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# Role of the Capsule and the O Antigen in Resistance of O18:K1 *Escherichia coli* to Complement-Mediated Killing

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Epidemiological data show that O18:K1 *Escherichia coli* is a common cause of neonatal bacteremia and meningitis. These bacteria were capable of multiplying in the bloodstream of newborn rats and were resistant to the bactericidal effects of complement in the absence of specific antibodies. The roles played by the O antigen and the K antigen in complement resistance were analyzed by comparing the bactericidal effects of normal sera and of sera deficient in various complement components or in immunoglobulins. These sera were tested on O18:K1 bacteria and on mutants lacking either the lipopolysaccharide O antigen or the K1 capsular polysaccharide. In addition, O1:K1 cells, which can cause pyelonephritis but which are rare in newborn meningitis and which do not multiply in the bloodstream of newborn rats, were also examined. Different mechanisms of protection against the alternative and classical pathways were recognized: K1-positive cells were resistant to the bactericidal activity of sera deficient in classical complement pathway components, whereas K1-negative cells were sensitive to these sera. Based on these results and on those from complement fixation assays, the K1 sialic acid polysaccharide impedes the activation of, and thus protects the bacteria against, the alternative complement pathway. Not only the K1-negative mutant cells but also O1:K1 bacteria and mutants lacking the O18 oligosaccharide repeating units of the lipopolysaccharide were sensitive to the classical complement pathway. These bactericidal effects were observed even in the absence of specific antibodies. It is proposed that both the K1 capsule and the O18 oligosaccharide restrict antibody-independent classical pathway activation by shielding deeper structures on the cell membrane that are capable of activating this pathway.

*Escherichia coli* is one of the common causes of neonatal septicemia and meningitis (26, 27). More than 80% of the *E. coli* strains isolated from the cerebrospinal fluid of newborns with meningitis synthesize the K1 capsular antigen (26), one of the more than 80 different known *E. coli* capsular antigens (21). Although there is convincing evidence that the K1 capsule is associated with the pathogenicity of invasive *E. coli* (9, 23, 25–28), the precise mechanism by which this antigen confers virulence is not known (25).

About half of the K1 bacteria isolated from diseased individuals are of the O (lipopolysaccharide [LPS]) antigen types O1, O7, or O18 (26). In a previous study (23), 95 O1:K1, O7:K1, and O18:K1 strains obtained from different sources, and from diseased as well as healthy individuals, were tested for their ability to cause bacteremia after colonizing the gut of newborn rats. With few exceptions, O7:K1 and O18:K1 strains were able to multiply directly in the bloodstream of the infected animals, whereas the O1:K1 bacteria were incapable of such multiplication. A comparable difference in virulence among these three serotypes was found upon analysis of the relative frequency of isolation of these bacteria from cases of meningitis in newborn children (23). In contrast, among isolates from pyelonephritis (B. Kusecek, H. Wloch, A. Mercer, V. Vaisanen, G. Pluschke, T. Korhonen, and M. Achtman, submitted for publication) O18:K1 bacteria are rare, and O1:K1 and O7:K1 bacteria are common. These three groups of bacteria thus seem to differ in the type of disease they can cause, as opposed to representing virulent and avirulent isolates.

In vitro experiments (G. Pluschke and M. Achtman, submitted for publication) showed that O7:K1 and O18:K1 bacteria were resistant to the bactericidal activity of adult rat serum, whereas O1:K1 bacteria were killed by the classical complement pathway of such sera. This observation may supply an explanation for their differential ability to multiply in the bloodstream of infected newborn rats. Complement is bactericidal to many gram-negative bacteria but often

does not kill disease isolates (7). The molecular basis of complement resistance is not well understood, but it is likely to be multifactorial. In various analyses, the presence of the somatic O side chains of LPS, the outer membrane protein composition, and the type and amount of capsular polysaccharide have been implicated (7). The K1 capsule has been shown to increase the serum resistance of rough *E. coli* strains (8, 30), and K1 *E. coli* isolated from the bacteremia was shown to be opsonized only via the classical complement pathway (4, 29). In other studies, sensitivity to the serum bactericidal system was not found to relate to differences in K antigen content (for a review, see reference 7). In most of these studies, strains were used which had not been defined in terms of animal infection studies and human epidemiology.

Previous experiments have revealed that O18:K1 strains isolated from humans are highly uniform in respect to virulence (23), resistance to rat serum (Pluschke and Achtman, submitted), LPS structure, K1 capsular polysaccharide content (Kusecek et al. submitted for publication), and numerous other properties (1). One group of O1:K1 strains is also highly uniform in these properties and differs from O18:K1 strains at the molecular level primarily in fimbriation and the chemical nature (but not amount or length) of the LPS synthesized (1; Kusecek et al., submitted for publication). Typical representatives of these two groups were chosen for comparison with two mutant strains with defects in the biosynthesis of either the K1 capsule or the O antigen. These four strains were used to analyze the specific contributions made by the capsule and the O antigen to the resistance of invasive *E. coli* to complement-mediated killing.

## MATERIALS AND METHODS

**Bacterial strains.** The *E. coli* strains used here have been described elsewhere (1, 23; Kusecek et al., submitted for publication). Strain RS228 (sequential number 103 in reference 1) is a representative O18:K1 strain, whereas strain A110 (sequential number 41 in reference 1) is a representative O1:K1 strain. Strain A691, a spontaneous mutant of strain RS228, synthesizes no detectable K1 capsule but is unaltered in other properties; strain A464 is a transposon-induced mutant which makes an intact lipid A core and a few short LPS chains and is otherwise unaltered in other properties (Kusecek et al., submitted for publication).

**Sera, media, and buffers.** Freshly drawn blood was allowed to clot for 1 h at room temperature, followed by 3 h at 4°C and then low-speed centrifugation. Samples stored at −70°C were thawed only once before use. Inactivated sera were obtained by heating for 30 min at 56°C.

Normal guinea pig serum (N-GPS) was pooled from the sera of 15 adult guinea pigs. Serum devoid of alternative pathway activity (classical pathway reagent) (CPR-GPS) was prepared from N-GPS as follows: the serum was adsorbed with bentonite (5 mg/ml) for 10 min at 0°C. This procedure removes properdin (10) and drastically lowers alternative pathway activity (31). Factor $\bar{D}$ was inactivated by treatment with 5 mM diisopropyl fluorophosphate (6) to completely abolish the remaining alternative pathway activity. The resulting serum had normal classical pathway activity as measured by hemolysis of sensitized sheep erythrocytes (see below) but caused no detectable lysis of untreated rabbit erythrocytes. C4-deficient guinea pig serum (C4d-GPS) and C2-deficient human serum (C2d-HS) were the kind gifts of J. Atkinson (St. Louis, Mo.). These sera have only alternative complement pathway activity. Colostrum-deprived calf serum (CD-CS) was obtained from a 2-day-old colostrum-deprived calf though the kind auspices of D. Matthiessen and T. Röhmann (Berlin). Radial immunodiffusion revealed no detectable immunoglobulin G (IgG) or IgM (less than 0.02 mg/ml). Serum from germfree BDIX rats (GF-RS) was a kind gift from N. F. Juhr (Berlin).

$DGVB^{2+}$ buffer contains 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 75 mM NaCl, 2.5 mM Veronal buffer, 0.05% gelatin, and 2.5% D-glucose (pH 7.5). $VBS^{2+}$ buffer contains 5 mM Veronal buffer, 0.15 M NaCl, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$ (pH 7.5). L broth contains 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of water.

**Bactericidal assay.** Bacteria were grown in L broth at 37°C to an optical density at 600 nm of 0.3, collected by centrifugation, and washed with and diluted in $VBS^{2+}$. After incubation of the cells with serum at 37°C, samples were diluted in $VBS^{2+}$, and the viable cell count was determined by plating on L agar plates.

In some experiments, monoclonal rat IgM, specific for the O18 antigen, was added to the assay mixture to a final concentration of 50 μg/ml. These antibodies were purified from the culture supernatant of a hybrid cell line produced by the fusion of mouse myeloma cells (Sp 2/0-Ag14) and spleen lymphocytes from rats immunized with boiled A691 bacteria.

**Complement fixation assays.** Reaction mixtures containing 90 μl of serum and 10 μl of bacteria in $VBS^{2+}$ ($2 \times 10^7$ cells) were incubated for 30 min at 37°C. The bacteria were removed by centrifugation, and the supernatants were assayed for residual complement activity by hemolysis of sensitized sheep erythrocytes (EA for N-GPS and EAC1,4 for C4d-GPS) as described (16). Briefly, the serum dilution necessary to yield 50% hemolysis with $1.25 \times 10^7$ erythrocytes in a total volume of 0.25 ml after 1 h at 37°C was determined. Hemolytic units are defined as the volume of undiluted serum calculated to be sufficient to lyse $0.625 \times 10^7$ erythrocytes under these conditions. Complement fixation was then expressed as the percentage of activity lost relative to a control serum incubated without bacteria.

**Intraperitoneal injection of newborn rats.** Litters of specific-pathogen-free, outbred Wistar rats were obtained from the Bundesgesundheitsamt, Berlin. Seven-day-old rats from several litters were pooled to randomize litter effects, injected intraperitoneally with 100 μl of bacterial suspension, and returned to the mother rats. Bacteria from a culture growing exponentially in L broth were washed with and diluted in phosphate-buffered saline. Ten rats were tested with each

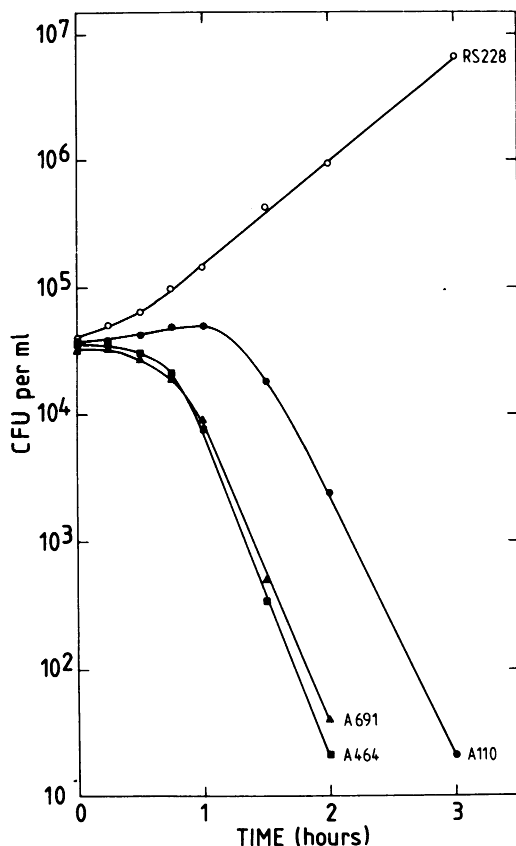


FIG. 1. Bactericidal effects of 90% N-GPS.

serial 10-fold dilution step. After 3 days, the number of surviving rats was determined, and the 50% lethal dose was calculated (24).

## RESULTS

**Bactericidal assay and virulence studies.** The two smooth, K1-encapsulated strains RS228 (O18:K1) and A110 (O1:K1) were compared with two mutants derived from strain RS228 lacking either the K1 capsule (strain A691) or the O antigen (strain A464). It has been shown previously that strain RS228 (O18:K1) can multiply in the bloodstream of newborn rats, whereas strain A110 (O1:K1) cannot (23). In contrast to the parental O18:K1 strain, both mutants were unable to cause bacteremia when given orally to newborn rats (data not shown). Differences were also found when the bacteria were tested for lethality to newborn rats after intraperitoneal injection. Strain RS228 (O18:K1) was associated with a 50% lethal dose of 20 cells, whereas the other three strains had much higher values ($3 \times 10^5$ for strain A464, $3 \times 10^6$ for strain A110, and over $10^7$ for strain A691).

The bactericidal activity of 90% N-GPS was analyzed using these four strains (Fig. 1). The O18:K1 strain was able to multiply extensively, whereas both the mutant strains and the O1:K1 strain were killed after a delay. (No such delay in killing is usually seen when laboratory strains of *E. coli* K-12 are tested [3].) Similar differences in sensitivity were observed when the initial bacterial concentration was 10 times higher or lower than that in the experiment shown. Comparable results were obtained with serum pools derived from animals from two different sources. In contrast, N-GPS from three different commercial sources caused only a transient decrease in viable counts of the sensitive strains, which was followed by growth, as if the killing capacity were being rapidly exhausted.

Figure 2 shows the numbers of bacteria found after 3 h of incubation in various serum concentrations relative to the numbers found in heat-inactivated serum. The four test strains were all able to multiply extensively in heat-inactivated serum. Pronounced reductions in viable counts were found in reaction mixtures containing more than 10% untreated N-GPS for the mutant strains and in mixtures containing more than 30% N-GPS for the O1:K1 strain. Similar to N-GPS, CPR-GPS, which has only classical pathway activity, killed both the mutants and the O1:K1 strain but not the O18:K1 strain (data not shown). In contrast, only the capsule-deficient mutant, and none of the capsule-positive strains, was sensitive to C4d-GPS, which has only alternative pathway activity (Fig. 2). Comparable results were obtained when other independently derived $K^-$ or $O^-$ mutants were tested.

For the following experiments, bacterial strains were scored as sensitive to a given serum when the viable counts had decreased to less than 1% of the original values and were scored as resistant when the viable counts had increased more than fivefold after 3 h of incubation in 90% serum. Bactericidal killing by sera has often been tested under less stringent conditions (short time periods and lower serum concentrations). Under these more common conditions, all four of our strains would probably have been scored as serum resistant. However, the strains scored as serum sensitive under our conditions were also not virulent in animal infection studies. Based on the results with guinea pig sera described above and summarized in Table 1, it is concluded that the O18:K1 strain is resistant to both complement pathways, whereas the $O^-$ mutant and the O1:K1 strain are sensitive to the classical pathway. The $K1^-$ mutant is sensitive to both complement pathways.

**Influence of specific antibodies on complement killing.** Two sera (CD-CS and GF-RS) which lack specific antibodies were used to determine whether antibodies play a role in the classical

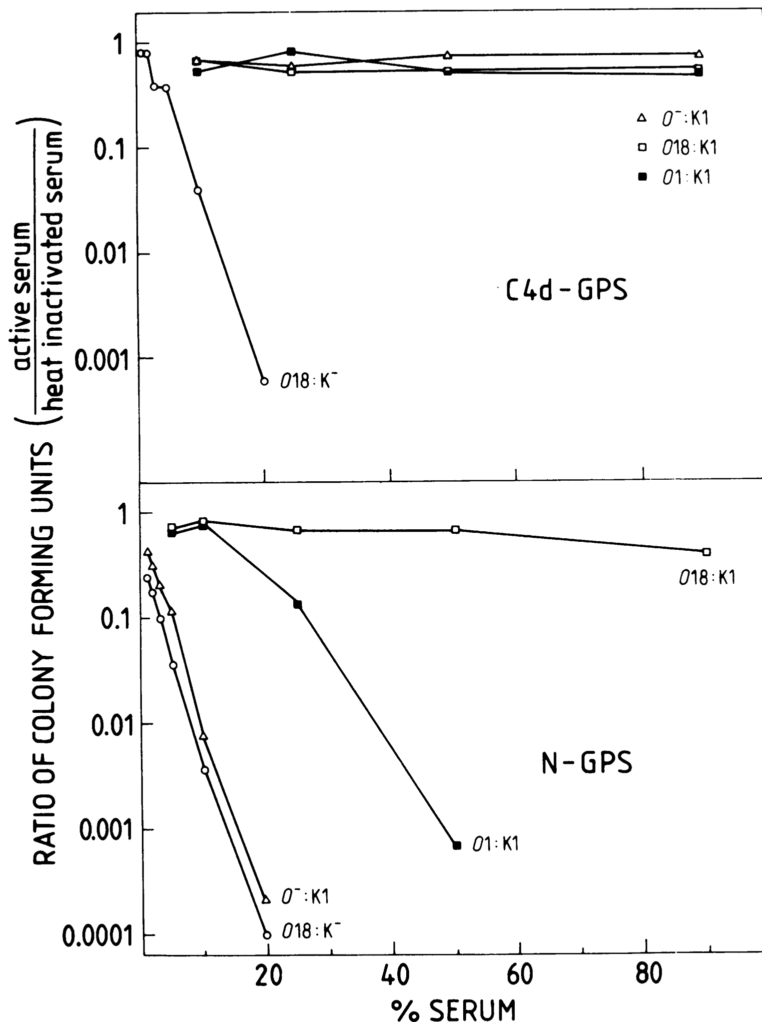


FIG. 2. Concentration dependence of the bactericidal activity of C4d-GPS and N-GPS. The incubation mixtures (150 μl) contained 2 × $10^4$ bacteria per ml and active or heat-inactivated serum diluted in $VBS^{2+}$. The ratio of CFU found in the active serum to that found in the comparable heat-inactivated serum, after 3 h of incubation at 37°C, is shown. Many of the individual data points were repeated on separate occasions with reproducible results.

TABLE 1. Bactericidal effects of different sera on *E. coli* strains[a]

| Bacterial strains | Serum (alternative pathway, classical pathway)[b] | | | | | |
|---|---|---|---|---|---|---|
| | C2d-HS (+, −) | C4d-GPS (+, −) | N-GPS (+, +) | CPR-GPS (−, +) | CD-CS (+, +) | GF-RS (+, +) |
| RS228 (O18:K1) | R | R | R | R | R | R |
| A464 ($O^-$:K1) | R | R | S | S | S | S |
| A691 (O18:$K^-$) | S | S | S | S | S | S |
| A110 (O1:K1) | R | R | S | S | S | S |

[a] Bacteria (2 × $10^3$) were incubated in 0.1 ml containing 90% serum, and the change in viable count was determined after 3 h at 37°C. R (resistant) indicates that these bacteria had multiplied at least fivefold. S (sensitive) indicates that less than 1% of the original inoculum survived the incubation. Intermediate survival was not observed in any of the experiments. All assays were performed at least three times, and the time course of killing was followed in all experiments. The killing curves resembled those shown in Fig. 1. Only minor differences, not affecting the conclusions shown here, were observed in the time course of killing in the different experiments.

[b] +, Pathway activity; −, lacks pathway activity.

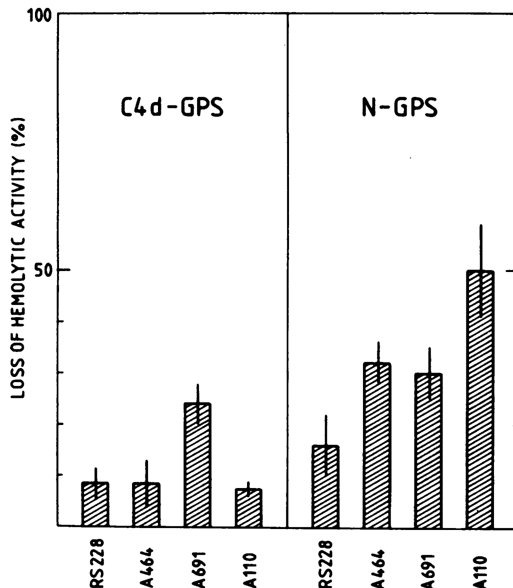


FIG. 3. Fixation of complement by bacterial cells. The residual hemolytic activity was measured in samples incubated with bacteria for 30 min. The percentage of activity lost relative to control samples incubated without bacteria is shown. The control activities were 19,600 U/ml for N-GPS and 19,000 U/ml for C4d-GPS. The values shown are the average values from four experiments. The vertical lines represent one standard deviation.

pathway killing observed. CD-CS, drawn from a colostrum-deprived calf, contained only trace amounts of antibodies, as expected for such sera (15). GF-RS, obtained from germfree rats, was shown by enzyme-linked immunosorbent assay and Western blotting techniques (Pluschke and Achtman, manuscript in preparation) to lack significant amounts of specific antibodies to O18:K1 and O1:K1 *E. coli* antigens. Both the mutant strains and the O1:K1 strain were sensitive to these two sera (Table 1), indicating that the bactericidal effects observed were antibody independent.

When monoclonal IgM specific for the O18 antigen was added to GF-RS, CD-CS, or N-GPS, strain RS228 (O18:K1) was killed efficiently, demonstrating that this strain is not resistant to killing by the membrane attack complex once the complement system has been triggered by immune complexes.

**Human sera.** Similar to the result with C4d-GPS, all three K1-positive strains were resistant to the bactericidal activity of C2d-HS, which lacks classical pathway activity (Table 1). Only the K1-negative mutant was killed efficiently by the alternative pathway.

In support of published data (20), our results with a limited number of sera from healthy adult humans indicate that human sera (but not the animal sera used here) contain agglutinating antibodies directed against the common O antigens of *E. coli*. These human sera also had bactericidal activity against O1:K1, O7:K1, and O18:K1 bacteria. Thus, the results obtained with animal sera which seem to reflect antibody-independent effects cannot be directly extrapolated to healthy humans. However, because the O1:K1 bacteria both were sensitive to antibody-independent killing by complement and are a rare cause of meningitis and septicemia in newborn children, it seems likely that antibody-independent complement activation provides an important defense mechanism for newborn children against invasion by gram-negative bacteria.

**Complement fixation tests.** The differences in sensitivity of the bacterial strains to the bactericidal effects of serum might reflect differential capacity to activate complement or differential sensitivity to the membrane attack complex. These alternatives were analyzed by complement fixation assays. A low level of comsumption of the hemolytic activity of C4d-GPS complement was observed with the three K1-positive strains, whereas the sensitive $K^-$ strain, A691, consumed about three times more hemolytic activity (Fig. 3). In N-GPS, the sensitive O1:K1 strain (A110) consumed more than three times as much activity as the resistant O18:K1 strain (RS228). Although the mutants (strains A464 and A691) were more sensitive to killing by N-GPS (Fig. 1 and 2) than the O1:K1 strain, they consumed only intermediate levels of hemolytic activity, indicating that strain A110 is less sensitive to activated membrane attack complex than the $O^-$ and $K^-$ mutants. These data suggest that the K1 capsule specifically restricts alternative pathway activation, whereas the O18 antigen interferes with classical pathway activation.

## DISCUSSION

Sialic acid is a frequent component of the capsule of invasive bacterial pathogens, suggesting that it contributes specifically to the virulence promoted by surface polysaccharides (26). In the case of K1 *E. coli*, there is convincing epidemiological evidence that the K1 capsule, a sialic acid homopolymer, is associated with the development of invasive disease in newborns (9, 23, 25–28). Because it was recently realized that O1:K1 and O18:K1 bacteria differ in their potential to cause newborn meningitis and to multiply in the bloodstream of infected newborn rats (23), experiments were initiated to determine whether both the capsular and the O antigen contribute specifically to complement resistance. The sensitivity of four *E. coli* strains which differ in their

surface polysaccharide content was tested with different normal and genetically deficient sera to assess the roles of the classical and alternative pathways in complement-mediated killing. Under the stringent assay conditions used, resistance or sensitivity to the bactericidal effects of normal rat (Pluschke and Achtman, manuscript in preparation) or guinea pig sera correlated with the differential ability of the bacterial strains to multiply in the bloodstream of infected newborn rats and to cause newborn meningitis.

Strain RS228, a respresentative O18:K1 strain (1, 23) selected from our collection of human K1 isolates (1), was found to be generally complement resistant in the absence of specific antibodies. The addition of monoclonal antibodies specific for the O18 antigen led to sensitivity, thus demonstrating that this strain is not totally resistant to killing by the membrane attack complex generated upon activation of the complement system by immune complexes. In the absence of antibodies, however, complement activation (even in 90% serum) was not efficient enough to cause killing of strain RS228.

Loss of the O antigen makes the rough derivative of strain RS228, strain A464, sensitive to the classical complement pathway in N-GPS and avirulent in the infant rat disease model. Because this strain was resistant to the alternative pathway (C2d-HS, C4d-GPS) but sensitive to sera lacking specific antibodies (CD-CS, GF-RS), it is concluded that the killing of strain A464 (or strain A110) observed in normal sera is due to antibody-independent classical pathway activity. It has recently been demonstrated that certain substances and organisms can activate the classical pathway in the complete absence of antibodies (2, 5, 13, 17, 18; N. L. Cooper, Fed. Proc. **42**:134–138, 1983). Complement fixation in N-GPS was increased with strain A464 relative to strain RS228. This indicates that the O18 antigen hinders deeper structures on the bacterial cell surface from activating the classical pathway.

Mutations resulting in the loss of the K1 capsule (e.g., strain A691) also caused loss of virulence and of complement resistance. In contrast to strain A691, all K1-positive strains, regardless of the LPS structure, were resistant to the alternative pathway bactericidal activity of sera deficient in classical pathway activity (C4d-GPS, C2d-HS). In agreement, it has been shown that K1 *E. coli* strains are only opsonized by the classical complement pathway (4, 29). A691 bacteria consumed nearly three times as much complement hemolytic activity from C4d-GPS as did the K1-positive strains. This indicates that the K1 capsule specifically interferes with alternative pathway activation. It has been suggested that sialic acid can either enhance the affinity of H (formerly β1H) or decrease the affinity of B for C3b (14, 22). In either case, this would impede the activation of the alternative pathway. Sialosylglycolipids are thought to restrict an undesirable amplification of the alternative pathway by host cell surface structures after initiation by spontaneous hydrolysis of C3 (19). The K1 capsule, a sialic acid homopolymer, seems to block amplification of the alternative complement pathway in a similar manner, thus inactivating one major nonspecific defense mechanism of the preimmune host against bacterial invasion.

The O18:K$^-$ mutant, strain A691, was also killed in CPR-GPS, which has only classical pathway activity. This suggests that the capsule (in addition to the O antigen) is required to prevent nonimmunological classical pathway activation by deeper structures on the cell surface. However, these results do not imply that any smooth LPS plus the K1 capsule will suffice to prevent complement activation. In fact, the O1:K1 strain A110 was insensitive to C4- or C2-deficient sera but sensitive to CPR-GPS, CD-CS, and GF-RS and thus triggers antibody-independent classical pathway activation although it has a smooth LPS. Other data (to be published) demonstrate that O1 LPS itself activates the classical pathway efficiently, whereas O18 LPS does not.

The O$^-$ and K$^-$ mutant derivatives of strain RS228 were killed more rapidly and at lower serum concentrations than the serum-sensitive O1:K1 strain, even though complement fixation by the O1:K1 strain was more efficient. This observation indicates that the loss of the O antigen or of the capsule not only affects complement activation, but also makes the cells more sensitive to attack by the terminal complex. In fact, studies with *Salmonella* strains have shown that the terminal complex of complement can bind to the surface of serum-resistant, smooth, gram-negative bacteria without penetrating the outer membrane (11, 12).

O18:K1 *E. coli* strains are responsible for many of the cases of *E. coli*-induced newborn meningitis (26). The experiments presented here demonstrate that both the K1 capsule and the O18 LPS are required for virulence and for complement resistance of O18:K1 *E. coli* in the absence of specific antibodies.

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