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# Phagocytic Resistance of *Escherichia coli* K-1 Isolates and Relationship to Virulence

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Blood culture isolates from 133 episodes of *Escherichia coli* bacteremia were typed for K-1 capsular antigen by immunodiffusion, utilizing equine antiserum raised against meningococcal group B polysaccharide. Twenty-six percent (34 of 133) of these isolates were positive for K-1 antigen. These 133 strains, 34 K-1 and 99 non-K-1, were tested for susceptibility to phagocytosis. K-1 strains were found to be more resistant to clearance (27%) than non-K-1 strains (71%) when tested in an in vitro opsonophagocytic/killing assay containing normal human granulocytes and plasma. Additional studies demonstrated that resistance was due to decreased phagocytosis rather than diminished intraleukocytic killing. K-1 strains obtained from stool showed a similar degree of resistance to phagocytosis when compared with K-1 blood isolates. A comparison of clinical data on non-neonatal patients with *E. coli* K-1 and non-K-1 bacteremia showed no significant differences in mortality for these two groups. The incidence of shock for patients bacteremic with K-1 strains (74%) was significantly greater than that for patients bacteremic with non-K-1 strains (33%). These differences are attributed to the increased resistance to phagocytosis observed for K-1 versus non-K-1 *E. coli* isolates.

The K-1 antigen, an acid polysaccharide, found on certain strains of *Escherichia coli* has been reported to be a virulence factor in neonatal meningitis and in experimental infections in animals. Neonatal *E. coli* meningitis is caused by K-1 strains in about 85% of cases, and meningitis due to the K-1 organisms has a greater morbidity and mortality than meningitis due to non-K-1 strains (17). The quantity of K-1 antigen and the length of time K-1 antigen is present in serum and cerebrospinal fluid are related to the clinical outcome (16). Mechanisms to explain the increased virulence of K-1 *E. coli* strains have not been deliniated. Evidence suggests that K-1 polysaccharide is a weak immunogen in humans and antibody deficiency may play a crucial role in the pathogenesis of these infections (11). *E. coli* containing K-1 antigen has been reported to be a frequent capsular type among urinary isolates of this species and is more often found on bacteria causing pyelonephritis than on those causing cystitis (8).

In a previous study of neutrophil function in gram-negative bacteremia, we observed that 11 of 30 isolates, all *E. coli*, were relatively resistant to opsonophagocytosis by normal human plasma and granulocytes (polymorphonuclear leukocytes [PMNs]) (18). Subsequently, we determined that many of these strains possessed the K-1 capsule. A relationship between K-1 antigen and non-neonatal *E. coli* bacteremia has not yet been reported. Therefore, we decided to study the relationship of K-1 antigen to opsonization and phagocytosis by normal human plasma and PMNs and then assess the significance of these findings on the outcome of *E. coli* bacteremia. In this report we have demonstrated that most K-1 strains of *E. coli* are more resistant to phagocytosis than non-K-1 strains. The records of patients (non-neonates) with *E. coli* bacteremia were analyzed for differences in morbidity and mortality between those infected with K-1 versus non-K-1 isolates.

(This study was presented in part at the 17th Interscience Conference on Antimicrobial Agents and Chemotherapy, New York, N.Y., October 1977.)

## MATERIALS AND METHODS

**Isolation of *E. coli* strains and K-1 typing.** One hundred thirty-three blood culture isolates of *E. coli* from the UCLA Center for the Health Sciences obtained between 1972 and 1976 were studied. They were identified as *E. coli* by standard criteria (2). K-1 typing was performed by an immunodiffusion technique, using equine group B meningococcal antiserum (horse 46) (kindly provided by John B. Robbins, Bureau of

Biologics, Food and Drug Administration, Bethesda, Md.) (17). A freeze-thaw extract of an 18-h growth of $10^9$ *E. coli* was used as the antigen. Purified group B meningococcal polysaccharide and capsular polysaccharide prepared from *E. coli* O7 K-1 were run as controls. A strain was designated as K-1 if it formed a precipitation band identical to that seen for the two control polysaccharides. Further confirmatory studies were carried out with the antiserum agar technique as described by Kaijser, using a 10% concentration of equine antiserum (9).

**Neutrophil function studies.** Forty milliliters of blood was obtained from a normal control (healthy laboratory personnel). It was heparinized (final concentration, 10 U of aqueous heparin per ml) and combined with 6% clinical dextran (average molecular weight, 70,000; Abbott Laboratories, Chicago Ill.) in a ratio of 2 volumes of blood to 1 volume of dextran. After 1 h of sedimentation at room temperature, the leukocyte-rich supernatant layer was separated, centrifuged, washed once, and suspended in 0.9% saline solution. After hypotonic lysis of erythrocytes, the preparation was again washed, centrifuged, and suspended in Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) with 0.1% gelatin. More than 95% of PMNs appeared viable by their ability to exclude 0.4% trypan blue. Quantitative and differential leukocyte counts were made, and the final suspension was adjusted to yield a concentration of $10^7$ PMNs/ml.

Quantitative PMN opsonophagocytic/killing assays were modified after the method of Hirsch and Strauss and Maaløe (5, 12). Duplicate plastic tubes containing 0.1 ml of plasma, 0.5 ml of the PMN mixture, 0.1 ml of a $10^8$ suspension of bacteria, and 0.3 ml of Hanks balanced salt solution were continuously rotated at 37°C. Reaction mixtures containing plasma and bacteria without PMNs were included to test for plasma sensitivity of the organism. At 0, 30, 60, and 120 min, samples were taken and diluted with distilled water, and the number of bacteria per milliliter was determined by the pour plate method. The final concentrations of PMNs and bacteria were $5 \times 10^6$ and $10^7$, respectively, yielding a ratio of bacteria to PMNs of 2:1 in 10% plasma. A laboratory test strain sensitive to opsonophagocytosis and killing by normal plasma and PMNs was used as a control in all experiments. The percentage of bacteria phagocytized and killed by PMNs was calculated by using the difference in counts from tubes with and without PMNs. Although there was some decrease in number of organisms at 30 min, maximum killing activity was noted at 60 min, and no further decrease was observed at 120 min. For this reason only the amount of bacteria cleared from the assay at 60 min is shown. All strains were tested against pooled plasma obtained from three healthy controls. The plasma was aliquoted into 2-ml volumes and stored at −80°C. A total of three different pools were used during the study.

**Assessment of complement activity in pooled plasma.** To ensure that the frozen plasma did not lose its heat-labile opsonic activity during the course of the study, each batch of pooled plasma was checked for both alternate and classical pathway complement activity. Fresh plasma obtained from normal healthy donors was used in the phagocytic system as described above. In some experiments $MgCl_2$ ethyleneglycol tetraacetic acid was added to plasma to chelate $Ca^{2+}$, a method which blocks classical pathway complement activity (3). Two *E. coli* strains, one opsonized by the classical and the other by the alternate pathway of complement activation, were defined by this method. These two *E. coli* strains were used in the phagocytic assay to monitor the activity of complement in the plasma pools, at the beginning and end of the study.

**Measurement of phagocytosis.** A visual index of phagocytosis was determined by combining $5 \times 10^6$ granulocytes and $2.5 \times 10^8$ viable bacteria in 10% pooled plasma and Hanks balanced salt solution. After 20 min of continuous mixing at 37°C, a cell monolayer was prepared, Wright stained, and examined under a light microscope. The percentage of granulocytes associated with bacteria and the average number of bacteria per granulocyte were determined by counting 200 cells. Monolayers were prepared for electron microscopy by a previously described method (F. J. Silverblatt and I. Ofek, Proceedings of the Third International Symposium on Pyelonephritis, London 1976, in press). They were examined under the electron microscope to see if the bacteria which were cell associated by light microscopy had been ingested by the granulocytes.

**Clinical studies.** The records of 133 non-neonates with *E. coli* bacteremia were reviewed retrospectively without knowledge of the pathogen's sensitivity to phagocytosis or the presence of K-1 capsular antigen. The occurrence of shock and the mortality from bacteremia were determined for each patient. Shock was considered to be present if there was a decrease in blood pressure to <90/60 or a >70-mm drop in hypertensive patients (15). Patients who died were considered to have shock only if the decrease in blood pressure was noted more than 12 h before their demise. Death was considered due to bacteremia in patients who died within 3 days of onset. In patients who died after a longer period, additional clinical features, pre- and postmortem cultures, and pathological findings were used to assess the cause of death. Patients were classified according to the severity of their underlying illness into those with rapidly fatal, ultimately fatal, and nonfatal underlying disease (14). Additional clinical information on patients was obtained, including the type of underlying disease, age, sex, and the source of bacteremia.

**Statistical analysis.** A comparison between K-1 and non-K-1 strains for their resistance to in vitro phagocytosis was analyzed by Student's *t* test. Difference among patients with K-1 and non-K-1 *E. coli* bacteremia and a comparison of the incidence of shock and death in these two groups were analyzed by chi-square (4).

## RESULTS

**K-1 typing.** One hundred thirty-three blood culture isolates of *E. coli* were tested for the presence of K-1 capsular antigen. This was performed by using an immunodiffusion technique with equine antiserum raised against meningococcal type B capsular polysaccharide. Ninety-

nine (74.4%) were non-K-1, whereas 34 (25.6%) contained the K-1 antigen of *E. coli*.

**Neutrophil function studies.** These 133 bacteremic isolates were tested in a quantitative PMN opsonophagocytic/killing assay. All K-1 strains and 97 of 99 non-K-1 strains were resistant to killing by plasma alone. With the addition of PMNs to the system, the mean percentage of bacteria killed in 1 h was 71% for non-K-1 blood isolates and 27% for K-1 isolates. This difference was significant at $P > 0.001$ by Student's $t$ test (Fig. 1). To determine if resistance to clearance in this assay was limited to those *E. coli* K-1 strains obtained from blood cultures, we tested 21 K-1 stool isolates in this system. The mean percentage of organisms phagocytized and killed at 60 min was the same for both blood and stool isolates (Fig. 1).

A visual index of phagocytosis was determined by examining a Wright-stained monolayer of PMNs, plasma, and bacteria under a light microscope. Five *E. coli* strains which showed a marked decrease in bacterial counts (82 to 98% killed) in the quantitative PMN opsonophagocytic/killing assay and five strains which showed a slight decrease in counts (5 to 33% killed) were examined under the microscope. Organisms more sensitive to killing showed a high degree of cell association and a large number of bacteria per PMN, whereas those organisms more resistant to killing tended to be non-cell associated and only a rare organism per PMN was noted (Table 1). To determine if cell-associated bacteria were ingested or simply attached to the surface of the granulocyte, electron micrographs were made. The electron micrographs confirmed that the majority of bacteria associated with PMNs had been ingested by the cell and were contained within phagocytic vacuoles of the granulocyte. An example of the light microscopy and the electron microscopy study of strain 4824 is shown (Fig. 2 and 3).

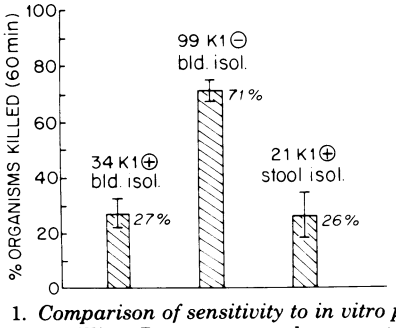


FIG. 1. *Comparison of sensitivity to in vitro phagocytosis and killing. Bars represent the percentage of organisms phagocytized and killed by normal plasma and PMNs after 1 h of incubation. Brackets are ± standard error of the mean. Difference in mean percent killed for 34 K-1 and 99 non-K-1 strains is significant by Student's t test ($P < 0.001$).*

TABLE 1. *Visual index of phagocytosis of E. coli: comparison of in vitro bacterial clearance with susceptibility to ingestion by PMNs for organisms sensitive and resistant to phagocytosis*

| Strain | % PMNs associated with bacteria | Bacteria per PMN | % Bacteria killed[a] |
|---|---|---|---|
| Sensitive | | | |
| 4824[b] | 88 | 20 | 96 |
| 4197 | 81 | 15 | 95 |
| 4185 | 75 | 11 | 98 |
| 3524 | 99 | 20 | 82 |
| 3343 | 92 | 14 | 96 |
| Mean ± SEM[c] | 87 ± 4.2 | 16.2 ± 1.7 | 91.4 ± 3.1 |
| Resistant | | | |
| 5008 | 0 | 0 | 5 |
| 5011 | 37 | 2 | 20 |
| 4120 | 10 | 1 | 33 |
| 3177 | 10 | 1 | 18 |
| 4310 | 19 | 2 | 5 |
| Mean ± SEM | 15.2 ± 6.2 | 1.2 ± .37 | 16.2 ± 5.2 |

[a] Percentage of organisms killed by normal PMNs and pooled plasma in 60 min.
[b] Light and electron microscopy of phagocytosis of this organism is shown in Fig. 2 and 3.
[c] SEM, Standard error of the mean.

**Assessment of complement activity in pooled plasma.** Mg-ethyleneglycol tetraacetic acid, which blocks classical pathway complement activation by chelating $Ca^{2+}$, was used to identify two strains of *E. coli*, one opsonized by the alternate (3102) and one by the classical (3599) pathway. Frozen plasma from each plasma pool was compared with fresh normal plasma (at the beginning and end of the study) for complement-dependent opsonic activity. Results show that frozen, pooled plasma was comparable to fresh plasma when tested for alternate and classical pathway opsonic activity (Fig. 4 and 5).

**Comparison of mortality and morbidity for K-1 and non-K-1 bacteremia.** A retrospective chart review of 133 episodes of *E. coli* bacteremia was undertaken. Thirty-four episodes or 26% of *E. coli* sepsis were due to K-1 strains. There was a slightly higher mortality for K-1 versus non-K-1 *E. coli* sepsis, but the difference was not significant (Table 2). Shock was noted in 74% of patients with K-1 compared to 33% of patients with non-K-1 bacteremia, and the difference was significant at $P < 0.01$. Because most of the patients who died also had shock, the incidence of nonlethal shock for both groups was calculated. Forty-four percent of pa-

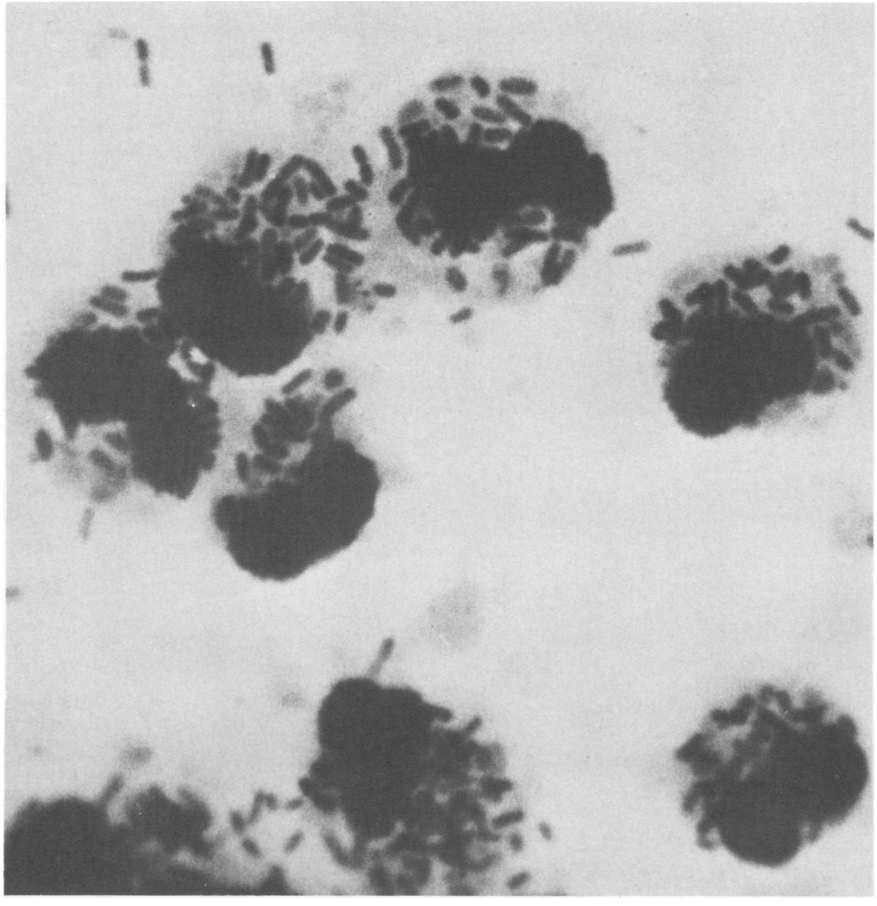

FIG. 2. *Photomicrograph depicting phagocytosis of E. coli 4824 by human granulocytes. Note the large number of organisms associated with PMNs. ×1,000.*

tients with K-1 bacteremia had nonlethal shock compared to 16% with non-K-1 bacteremia ($P < 0.01$). Patients were subdivided into those with rapidly fatal, ultimately fatal, and nonfatal underlying disease, and the incidence of death, shock, and nonlethal shock was calculated for each group. Among patients with rapidly fatal disease there were no differences noted for the three parameters. For patients with ultimately fatal and nonfatal disease the incidence of shock was greater when bacteremia was due to K-1 strains (Table 2).

We next compared the outcome of bacteremia for K-1 and non-K-1 strains that had a similar degree of resistance to phagocytosis. Twenty-eight K-1 strains with a mean of 18% phagocytosis (range, 0 to 66%) and 35 non-K-1 strains with a mean of 24% phagocytosis (range, 0 to 68%) were selected. This represents all bacteremic *E. coli* isolates with less than 75% of organisms phagocytized at 1 h. The use of 75% as a cutoff is based on the fact that sensitive laboratory strains, used as controls in this and in a previous study, consistently showed greater than 75% of these organisms phagocytized and killed by normal PMNs and plasma (18). For bacteremia due to these two groups of *E. coli,* matched for their resistance to phagocytosis, the incidence of death and shock was not significantly different. Death was noted in 41% and shock in 79% of patients bacteremic with K-1 strains and for patients bacteremic with non-K-1 strains the incidence of death and shock was 39 and 62%, respectively.

**Clinical characteristics of study population.** A comparison of the source of *E. coli* bacteremia for K-1 and non-K-1 isolates showed that approximately half the episodes originated in the urinary tract for both groups. In addition, wound and skin, biliary, pulmonary, and gastrointestinal foci were comparable for K-1 and non-K-1 strains (Table 3). The median age and

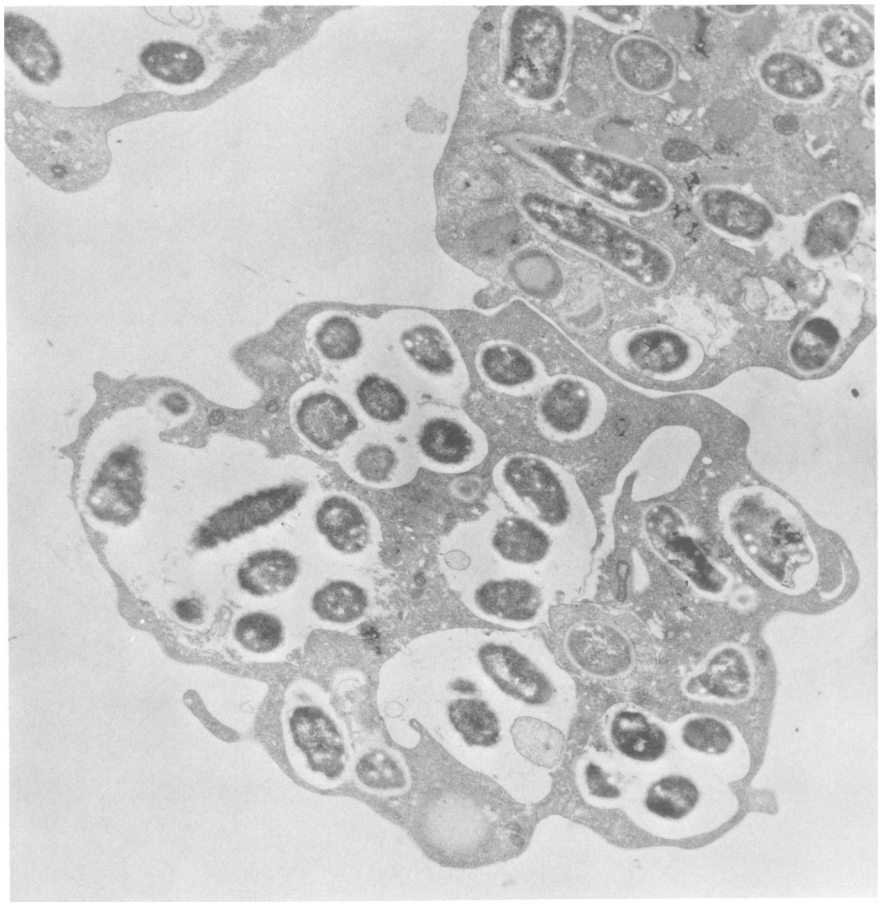

FIG. 3. *Electron micrograph of granulocytes and E. coli 4824. Note that each phagocytic cell has ingested large numbers of bacteria which are contained within phagocytic vacuoles. ×7,000.*

male-to-female ratio were comparable between the K-1 and non-K-1 groups (18 female, 16 male, and 45.2 years versus 57 female, 42 male, and 44.9 years, respectively). A similar percentage of K-1 isolates and non-K-1 isolates accounted for bacteremia in patients with rapidly fatal, ultimately fatal, and nonfatal disease. Acute leukemia was noted in 18% of cases with K-1 and 22.5% of cases with non-K-1 sepsis, and other malignancies made up an additional 20 to 25% of the patients in each group.

## DISCUSSION

We have demonstrated an association between *E. coli* K-1 capsular antigen and resistance to clearance in an in vitro opsonophagocytic/killing assay. By using three separate plasma pools, obtained from healthy persons, we minimized differences in plasma as a variable in this system. The frozen, pooled plasma retained its complement activity for both classical and alternate pathway opsonization. There was a close correlation between a visual index of cell-associated bacteria and degree of clearance in the assay. Examination of monolayers by electron microscopy revealed that the majority of cell-associated bacteria had been ingested and were located within phagocytic vacuoles. In an earlier study we noted that intraleukocytic killing was identical for both resistant and sensitive strains of *E. coli*. These results are consistent with a previous study and show that K-1 strains of *E. coli* are resistant to phagocytosis rather than to intraleukocytic killing (6). A recent report has shown that K-1 isolates, resistant to phagocytosis, become sensitive when opsonized by plasma containing antibody raised against the K-1 capsule (W. Welsh, W. Martin, L. S. Young, and P. Stevens, Abstr. Annu. Meet. 1977, Am. Soc. Microbiol. E44, p. 88).

Protection against *E. coli* K-1 infection in an

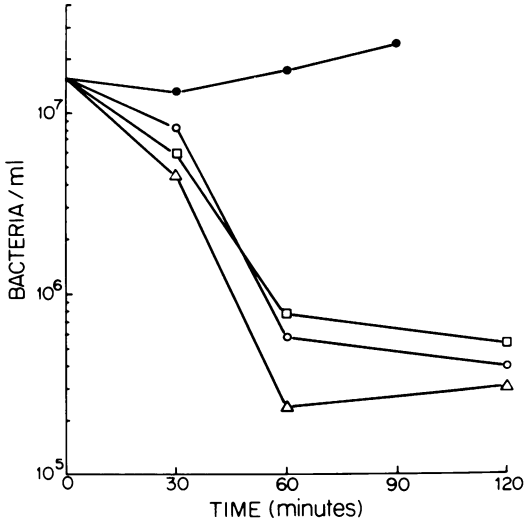


FIG. 4. *Pooled plasma and fresh plasma compared for alternate pathway complement activity. All experiments contain plasma, PMNs, and bacteria (E. coli 3102). Symbols: (△) 10% fresh plasma, 98% killed; (●) 10% fresh plasma heated at 56°C for 30 min, 0% killed; (○) 10% fresh plasma with 10 mM Mg-ethyleneglycol tetraacetic acid (EGTA), 96% killed; (□) 10% frozen, pooled plasma with 10 mM Mg-EGTA; 94% killed (range, 92 to 99%). This is a mean of 12 assays run on three separate plasma pools.*

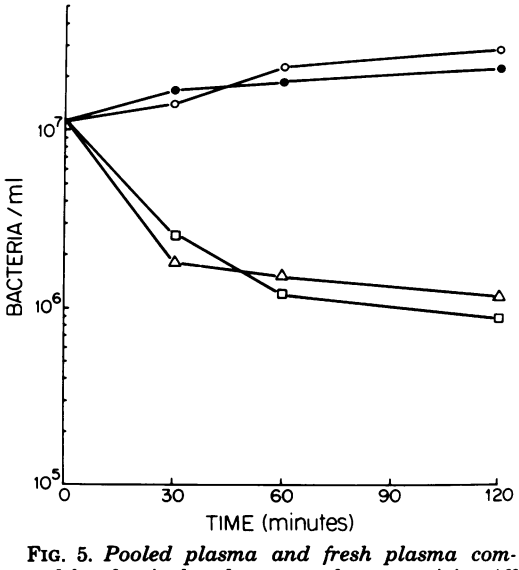


FIG. 5. *Pooled plasma and fresh plasma compared for classical pathway complement activity. All experiments contain plasma, PMNs, and bacteria (E. coli 3599). Symbols: (△) 10% fresh plasma, 89% killed; (●) 10% fresh plasma heated at 56°C for 30 min, 0% killed; (○) 10% fresh plasma with 10 mM Mg-ethyleneglycol tetraacetic acid, 0% killed; (□) 10% frozen, pooled plasma, 91% killed (range, 83 to 99%). This is a mean of 10 assays run on 3 separate plasma pools.*

TABLE 2. *Morbidity and mortality of E. coli bacteremia*

| Patients[a] (no. tested) | No. of patients | Deaths (%) | Shock, total[b] (%) | Nonlethal shock (%) |
|---|---|---|---|---|
| All (133) | | | | |
| K1+ | 34 | 11 (32) | 25 (74) | 15 (44) |
| K1− | 99 | 24 (24) | 33 (33) | 16 (16) |
| *P*[c] | | NS[d] | <0.01 | <0.01 |
| With RFD (29) | | | | |
| K1+ | 6 | 3 (50) | 5 (83) | 3 (50) |
| K1− | 23 | 15 (65) | 13 (57) | 4 (17) |
| *P* | | NS | NS | NS |
| With UFD (43) | | | | |
| K1+ | 16 | 8 (50) | 14 (88) | 6 (38) |
| K1− | 27 | 6 (22) | 10 (37) | 4 (15) |
| *P* | | NS | <0.01 | NS |
| With NFD (61) | | | | |
| K1+ | 12 | 0 (0) | 6 (50) | 6 (50) |
| K1− | 49 | 3 (6) | 10 (20) | 8 (16) |
| *P* | | NS | <0.05 | <0.05 |

[a] RFD, Rapidly fatal disease; UFD, ultimately fatal disease; NFD, nonfatal disease.

[b] Not all patients who died had shock as defined in the text.

[c] Analyzed by chi-square.

[d] NS, Not significant.

TABLE 3. *Sources of E. coli bacteremia*

| Source | K-1 | | Non-K-1 | |
|---|---|---|---|---|
| | No. | % | No. | % |
| Urinary tract | 17 | 50 | 52 | 53 |
| Wound or skin | 7 | 21 | 11 | 11 |
| Biliary | 3 | 9 | 8 | 8 |
| Pulmonary | 0 | 0 | 4 | 4 |
| Gastrointestinal | 1 | 3 | 5 | 5 |
| Unknown | 6 | 17 | 19 | 19 |

animal model is afforded by antibody to group B meningococcal polysaccharide (closely related or identical to K-1 antigen) (17). A protective role for anti-K-1 antibody has also been reported (10). However, since both K-1 antigen and meningococcal type B polysaccharide are poor immunogens, it is not surprising that normal human plasma had weak opsonic activity against most K-1-containing *E. coli* (both blood and stool isolates).

K-1 bacteremia accounted for 26% of *E. coli* blood stream infections in our study. This figure is virtually identical with the incidence of K-1 *E. coli* found in stool cultures (7). This is in contrast to the high incidence of *E. coli* K-1 meningitis (84%) and the modest increase in bacteremia (39%) seen in neonates (17). Our inability to detect a marked increase in the percentage of K-1 isolates causing *E. coli* bac-

teremia in adults is in agreement with previous reports (13). Of interest, we found that approximately 50% of *E. coli* K-1 and non-K-1 bacteremic isolates originated in the urinary tract.

A comparison of morbidity and mortality of *E. coli* bacteremia showed a significant increase in shock but not death for patients infected with K-1 versus non-K-1 strains. A majority of the deaths occurred among the 29 patients with rapidly fatal disease, and the incidence of death for this group as a whole was >60%. The overall incidence of shock and nonlethal shock was significantly increased for patients bacteremic with K-1 strains, but there were no differences in either parameter for patients with rapidly fatal disease. These data suggest that K-1 strains are more virulent than non-K-1 strains in patients with *E. coli* bacteremia. In addition, our findings underscore the importance of the underlying illness in determining the outcome of serious infection. The incidence of shock and death was similar for patients bacteremic with K-1 and non-K-1 strains which were matched for their resistance to phagocytosis. This shows that resistance to phagocytosis, rather than the presence of K-1 antigen per se, is responsible for increased virulence noted for K-1 strains of *E. coli*.

*E. coli* isolates which lack K-1 antigen and resist phagocytosis are an interesting subgroup which needs further study. Capsular antigens other than K-1 may be responsible for this property. Alternatively, certain O antigens or specific combinations of O and K serotypes may confer increased virulence.

An association among capsular antigen, resistance to phagocytosis, and increased virulence is not limited to *E. coli*. The type III pneumococcus, one of the more virulent strains, possesses a large polysaccharide capsule and is resistant to phagocytosis (19). The VI capsule of *Salmonella typhi* has been shown to confer resistance to phagocytosis (1). A precise mechanism responsible for the weak opsonic activity of normal plasma for K-1 *E. coli* strains is not known. One possibility is that this capsular substance physically obscures receptor sites for immunoglobulin or complement on the cell wall. In addition, opsonization of K-1 *E. coli* appears to require specific antibody for classical pathway complement activation, whereas non-K-1 strains may be opsonized in the absence of specific antibody by the alternate pathway (P. Stevens, S. Huang, W. Welch, and L. S. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E45, p. 88). Also, K-1 is only weakly immunogenic and normal human plasma often lacks anti-K-1 antibody. Thus, antibody deficiency alone, or in concert with the two previously mentioned mechanisms, may be responsible for the increased resistance to phagocytosis seen among *E. coli* K-1 strains.

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