ESCHERICHIA COLI K ANTIGEN IN RELATION TO SERUM-INDUCED LYSIS AND PHAGOCYTOSIS

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Both non-specific immunity and specific humoral immunity mediated by antibodies to bacterial cell envelope components are important host defence mechanisms against invasion by gram-negative bacilli. The presence of depressed levels of polymorphonuclear leucocytes (PMNs) or impaired phagocytic function have been found to correlate with an increased susceptibility to infections (Bodey et al., 1966; Winkelstein and Drachman, 1974). Also the presence of low serum levels of opsonising antibodies directed against bacterial envelope antigens correlate with the occurrence of a high morbidity in septicaemia caused by gram-negative bacilli (Young et al., 1977a; Zinner and McCabe, 1976). There are conflicting reports regarding the specificity of these protective antibodies.

The cell envelope of members of the Enterobacteriaceae consists of an outer membrane, containing lipopolysaccharide (LPS) and lipoproteins, and a rigid inner layer containing peptidoglycan. LPS contains the O antigen linked to lipid A through a common core structure (Lüderitz, Staub and Westphal, 1966). Antibodies against antigenic determinants in the core region have been reported to be protective against the sequelae of bacteremia due to gram-negative bacilli (McCabe, 1972; Young, Stevens and Ingram, 1975). These antibodies seem to act primarily as antitoxins rather than as opsonins (Braude, Douglas and Davis, 1973). Antibodies against O-antigenic side chains seem to enhance phagocytosis (Medeavaris, Camitta and Heath, 1968; Young, 1974). Several other studies indicate that only capsular polysaccharides (K antigens) have antiphagocytic activity and suggest that strains possessing K antigens have enhanced virulence (Dri et al., 1976; Young et al., 1977b). Meanwhile Howard and Glynn (1971) demonstrated that K antigens also inhibit the complement-dependent bactericidal activity of serum.

Recent workers, however, were unable to find a correlation between the presence of K antigen and the capacity of Escherichia coli to resist phagocytosis and the bactericidal activity of serum (Taylor, 1975; Björksten et al., 1976); McCabe et al. (1975) could not demonstrate a relationship between the amount of K antigen produced by different E. coli strains and their virulence in man.

The relation between the presence of K antigen and resistance to phagocytosis and the bacteriolytic action of serum has been re-investigated in this study by examination of E. coli strains isolated from faeces and blood.

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MATERIALS AND METHODS

Bacteria. Twelve strains of *E. coli* were isolated from cultures of stool obtained from healthy volunteers and 14 were isolated from blood cultures of patients suffering from *E. coli* bacteremia. *E. coli* strain O111 B4 and its UDP-galactose 4-epimerase-deficient mutant *E. coli* strain J5 together with *E. coli* strain F464 and its *rfa* mutant F583 (kindly provided by Dr E. J. J. Lugtenberg) were included in the study.

Preparation of K antigen was carried out according to Glynn and Howard (1970). Young (4-h) cultures of *E. coli* were used to inoculate three nutrient-agar plates. After incubation overnight at 37°C, the growth on the three plates was harvested with 5 ml of phosphate-buffered saline, pH 7.4 (PBS). The suspended bacteria were precipitated by addition of three volumes of cold acetone and were harvested by centrifugation at 1600 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 PBS and exposed to ultrasonic disruption for 3 min. at 4°C (Sonifier Cell Disruptor B12, Branson Sonic Power Co, Danbury, Co, USA), after which the particulate material was removed by centrifugation (1600 g for 30 min.). K antigen was precipitated from the resulting supernate by addition of three volumes of cold ethanol (96% v/v) and was harvested by centrifugation (1600 g for 10 min.). The sediment was resuspended in 1.0 ml of PBS, dialysed overnight against PBS and the volume was adjusted to 2.0 ml.

Determination of K antigen by haemagglutination inhibition (HAI) activity. The HAI technique of Glynn and Howard (1970) was used as follows: rabbit anti-sheep erythrocyte antiserum (Rijks Instituut voor de Volksgezondheid, RIV, Bilthoven, Netherlands) was titrated in serial two-fold dilutions in 0.25 ml volumes and 0.05 ml of 2.5% (v/v) ovine erythrocytes was added to each tube. Titrations with ovine erythrocytes that had been incubated for 30 min. at 37°C with dilutions (ranging from 1 in 2 to 1 in 64) of the K antigen preparation were set up in parallel. The inhibitory titre of K antigen was taken as the dilution of the K antigen preparation that produced a two-fold reduction in the haemagglutinating activity of the anti-sheep erythrocyte antiserum. K1 antigen was detected by countercurrent immuno-electrophoresis (Robbins et al., 1974); glass slides (LKB 2117-402, 8.4 × 9.4 cm) were coated with 1% agarose in barbitone buffer, pH 8.8 and meningococcal group B antiserum (Wellcome, Beckenham, Kent) was used as a reference antiserum.

[3H]-labelled bacterial suspensions. Single colonies from nutrient agar plates were inoculated into 5-ml of Mueller-Hinton broth (Difco, Detroit, Mi, USA) containing 0.02 mCi [3H]-methyl-thymidine (specific activity 5 Ci per mmol, Radiochemicals, Amersham, England) and 1.25 mg of Deoxyadenosine (British Drug Houses). After growth for 18 h at 37°C, the bacteria were washed three times in PBS and adjusted to a final bacterial concentration of 2.5 × 10⁸ colony forming units (c.f.u.) per ml as previously described (Verhoef et al., 1977).

Serum. Sera from 10 healthy donors was pooled and stored in 0.5-ml portions at −70°C. For opsonisation and serum-lysis studies, serum was thawed shortly before use and diluted in Hank’s Balanced Salt Solution containing 0.1% gelatin (HBSS).

Serum bactericidal assay. The bactericidal activity of serum was tested by the method of Friedlander (1975). [3H]-thymidine-labelled bacterial culture (5 × 10⁷ c.f.u.) were mixed with different dilutions of human serum in polypropylene vials (Biovials, Beckman, Chicago, Ill, USA) in a total volume of one ml. At time zero and at various intervals after incubation in a water-bath at 37°C, vials were placed in ice. After centrifugation (1600 g for 20 min.) the radioactivity in the sediment and supernate was measured in a liquid scintillation counter (Mark II, Nuclear Chicago, Chicago, Ill, USA) after the addition of 2.5 ml volumes of scintillation fluid (toluene containing fluoralloy, TLA, Beckman, and 20% Biosolve-3, Beckman) to the pellets; the supernates were each suspended in 10 ml of scintillation liquid. Percentage lysis was expressed as the percent of total radioactivity that was released into the supernate.

Leucocytes. Blood samples from healthy volunteers, aged 18–69 years (mean age 28) were collected in heparinised syringes (10U heparin per ml of blood). PMNs were prepared by a method modified from that of Böyum (1968) as described by Verbrugh et al., (1978).
K ANTIGEN AS A VIRULENCE FACTOR

The PMNs were resuspended in HBSS and total and differential counts were performed; the leucocyte suspension was adjusted to contain $5 \times 10^6$ PMNs per ml.

**Opsonisation of E. coli strains.** Bacterial suspensions (0.3-ml volumes) containing $7.5 \times 10^7$ c.f.u., were placed in plastic tubes (12 × 75 mm, Falcon Plastics, Oxnard, Ca) and incubated in the presence of 1.2 ml of 5% normal human serum for 30 min at 37°C. Serum was removed by centrifugation (15 min at 1600 $g$) and the bacteria were resuspended in HBSS to a final concentration of $5 \times 10^7$ c.f.u. per ml.

**Phagocytic mixtures and determinations of phagocytosis.** Phagocytosis assays were performed by a slight modification of a previously described method (Verhoef et al., 1977). Leucocyte suspensions (0.2 ml-volumes) were added to 0.2-ml portions of opsonised radio-labelled bacterial suspension. Phagocytosis mixtures were shaken at 150 r.p.m. at an angle of 30°C in a water-bath at 37°C and sample vials were removed at 2, 6, and 12 min.; they were placed in an ice-bath and 2.5 ml of ice-cold PBS was added to each vial. After centrifugation (160 $g$ for 5 min. at 4°C) leucocyte pellets were washed twice with ice-cold PBS to remove non-leucocyte-associated bacteria; these pellets, containing leucocyte-associated bacteria, were dissolved in 2.5 ml of scintillation fluid.

To determine total sedimentable radioactivity, representing leucocyte-associated plus non-leucocyte-associated bacteria, 2.5 ml of ice-cold PBS was added to a single control vial and the pellet obtained after centrifugation (1600 $g$ 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.

**RESULTS**

*Sensitivity of E. coli strains to serum*

From 26 strains of *E. coli* (12 from stool cultures and 14 from blood cultures) only four were sensitive to the bacteriolytic activity of normal serum (table I).

Three of these sensitive strains had no detectable capsular antigen. Only four of the 12 strains of *E. coli* isolated from stool cultures of healthy volunteers had detectable amounts of K antigen; on the other hand 12 of 14 strains isolated from blood cultures had detectable amounts of capsular antigen.

Because most of the K+ and K- strains were resistant to the lytic action of normal serum, the lytic action of serum on four well defined strains lacking K antigen or having incomplete lipopolysaccharide was determined. As can

<table>
<thead>
<tr>
<th>Serum reaction†</th>
<th>Reaction of K- strains/total no. of K- strains isolated from stools</th>
<th>Reaction of K+ strains/total no. of K+ strains isolated from blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive to lysis</td>
<td>2/8</td>
<td>1/2</td>
</tr>
<tr>
<td>Resistant to lysis</td>
<td>6/8</td>
<td>1/2</td>
</tr>
</tbody>
</table>

* Percent lysis was determined as the percent of bacterial radioactivity that was released in the supernate after incubation in 20% serum at 37°C.
† Strains were considered to be serum-sensitive when more than 50% of the bacterial radioactivity was released. None of the serum-resistant strains released more than 20% of their radioactivity.
be seen in the figure the R mutants J5 and F583 (strains lacking O-antigenic side chains) of the parent strains O111 B4 and F464 respectively, were highly sensitive; between 45% and 75% lysis occurred in the presence of 10% serum. Almost no lysis was observed when the parent strains were incubated in 10% serum.

**Phagocytosis of E. coli by human polymorphonuclear leucocytes**

After opsonisation in 5% normal human serum, *E. coli* strains were incubated with PMNs. The uptake of bacteria after 12 min. incubation with PMNs is shown in table II. Only 4 strains isolated from blood cultures were readily ingested by PMNs showing greater than 50% uptake after 12 min. Two of these strains were the only ones lacking detectable amounts of K antigen. All 10 K− strains (2 blood isolates and 8 stool isolates) were readily phagocytosed.

Seven of 16 K+ strains had K1 antigen and each K1 strain exhibited less than 50% uptake in the phagocytosis test; indeed six of the K1 strains showed less than 20% uptake. When strains were ranked according to the amount of K antigen present, as measured by the haemagglutination test, it was found (table III) that 8 of the 10 strains rich in K antigen (10 HA1 units) were resistant to ingestion by PMNs. By contrast only two of six strains with low amounts of K antigen were resistant to phagocytosis.

![Figure](image_url)
TABLE II

Phagocytosis of strains of E. coli by human polymorphonuclear leucocytes

<table>
<thead>
<tr>
<th>Extent of phagocytosis*</th>
<th>Reaction of K⁻ strains/total no. of K⁻ strains isolated from stools</th>
<th>Reaction of K⁺ strains/total no. of K⁺ strains isolated from blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 50%</td>
<td>5/8</td>
<td>1/4</td>
</tr>
<tr>
<td>20-50%</td>
<td>3/8</td>
<td>0/4</td>
</tr>
<tr>
<td>&lt; 200%</td>
<td>0/8</td>
<td>3/4(2)</td>
</tr>
</tbody>
</table>

* Phagocytosis was measured after 12 min. incubation of PMNs with pre-opsonised bacteria at 37°C (see Materials and Methods).
† Figures in parenthesis indicate number of strains possessing K1 antigen.

TABLE III

Susceptibility to phagocytosis in relation to amount of K antigen

<table>
<thead>
<tr>
<th>Extent of phagocytosis*</th>
<th>Reaction of strains/total no. of strains grouped according to the amount of K-antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K⁻ (O HAI)†</td>
</tr>
<tr>
<td>&gt; 50%</td>
<td>7/10</td>
</tr>
<tr>
<td>20-50%</td>
<td>3/10</td>
</tr>
<tr>
<td>&lt; 20%</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Phagocytosis was measured after 12 min. incubation of PMNs and pre-opsonised bacteria at 37°C (see Materials and Methods).
† HAI unit is the reciprocal of the dilution of the K-antigen preparation that produces a two-fold reduction of the haemagglutination titre of antiserum to ovine erythrocytes (see Materials and Methods).

DISCUSSION

This study provides indirect evidence that both the bacteriolytic activity of serum and phagocytosis are important factors in host resistance to invasive strains of E. coli; strains that are resistant to the lytic action of serum and that escape phagocytosis by PMNs are able to cause generalised infections. Only 4 of the 14 strains isolated from blood cultures of patients with E. coli bacteremia were readily phagocytosed. In contrast, only three out of 12 strains isolated from stools of healthy volunteers were not ingested. All the strains that were resistant to phagocytosis had detectable amounts of K antigen and those strains that possessed high amounts of K antigen were the most resistant to phagocytosis. None of the strains that were readily taken up by PMNs had high amounts of K antigen. These in-vitro findings therefore support the view that K antigen is an important virulence factor. Inhibition of phagocytosis seems to be related to the reduced ability of phagocytes to recognise K⁺ strains and to the impairment of attachment to the leucocyte surface (Dri et al., 1976).

According to Kaijser (1973) antibodies to K antigen are protective. Also others (Young et al., 1977b) concluded that opsonisation of K⁺ strains requires...
specific antibodies, whereas strains of *E. coli* lacking K antigen are opsonised in the absence of specific antibodies, the latter strains being able to activate complement by the alternative route. Howard and Glynn (1971) showed that the amount of capsular polysaccharide is inversely proportional to the rate of clearance of organisms from experimentally infected mice. Also K-antigen rich strains were found to be more likely to produce renal involvement than isolates containing small quantities of K antigen (Kaijser, 1973) and antigenic K1 polysaccharide was found to be particularly important as a virulence factor. These findings however are disputed by others (Medeavaris *et al.*, 1968; Björksten *et al.*, 1976), who have concluded that the presence or absence of specific sugars in the O-antigenic side chains in the LPS of *E. coli* are the determinants of antiphagocytic capacity and virulence. It is notable that Medeavaris and co-workers (1968) used *E. coli* strain O111 B4 and a mutant derivative J5, that lacked O-antigenic side chains; both of these strains lacked detectable amounts of K antigen. While the parent strain was resistant to phagocytosis, the mutant, J5, was readily ingested. This observation was confirmed by us (unpublished data). However, we were unable to isolate, from either stool cultures or blood cultures, any strains similar to *E. coli* strain O111 B4 (i.e. strains that lacked K antigen and were resistant to phagocytosis). This suggests strongly that the Medeavaris strain is not representative of strains isolated from blood and stool cultures.

It must be pointed out that Björksten and co-workers (1976) could not find any correlation between the presence of K1 antigen and resistance to phagocytosis or sensitivity to complement-lysis. Also McCabe *et al.* (1975) failed to demonstrate greater virulence of *E. coli* strains with large quantities of K antigen.

Although in the present study we found that strains of *E. coli* with large amount of K antigen were highly resistant to phagocytosis, the relationship between K antigen and resistance to complement killing was much less clear. Data from Howard and Glynn (1971) that *E. coli* strains rich in K antigen were resistant to complement killing by serum were confirmed, but many strains without detectable capsular polysaccharide were also found to be resistant.

Preliminary evidence suggested that O-antigenic side chains of the LPS are important for serum resistance, because two mutants devoid of O antigen exhibited much more susceptibility to complement-mediated lysis than the parent strains that had intact O-antigenic side chains. Further studies are needed to determine to which antigens (K antigens, O antigens or both) opsonic antibodies are formed; and the protective value of these antibodies has yet to be elucidated.

**SUMMARY**

The presence of capsular polysaccharides (K antigens) and their relation to phagocytosis and sensitivity to the lytic action of serum of 26 strains of *E. coli* isolated from stools of healthy volunteers and from blood cultures were studied. Four of 12 strains isolated from stool cultures and 12 (86%) of the 14 strains isolated from blood cultures possessed K antigen. Three of the 12 strains isolated from stool cultures and seven of the 14 isolated from blood cultures
were resistant to uptake by polymorphonuclear leucocytes; these resistant strains contained large amounts of K antigen. By contrast 10 strains, three with low amounts of K antigen and seven without detectable amounts of K antigen, were readily phagocytosed. Thus it appears that K antigen renders E. coli resistant to phagocytosis. Only four (15%) of the 26 strains were sensitive to serum lysis and there was no correlation between the presence of K antigen and the resistance to serum lysis.

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