molecule of lipid A core[c]
	Colitose	KDO	LPS	O-antigen capsule	
CL99	303 ± 15	0.81 ± 0.11	53	47	9.6
1-2	286 ± 21	1.22 ± 0.22	>99	<1	11.7
1-1	357 ± 13	1.14 ± 0.17	46	54	9.0

[a] Values were determined in quadruplicate, and the estimated standard deviation, $\bar{\sigma}$, was calculated.

[b] Values were determined after phenol extraction of total O antigen and separation of fractions I and II on cesium chloride gradients.

[c] Values were calculated from the data in Fig. 1.

increase in lipid A core. Thus strain 1-2 contained 50% more 2-keto-3-deoxyoctonate (a measure of lipid A core) per mg of protein than did strain CL99 (Table 4). Strain 29552 also contained fewer lipid A core molecules devoid of O antigen (10% versus 23% in strain CL99), whereas strain 12015 was similar to CL99 (27% of lipid A core molecules devoid of O antigen).

Strains Stoke W and 1-1 were partially resistant to serum killing (Tables 1 and 3, respectively) and still made normal amounts of O-antigen capsule. The average number of O-antigen repeats per molecule of lipid A was 9.0 for strain 1-1 (Table 4), and 26% of the lipid A-cores were devoid of O antigen (Fig. 1B). Strain 1-1 contained significantly more O-antigen colitose per mg of protein than did the parent CL99; this is accounted for by a 1.4-fold increase in total molecules of LPS, assayed by 2-keto-3-deoxyoctonate (Table 4). Thus, strain 1-1 differs from strain CL99 by making more LPS, but this LPS is nearly identical to that of CL99 with respect to the average number of O units per molecule of lipid A core and the degree of coverage of lipid A core with O antigen. O-antigen capsule is still made in nearly normal amounts (Table 4). Similar results were obtained for strain Stoke W (data not shown).

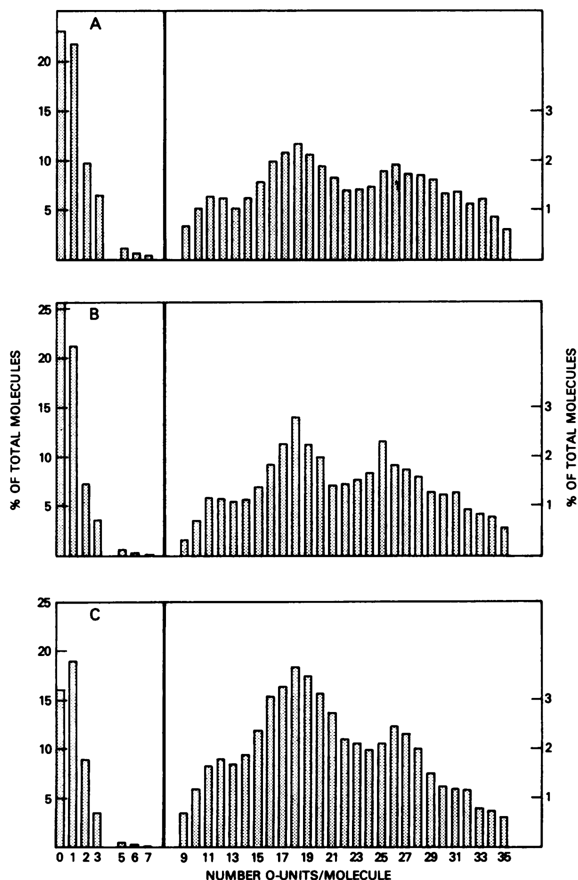

FIG. 1. O antigen distribution in LPS from strain CL99 (A) and serum-resistant variants 1-1 (B) and 1-2 (C). LPS was labeled with [^{33}P]orthophosphoric acid and subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Gels were dried and exposed to X-ray film, and the film was photographed for computer analysis. The percentage of total LPS represented by each species (lipid A core, lipid A core plus 1 O unit, ... , lipid A core plus 35 O units) was calculated. Note the two different scales for percentage of total LPS. The results for each strain are averages of two separate measurements.

Analysis of outer membrane proteins. Outer membranes were isolated from *E. coli* O111 strains and compared by gel electrophoresis in sodium dodecyl sulfate (Fig. 2). Only minor differences were observed between strains CL99, 1-1, and 1-2 (Fig. 2, lanes A, B, and C, respectively; note the slight differences in protein in the >68,000-dalton region). Strains 12015 and 29552 contained more of a 20,000-dalton protein than did the other strains, and strain 29552 also appeared deficient in production of 80,000- and 28,000-dalton proteins (Fig. 2). However, there was no apparent correlation between outer membrane protein composition and the degree of serum resistance.

DISCUSSION

Our results show that serum-resistant mutants of *E. coli* O111 are of two types: (i) those that have increased amounts of LPS, but normal coverage of lipid A core with O antigen, and (ii) those that have increased amounts of LPS and

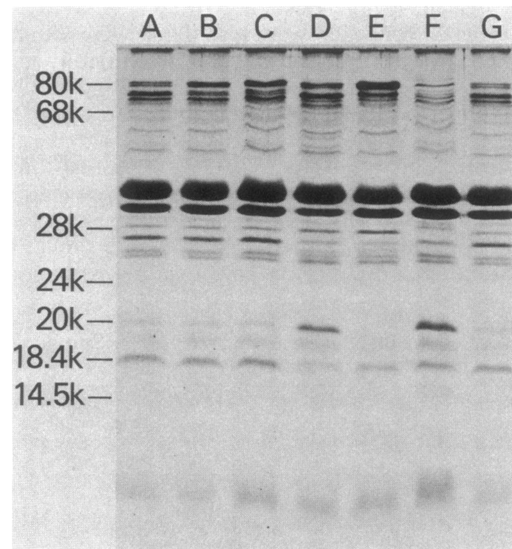

FIG. 2. Analysis of outer membrane protein. Outer membranes were purified and prepared for electrophoresis in a 12.5% polyacrylamide gel containing sodium dodecyl sulfate. Each lane contained 30 μg of protein, and gels were stained with Coomassie blue. Outer membrane protein was from strain CL99 (lanes A and G), strain 1-1 (lane B), strain 1-2 (lane C), strain 12015 (lane D), strain Stoke W (lane E), and strain 29552 (lane F). The positions of molecular weight markers are given on the left.

increased coverage of lipid A core by O antigen. Mutants of the latter type have undergone an additional major change in having lost their O-antigen capsule; all O antigen is now linked to lipid A core. In contrast, only minor differences were observed in the outer membrane protein composition of *E. coli* O111 strains, and these changes showed no correlation with serum resistance.

We have not as yet analyzed these strains genetically to determine whether they are altered in one or more than one locus; because of the multiple cycle selection method, we cannot unequivocally conclude that each reflects a single-step mutation. However, the data of Table 2 suggest that one, or at most two, mutations could have been selected, because by cycle 3 virtually complete viability remained after serum treatment. Furthermore, no mutagen was used, so these represent spontaneous mutations with predicted low frequencies. However, further experiments will be required before we can draw conclusions as to the number of mutational events involved.

Lacking genetic analysis, it is somewhat premature to speculate on the biochemical steps that may be altered in these mutants, but our analysis of the compositional changes of the cell surface suggests possible mechanisms. Mutants that still make O-antigen capsule, but which have increased amounts of LPS with a parental distribution of O antigen, may simply reflect the selection of cells that make more lipid A core. Regulation of O-antigen distribution might be normal. Mutants that no longer make O-antigen capsule and cover more lipid A core with O antigen suggest an alteration in control of O-antigen distribution. Polymerization of O antigen or transfer of O antigen to lipid A core may have been affected in this type of mutant.

In *Salmonella minnesota* (10, 11) and *E. coli* (8), resistance to complement killing is due to the inability of the membrane attack complex (C5b-9) to insert into hydrophobic membrane sites, rather than to lack of complement activation. Apparently the presence of O antigen in *S. minnesota* precludes access of C5b-9 to lethal membrane sites. In contrast, C5b-9 does insert stably into the membrane of rough mutants, which lack O antigen, and thus causes killing (10, 11).

Antibody is required for complement killing of *E. coli* O111. Resistance, in the absence of antibody, is mechanistically similar to resistance in *Salmonella* spp. strains that contain O antigen; the terminal membrane attack complex does not insert into the membrane, even though complement is consumed and initially deposited on the cell surface (9a, 9b). Antibody must be present before or at the time of C5 convertase formation on the cell surface to effect killing and may function to focus C5b-9 deposition at a critical membrane site; antibody is not simply increasing the amount of complement components bound to the cell (9a, 9b) or the distribution of covalent C3 attachment between O-antigen capsule and LPS (9c).

The mutants isolated from *E. coli* O111 bind antibody and activate complement, but are resistant to complement killing. Since antibody apparently allows access of terminal components to critical membrane sites (9a, 9b, 9c), we conclude that the critical sites remain inaccessible in these variants. The mutant type that simply contains more LPS (partially resistant mutants) may be resistant due to greater coverage of the cell surface with LPS. We have not determined whether there is actually an increase in LPS per outer membrane unit or rather an increase in total outer membrane amount per cell. Since LPS is known to influence the hydrophobic permeability of the outer membrane (7, 12), the former mechanism seems more effective for excluding the terminal attack complex. Mutants of the type that contains more LPS and greater coverage of lipid A core with O antigen (this type is completely resistant to killing) are probably resistant due to a similar mechanism, restricted access of C5b-9 to critical hydrophobic membrane sites. Preliminary experiments from our laboratories show that when complement is added to whole bacteria, C3b preferentially attaches to the longest O-antigen chains in LPS (Joiner, Grossman, and Leive, manuscript in preparation); thus increased coverage of lipid A core with O antigen may also serve to direct complement deposition to sites distant from the cell surface where a lethal event cannot occur.

ACKNOWLEDGMENT

We thank Penny Hitchcock for advice on using RNase A and proteinase K during preparation of LPS for gel electrophoresis.

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