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Role of *Escherichia coli* K Capsular Antigens During Complement Activation, C3 Fixation, and Opsonization

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Escherichia coli strains with K capsular polysaccharides are relatively resistant to phagocytosis by polymorphonuclear leukocytes, in contrast to *E. coli* strains without K antigens. This inhibition of phagocytosis is related to an impaired recognition of the K^+ strains by the phagocytes due to ineffective opsonization. All five strains without K antigens were readily phagocytized after opsonization in 5% normal serum, compared with no uptake of the K^+ strains. Evidence is presented that the decreased opsonization of the K^+ strains in normal serum is caused by a low rate of complement activation of the strains, with subsequent absence of C3b fixation or C3d fixation or both to the cell wall of the bacteria. After removal of the K^+ antigens by heating of a K^+ *E. coli* strain, the strain was able to activate complement, to bind C3b or C3d or both, and to become opsonized. Complement was then activated via the classical and alternative pathways, which was comparable to the complement consumption by K^- *E. coli*.

Antibodies to bacterial cell envelope components and C3 bound to the bacterial cell surface after activation of the serum complement system are important opsonins for efficient phagocytosis, a process crucial in host defense against invasion by microorganisms (31, 32). Conflicting data exist as to the specificity of the opsonic antibodies and to the route of complement activation. Johnston and co-workers (17) showed that the opsonic activity of immune serum was only amplified by complement, whereas opsonization in nonimune serum was primarily mediated by complement. Opsonically active fragments of complement can be generated by bacteria via the classical, antibody-dependent pathway and via the alternative pathway in the absence of specific antibodies (2, 6, 16, 37).

Differences in the opsonic requirements for phagocytosis have been observed among various microorganisms (1, 9, 35; P. Stevens, S. N.-Y. Huang, W. D. Welch, and L. S. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E45, p. 88). In some cases these differences were correlated with a difference in virulence of the microorganisms (Stevens et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E45, p. 88). Björksten and co-workers (1) described differences in opsonic requirements among *E. coli* strains. Some of the *E. coli* strains activated complement via the alternative pathway, and other strains could only activate complement via the classical pathway. Since many *E. coli* strains isolated from clinical material such as blood, cerebrospinal fluid, and urine contain K capsular polysaccharides and are poorly phagocytized compared with the large majority of *E. coli* strains isolated from stool cultures (11, 15, 18, 24, 33), the opsonic requirements of clinical isolates of *E. coli* strains were compared with the opsonic requirements of *E. coli* strains isolated from stool cultures. Polymorphonuclear leukocyte (PMN) uptake of [^{3}H]thymidine-labeled *E. coli* strains was measured after opsonization in normal serum, heat-inactivated serum, and serum chelated with ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid in the presence of $MgCl_2$ (MgEGTA-chelated serum). Opsonization was compared with hemolytic complement consumption by the *E. coli* strains and with bacterial C3 fixation. Evidence is presented that the decreased opsonization of strains containing capsular polysaccharides is caused by a low rate of complement activation and subsequent absence of C3b or C3d fixation to the cell wall of the bacteria.

MATERIALS AND METHODS

Bacterial strains. All isolates were identified as *E. coli* strains by using standard clinical bacteriological criteria. Strains were serotyped by P. A. M. Guinée (Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands) by a micro-agglutination technique, as described previously (13, 14). Strains F1 (serotype O78), F4 (O88), F8 (O156), F10 (nontypable), and F11

(nontypable) did not contain K antigen and were unselectively isolated from stool cultures from healthy volunteers. Strains J (serotype O5:K1), W (O75:K1), G (O7:K?), 5176 (O18:K1), and B (O18:K?) contained K antigen and were isolated from blood cultures of five consecutive patients with *E. coli* bacteremia, as described previously (33).

Determination of K antigen. K antigens were also determined by the hemagglutination inhibition technique of Glynn and Howard (12). In brief, 4-h cultures of *E. coli* strains were used to inoculate three agar plates. After incubation overnight at 37°C, the growth on the three plates was harvested with 5 ml of phosphate-buffered saline (PBS), pH 7.4. The suspended bacteria were precipitated by addition of 3 volumes of cold acetone and harvested by centrifugation (1,600 × *g* for 10 min). The bacteria were washed twice more with acetone, filtered through no. 3 Whatman filter paper, dried, and weighed. Dried bacteria (20 mg) were suspended in 2.0 ml of PBS and homogenized ultrasonically for 3 min at 4°C (B12 Sonifier cell disruptor; Branson Sonic Power Co., Danbury, Conn.), after which the particulate material was removed by centrifugation (1,600 × *g* for 30 min). K antigen was precipitated from the supernatant by the addition of 3 volumes of cold ethanol (96%, vol/vol) and harvested by centrifugation (1,600 × *g* for 10 min). The sediment was resuspended in 1.0 ml of PBS, dialyzed overnight against PBS, and reconstituted to 2.0 ml. Serial twofold dilutions of rabbit anti-sheep erythrocyte antiserum (Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands) were added to ovine erythrocytes that had been incubated for 30 min at 37°C with dilutions of the K antigen ranging from undiluted to 1:100. Titrations of antiserum with untreated erythrocytes were set up in parallel. The inhibitory titer of K antigen was taken as the dilution of the K antigen preparation that produced a twofold reduction in the hemagglutination titer of the antiserum to ovine erythrocytes. K1 antigen was also detected by countercurrent immunoelectrophoresis (28); glass slides (LKB Instruments 2117-402; 8.4 by 9.4 cm) were coated with 1% agarose in barbitone buffer salt, pH 8.8, and meningococcal group B antiserum (Wellcome Research Laboratories, Beckenham, England) was used as a reference antiserum.

Radioactive labeling. Single colonies from nutrient agar plates were inoculated into 5 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 0.02 mCi of [*methyl*-^{3}H]thymidine (specific activity, 5 Ci/mmol; The Radiochemical Centre, Amersham, England) and 1.25 mg of deoxyadenosine (BDH Chemicals Ltd., Poole, England). After 18 h of growth at 37°C, the bacteria were washed three times in PBS and adjusted to a final bacterial concentration of 2.5 × 10^8 colony-forming units (CFU) per ml as previously described (36).

Leukocytes. Blood samples from healthy volunteers were collected in heparinized syringes (10 U/ml of blood). PMNs were prepared by a method modified from that of Böyum (3), as described recently (34). The PMNs were resuspended in Hanks balanced salt solution with 0.1% gelatin, and total and differential counts were performed. The final leukocyte suspension was adjusted to 5 × 10^6 PMNs per ml.

Opsonins. Sera from 10 healthy donors were pooled and stored in 0.5-ml portions at −70°C. No agglutinating antibodies against any of the strains used were detected in the serum. To study opsonization in the absence of the classical complement pathway, undiluted serum was chelated with MgEGTA (final concentration, 10 mM) as previously described (5).

Opsonization of bacteria. In plastic tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.), 0.3 ml of each bacterial suspension (containing 2.5 × 10^8 CFU/ml) and 1.2 ml of serum were incubated for 30 min at 37°C. The serum was removed by centrifugation (15 min at 1,600 × *g*), and the opsonized bacteria were resuspended in Hanks balanced salt solution to a final concentration of 5 × 10^7 CFU/ml.

Phagocytic mixtures and determinations of phagocytosis. Phagocytosis assays were performed by a slight modification of a previously described method (36). A 0.2-ml sample of the leukocyte suspension was added to 0.2 ml of the opsonized bacterial suspension in polypropylene vials (Biovials; Beckman, Chicago, Ill.). Phagocytosis mixtures were shaken at 150 rpm at an angle of 30 degrees in a water bath at 37°C for 2, 6, and 12 min. To determine leukocyte-associated radioactivity, vials were removed at indicated times and placed in an ice bath, and 2.5 ml of ice-cold PBS was added. After centrifugation (160 × *g* for 5 min at 4°C) leukocyte pellets were washed twice with ice-cold PBS to remove non-leukocyte-associated bacteria and were solubilized in 2.5 ml of scintillation fluid. To determine total radioactivity (representing leukocyte-associated plus non-leukocyte-associated bacteria), 2.5 ml of ice-cold PBS was added to one vial and the pellet obtained after centrifugation (1,600 × *g* for 15 min) was resuspended in 2.5 ml of scintillation liquid. Radioactivity was measured in a liquid scintillation counter, and phagocytosis was expressed as a percentage of the total added radioactivity. This method uses the percentage of leukocyte-associated bacteria as a measure for phagocytosis. Previous studies have shown that over 90% of the leukocyte-associated bacteria were ingested and killed by PMNs (33, 34, 36).

Measurement of complement consumption. To 0.2 ml of the bacterial suspensions (containing 5 × 10^8 CFU/ml) 0.8 ml of serum (20%) was added. At intervals the total hemolytic complement remaining in the mixtures was titrated with optimally sensitized sheep erythrocytes by the method of Mayer (21). The consumption of complement in each test sample was expressed as a percentage of the hemolytic complement remaining in a control sample of serum incubated with buffer alone.

Measurement of C3 fixation. The amount of C3 fixation to bacteria was quantitatively measured by a fluorescent immunoassay modified from Gillis and Thompson (10). At indicated times the process of C3 fixation was stopped by the addition of 2.5 ml of PBS containing 10 mM ethylenediaminetetraacetic acid to the incubation vial containing 0.2 ml of the bacterial suspension (5 × 10^8 CFU/ml) and 0.8 ml of serum (20%). Bacteria were then washed three times with ice-cold PBS. The pellets were incubated with 500 μl of 1:20 fluorescein isothiocyanate-conjugated antiserum specific for human C3 (Wellcome) for 15 min at

room temperature. To remove nonreacted conjugate, the test material was washed three times with 2.5 ml of PBS and finally resuspended in 2.5 ml of PBS. Fluorescence was measured with a Perkin-Elmer 204 fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) at excitation and analyzing wavelengths of 485 and 525 nm, respectively. The results are given as percentages ranging from 0 (control of test material incubated with buffer) to 100% (maximal emission intensity in the series tested).

RESULTS

Opsonization of *E. coli* strains in normal and in MgEGTA-chelated sera. To study the opsonic requirements of five *E. coli* strains isolated from blood cultures and five strains isolated from stools, the uptake of these strains by PMNs after incubation in normal and MgEGTA-chelated sera was measured. Strains W and 5176 contained serotype K1 antigen. All five blood isolates produced over 10 hemagglutination inhibition units of K antigen. Figure 1 illustrates the uptake of the strains when opsonized in different dilutions of normal and MgEGTA-chelated sera. After opsonization in 5% serum 80 to 95% of the bacteria without K antigen were taken up by PMNs (Fig. 1A), in contrast to only 3 to 20% of the bacteria with K antigen (Fig. 1B). *E. coli* K^- strains were also efficiently taken up by PMNs after opsonization in 1% normal serum, whereas even after opsonization in 20% normal serum, only two of five *E. coli* K^+ strains were taken up. When opsonized in 20% MgEGTA-chelated serum, 45 to 82% of the K^- bacteria were taken up, compared with essentially no uptake of K^+ bacteria. Even after opsonization in 50% MgEGTA-chelated serum no uptake of K^+ *E. coli* strains was observed. Opsonization in normal serum occurred at a faster rate than opsonization in MgEGTA-chelated serum, with only the alternative complement pathway intact (Fig. 2). After a 1-min incubation in MgEGTA-chelated serum, only 5% of bacteria were taken up by PMNs, compared with 62% of the bacteria after a 1-min incubation in normal serum.

Kinetics of complement consumption by K^+ and K^- *E. coli* strains. Only one of the nine strains tested was taken up by PMNs after opsonization in 20% heat-inactivated serum, indicating that complement is essential for optimal phagocytosis of most of the strains (data not shown). Therefore, the kinetics of complement consumption by *E. coli* K^+ and K^- strains were studied. *E coli* strains were incubated in 20% normal serum and 20% MgEGTA-chelated serum. After 15, 30, 60, and 120 min the total hemolytic complement remaining in the mixtures was determined (Fig. 3). After a 60-min incubation 40 to 85% of the complement had been consumed by K^- strains (mean, 72%; Fig. 3A), whereas only 10 to 50% of the complement was activated by K^+ strains (mean, 23%; Fig. 3B). In MgEGTA-chelated serum complement consumption occurred at a slower rate (5 to 12% after a 15-min incubation compared with 9 to 38% in normal serum). Only two K^+ strains did consume complement in MgEGTA-chelated serum.

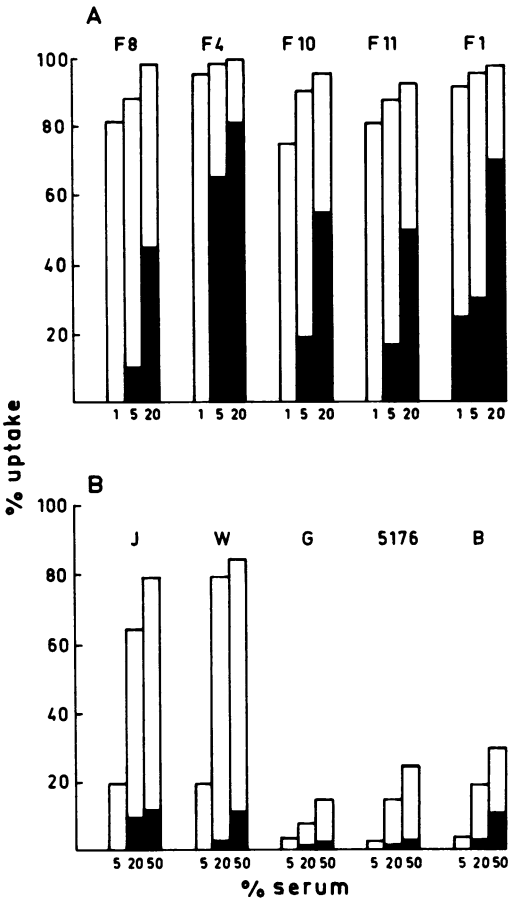

FIG. 1. *Opsonization of E. coli strains with (B) and without (A) K antigen in normal serum and in MgEGTA-chelated serum. K^- strains were opsonized in 1, 5, and 20% normal serum (□) and in 1, 5, and 20% MgEGTA-chelated serum (■), after being added to PMNs. K^+ strains were opsonized in 5, 20, and 50% normal (□) and 5, 20, and 50% MgEGTA-chelated serum (■). Uptake was measured after 12 min of incubation with PMNs.*

C3 binding by *E. coli* strains. Since complement consumption by K^- strains was much greater than that by K^+ strains, the amount of activated C3 fixed to *E. coli* K^- and K^+ strains was studied kinetically in the fluorescent immunoassay. Figure 4 illustrates the kinetics of

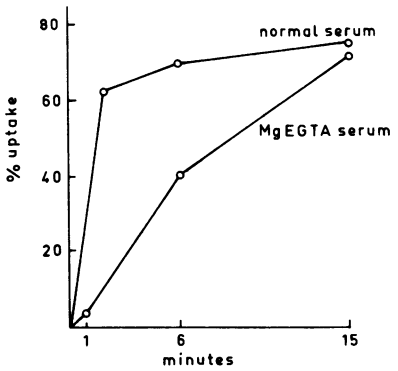

FIG. 2. *Uptake of E. coli F8 by PMNs after opsonization in 20% normal and in 20% MgEGTA-chelated serum. E. coli F8 was opsonized for 1, 6, and 15 min and then added to PMNs. Uptake was measured after 12 min of incubation with PMNs.*

C3 fixation after incubation of these strains in normal serum and in MgEGTA-chelated serum. Maximal immunofluorescence (taken as 100% C3 fixation) was observed with strain F8 after incubation in normal serum. After a 15-min incubation C3 fixation by K^- strains varied between 40 and 70% (Fig. 4A), compared with only 7 to 40% in K^+ strains (Fig. 4B). When MgEGTA-chelated serum instead of normal serum was used, fixation of activated C3 proceeded at a slower rate.

Opsonization of K^+ strain W after heat destruction of K antigen. From the foregoing it could be concluded that K capsular antigens interfered with opsonization. Therefore, *E. coli* K1 strain W was heated for 15 min at 80°C to remove the K antigen before opsonization in 5% normal serum (Fig. 5). After heat treatment no K antigen could be detected by hemagglutination inhibition or by counterimmunoelectrophoresis. The uptake of the heat-killed strain was compared with the uptake of Formalin-killed bacteria (containing K antigen) and with the uptake of the untreated strain. The heat-killed strain (without K antigen) was efficiently taken up by PMNs (70% phagocytosis after 12 min), whereas less than 20% of the Formalin-killed and untreated *E. coli* (both with K antigen present) were phagocytized. Complement consumption and C3 fixation by the heat-killed (K^-) bacteria varied between 60 and 80% after a 30-min incubation in 20% normal serum, compared with only 20% by the untreated (K^+) bacteria (data not shown).

DISCUSSION

Stevens et al. (30; Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E45, p. 88), Young et al. (38), and Van Dijk et al. (33) showed that many *E. coli* strains isolated from blood cultures of patients with *E. coli* bacteremia possessed K antigen and were poorly phagocytized. This inhibition of phagocytosis seems to be related to an impaired recognition of the K^+ strains by the phagocytes due to ineffective opsonization rather than to resistance to intracellular killing (29, 33). All five strains without K antigen used in our study were readily phagocytized after opsonization in 5% normal serum, whereas none of the K^+ strains was taken up by the PMNs after opsonization in 5% normal serum. When the strains were incubated in serum, it was shown that the K^- strains consumed more complement and did so at a much faster rate than did the K^+ strains. All K^- strains and only one K^+ strain were able to consume complement via the alternative pathway. The ability to consume complement and to become opsonized was paralleled by fixation of activated C3 to the cell

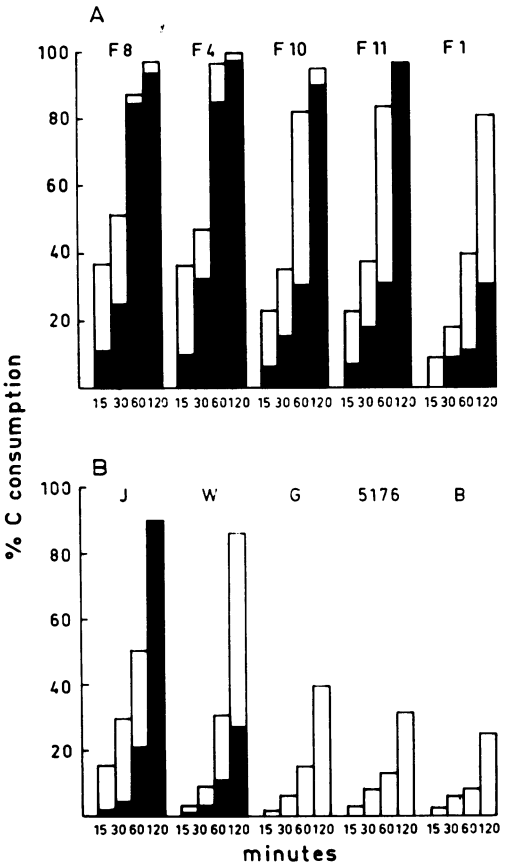

FIG. 3. *Consumption of hemolytic complement (C) by E. coli strains with (B) and without (A) K antigen in normal serum* (□) *and in MgEGTA-chelated serum* (■).

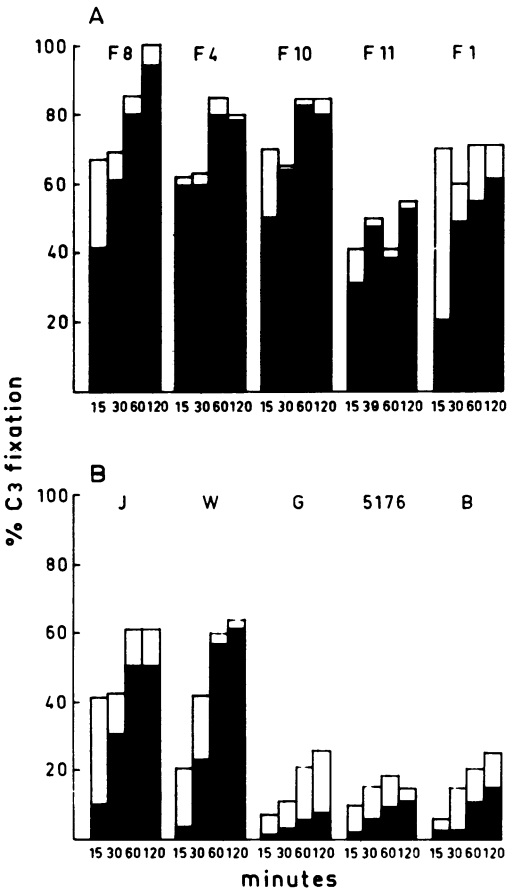

FIG. 4. *C3 fixation of K^+ and K^- E. coli strains in normal and MgEGTA-chelated serum. C3 fixation was measured after 15, 30, 60, and 120 min of incubation of K^- strains (A) and K^+ strains (B) in normal serum (□) and in MgEGTA-chelated serum (■).*

walls of the bacteria. C3 fixation proceeded rapidly in K^- strains. In contrast, only two of the five K^+ strains had C3 bound to their cell walls and the binding was to a lesser extent than in the K^- strains. These two K^+ strains were also the only K^+ strains that were able to consume some complement and were phagocytized.

The assumption can be made that K^+ strains resist phagocytosis due to their inability to consume complement and to become opsonized with C3b molecules. Since it is now well established that *E. coli* cell wall lipopolysaccharide can activate complement without immunoglobulin G (6–8, 20, 26), it is tempting to speculate that uncapsulated *E. coli* strains become opsonized in normal serum through the direct activation of complement via the classical and alternative pathways by lipopolysaccharide and that K antigens in capsulated *E. coli* strains prevent complement consumption by lipopolysaccharide. It is possible, however, that naturally occurring antibodies to cell wall antigens in K^- strains contribute to opsonization and complement consumption as well, and that in normal serum no antibodies to the K and O serotypes of the capsulated strains are found. Since our *E. coli* strains differed in O serotype, it cannot be ruled out that the difference in opsonization between K^+ and K^- strains was due to the presence of opsonic antibodies, to the O serotypes of the K^- strains, and to the absence of antibodies to the O serotypes of the K^+ strains. However, we were not able to detect agglutinating antibodies to any of the strains used. Although it is known that some polysaccharides do not consume complement (2), it is unlikely that the lipopolysaccharides in the cell walls of the K^+ strains are from a type that does not activate complement. We found that when the K antigen was removed by heating, the microorganisms were able to activate complement and to bind opsonic C3 molecules. However, the possibility exists that heating of the *E. coli* strains also interferes with other cell envelope components.

In contrast to our findings, Stevens et al. (30) were not able to detect a difference in the ability to consume complement between K^+ and K^- strains. These findings, contradictory to those obtained by using the opsonophagocytic assay, were explained by the differences in the experimental design of each method in that a larger concentration of bacteria is necessary to demonstrate significant depletion of complement by the total hemolytic complement assay. In our assay 10^8 microorganisms were used; Stevens et al. (30) used between 10^8 and 10^9 microorganisms.

Also, Howard and Glynn (15) concluded that K^+ strains were able to activate complement.

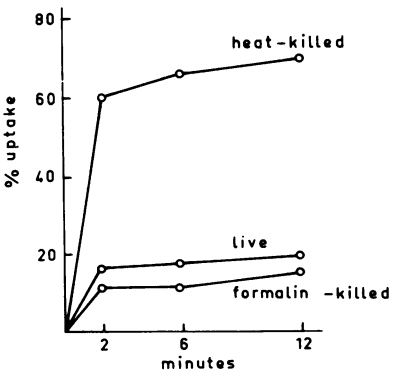

FIG. 5. *Phagocytosis of K^+ E. coli strain W by PMNs. Bacteria were either heat killed, Formalin killed, or untreated before being opsonized in 5% normal serum.*

But this occurred at a distance from its substrate and in their experiments no complement was fixed by the bacterial cell wall. It is possible that differences in the methods used and differences in bacterial strains used are responsible for the discrepancy between the different studies.

Our studies support the view that K antigen is an important virulence factor. Howard and Glynn (15) showed that the amount of capsular polysaccharide was inversely proportional to the rate of clearance from experimentally infected mice. K antigen-rich strains were found to be more likely to produce renal involvement than isolates containing small quantities of K antigen (18).

Kayser and Ahlstedt (19) showed that antibodies against the capsular K1 and K13 antigens had a higher protective capacity in intraperitoneally infected mice than did antibodies to O antigens. Other groups of investigators (4, 22, 38) have shown that antibodies against the core glycolipids of cell wall polysaccharides are protective against various sequellae of gram-negative bacteremia, such as shock and disseminated intravascular coagulation.

McCabe and co-workers (23) could not demonstrate a correlation between the K antigen content of blood culture isolates and the severity and outcome of the bacteremia. The amount of K antigen of blood culture isolates was not significantly greater than that of strains isolated from feces. Pitt (27) concluded from his studies that K1 strains were even more easily cleared from the bloodstream by adults than are other *E. coli* strains. It was not stated, however, whether the non-K1 strains were encapsulated strains.

It should be added that Medeavaris and coworkers (25) concluded that the presence of specific sugars in the O antigenic side chains of the lipopolysaccharide of *E. coli* is responsible for the antiphagocytic capacity of some *E. coli* strains.

Therefore, we are currently studying the identity of opsonic and protective antibodies for *E. coli*. Furthermore, we will study the capacity of complement consumption of K^- variants of K^+ strains by using normal, hyperimmune, and agammaglobulinemic sera.

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