ESCHERICHIA COLI ANTIBODIES IN OPSONISATION AND PROTECTION AGAINST INFECTION

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SUMMARY. The opsonic and protective capacities of rabbit antisera against *Escherichia coli* O, K and core-glycolipid cell-wall antigens were compared with specific antibody titres as measured by agglutination and enzyme-linked immunosorbent assay. Anti-O antisera were opsonic and protective against two noncapsulate strains. Only anti-K antisera were opsonic and protective against a K-antigen-containing strain. In a mouse model anti-core-glycolipid antiserum was not protective against challenge even by a strain bearing only core glycolipid.

INTRODUCTION

Strains of *Escherichia coli* with K-capsular antigens have been regarded as virulent because of the higher incidence of their involvement in invasive infections (McCabe *et al.*, 1975; van Dijk *et al.*, 1979a). Strains of *E. coli* bearing the K1 capsular antigen may cause acute pyelonephritis in children (Kaijser, 1973; Kaijser *et al.*, 1977) and meningitis in neonates (Robbins *et al.*, 1974). The most common K-antigen types found in isolates from patients with septicaemia are different from those common in acute pyelonephritis or neonatal meningitis. Many of the former have untypable K antigens (McCabe *et al.*, 1978). The virulence of the strains containing K antigen seems to be related to the antiphagocytic activity of the K antigen. Whereas antibodies are not required for the phagocytosis of noncapsulate strains, specific antibodies against K antigen appear to be necessary for optimal opsonisation of the strains bearing K antigen (Kaiser, Holmgren and Hanson, 1972; Kaijser and Ahlstedt, 1977; Welch and Stevens, 1979; Welch *et al.*, 1979). Several studies have shown that these opsonic anticapsular antibodies are protective *in vivo* (Wolberg and DeWitt, 1969; Kaijser and Ahlstedt, 1977; Kaijser *et al.*, 1977). However, in other studies (Wolberg and DeWitt, 1969) antisomatic O antibodies, raised against the O-antigenic polysaccharide side chains of the lipopolysaccharide were

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opsonic and protective for a strain containing K antigen. In addition, several investigators have reported that antisera raised in horses and rabbits against rough mutants of Enterobacteriaceae (strains with lipopolysaccharides lacking O-antigenic side chains) and containing antibodies against the core-glycolipid antigen, could passively transfer broad-spectrum protection against parenteral challenge with smooth strains (with O-antigenic side chains) of Enterobacteriaceae (McCabe, 1972; Ziegler *et al.*, 1973; Young, Stevens and Ingram, 1975). These antibodies appear to act primarily as anti-endotoxic antibodies rather than as opsonins (Braude, Douglas and Davis, 1973). In contrast, Ng *et al.*, (1976) and Greisman, Du Buy and Woodward (1978) were unable to confirm the protective capacity of core-glycolipid antibodies against challenge with smooth gram-negative bacteria.

Because of the controversy about the role of anti-K-, anti-O- and anti-core-glycolipid antibodies in opsonisation and protection against infection by *E. coli*, the protective and opsonic capacities of antisera raised in rabbits against *E. coli* strains varying in cell-wall composition were investigated. These properties were compared with levels of agglutinating antibodies and of specific IgG antibodies measured with an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Bacteria. *E. coli* O75K was isolated from the blood of a patient with septicaemia. It contained K antigen as measured by haemagglutination inhibition (van Dijk *et al.*, 1979a); the K antigen was untypable. *E. coli* F11, which was isolated from the faeces of a healthy person, was untypable and did not contain K antigen as measured by haemagglutination inhibition. *E. coli* J5 a UDP-gal-epimerase deficient mutant (with lipopolysaccharide containing only core-glycolipid) and the parent strain O111B4 (with complete lipopolysaccharide) were kindly supplied by Dr B. Lugtenberg (Department of Microbiology and Molecular Biology, State University Utrecht, The Netherlands). Serotyping was done by Dr P. A. M. Guinee (Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands).

Immunisation. *E. coli* strains were grown overnight at 37°C in Mueller-Hinton broth, washed in phosphate-buffered saline (PBS) and suspended to a concentration of 10¹⁰ colony forming units (cfu)/ml in PBS. To prepare *E. coli* J5, O111B4, F11 and O75 vaccines, the bacteria were killed by heating for 30 min at 80°C and washed twice more in PBS. To raise O75K antiserum containing antibodies against both O and K antigens, the vaccine was prepared by formalin treatment of *E. coli* O75K suspension which leaves the K antigen intact (Kaijser and Ahlstedt, 1977). Vaccines were stored at -70°C and thawed just before use. Four to six 1-kg New Zealand rabbits were subjected to the following intravenous immunisation schedule: day 1, 0.1 ml; day 4, 0.2 ml; day 7, 0.3 ml; day 11, 0.3 ml; day 14, 0.4 ml; day 30, 0.5 ml of vaccine. One week after the last injection the rabbits were bled by heart puncture, serum was collected and stored in 1-ml volumes at -70°C until used. Normal rabbit serum was collected from five normal rabbits, pooled, and stored at -70°C. Serum samples were thawed shortly before use.

Absorption studies. O75K antiserum was absorbed with heat-killed *E. coli* O75K (without K antigen) and live *E. coli* O75K (with K antigen). Bacteria were grown in Mueller-Hinton broth, washed and suspended to 10¹¹ cfu/ml in PBS. Part of the suspension was heated for 30 min at 80°C and washed again twice; 0.1 ml of the heat-killed and 0.1 ml of viable bacterial suspension were each incubated in 5 ml of O75K antiserum for 2 h at 37°C. As a control, 5 ml of serum were incubated with 0.1 ml of PBS. After incubation, serum samples were centrifuged for 25 min at 1600 g. The supernate was used in opsonisation studies.

Antibody titres in rabbit sera. Bacterial antibody titres were measured by the tube agglutination method (Campbell *et al.*, 1970) and by ELISA modified from Rissing *et al.* (1978). Vinyl microtitre plates (Vinyl M25, Nutacon BV, 1172 N Schiphol, The Netherlands) were coated with 0.2 ml of heated bacterial suspension containing 2.5×10^8 bacteria/ml PBS, by incubation for 2 h at 37°C. Unbound antigen was washed out and the plates were incubated for 1 h at 37°C with serial tenfold dilutions of serum which had been heated for 30 min at 56°C. Unbound serum was removed by washing. As conjugate, 0.2 ml of a 1 in 2000 dilution of goat peroxidase-labelled anti-rabbit IgG (Miles Yeda Ltd) was added to each cup. Plates were incubated for 2 h at 37°C. The substrate was 2-amino-5-hydroxybenzoic acid (Merck-Schuchart, 8011 Hohenbrunn bei München), 80 mg/100 ml in water containing 0.005% H₂O₂. To each cup 0.2 ml of the substrate was added, and plates were incubated for 1 h at 37°C. The reaction was stopped by adding 50 µl 1N NaOH to each cup. The ELISA titre was taken as the dilution that just gave a visible colour reaction as compared with the control incubated without serum.

Leucocytes. Isolation of polymorphonuclear leucocytes (PMN) from heparinised human normal donor blood was done by dextran sedimentation and centrifugation over a Ficoll isopaque gradient as previously described (Verbrugh *et al.*, 1978).

Opsonisation assay. Bacterial opsonisation was measured in a quantitative phagocytosis system with ³H-thymidine radiolabelled *E. coli* and isolated PMNs as described previously (van Dijk *et al.*, 1979b; Verbrugh *et al.*, 1978). Briefly, 0.3 ml of radioactive bacterial suspension containing 2.5×10^8 cfu/ml was incubated in 1.2 ml of serum diluted with Hanks's balanced salt solution (HBSS). After incubation at 37°C the bacteria were washed by centrifugation for 15 min at 1600 *g* and were resuspended in 1.5 ml of HBSS to obtain a final bacterial concentration of 5×10^6 cfu/ml. Washed pre-opsonisation bacteria, 0.2 ml, were incubated with 0.2 ml PMN suspension containing 5×10^6 PMN/ml HBSS in a 37°C shaking waterbath for 12 min. Phagocytosis was stopped by adding 2.5 ml of ice-cold PBS. Leucocyte-associated bacteria were then washed free from non-leucocyte-associated bacteria by three cycles of differential centrifugation for 5 min at 600 *g* and 4°C. Leucocyte-associated radioactivity was measured in a liquid scintillation counter (Mark II, Nuclear Chicago, Chicago, Ill, USA) and expressed as a percentage of the total added radioactivity, *i.e.*, percentage uptake of *E. coli*. Total added radioactivity was determined in one incubation mixture after centrifugation at 1600 *g* for 15 min. *E. coli* taken up by PMN after incubation for 12 min was taken as a measure of bacterial opsonisation, *i.e.*, as opsonic capacity of the serum sample.

Mouse-protection experiments. Swiss male albino mice 4–6 weeks old and weighing 15–20 g were used. LD50 measurements were made according to the method of Reed and Muench (1938). For protection studies 0.5 ml of a 1 in 2 dilution of immune or normal rabbit serum heated for 30 min at 56°C was injected intraperitoneally just before intraperitoneal challenge with 0.5 ml of a suspension containing $3 \times$ LD50 doses of live *E. coli*. Groups of mice consisted of seven animals. Protection studies were repeated two or three times. Percentages of survival after 48 h were calculated from all the experiments taken together.

RESULTS

Antibody titres in rabbit sera

Antibodies were raised in rabbits with heat-killed *E. coli* J5, O111B4, F11 and O75K. Because heat treatment destroys the capsular K antigen, antibodies were also raised against formalin-treated *E. coli* O75K with intact K antigen. Antibody levels were measured by the ELISA technique (table I). Titres ranged from 6000 to 24 000. In O75 antiserum and in O75K antiserum high levels of specific IgG anti-O75 antibodies could be detected. The agglutination titres are also given in table I. Agglutinating antibodies

TABLE I
Antibody titres in rabbit sera measured by ELISA and agglutination reaction

Antigen	Serum	Antibody titre* measured by	
		ELISA	agglutination
<i>E. coli</i> J5†	Anti J5	12000	NT
	NRS	200	NT
<i>E. coli</i> O111B4†	Anti O111B4	6000	1280
	NRS	100	10
<i>E. coli</i> F11†	Anti F11	24000	2560
	NRS	200	10
<i>E. coli</i> O75†	Anti O75	24000	2560
	Anti O75K	24000	2560
<i>E. coli</i> O75K‡	NRS	200	10
	Anti O75	NT	40
	Anti O75K	NT	160
	NRS	NT	10

* Titres are given as the serum dilution that gave a visible colour change in the ELISA reaction and just-visible agglutination in the agglutination reaction.

† Heat-killed bacteria as antigen.

‡ Live bacteria as antigen

NT = not tested; NRS = normal rabbit serum.

could not be measured for *E. coli* J5 because this strain was auto-agglutinating. Specific agglutinating antibody titres measured with the respective heat-killed strain as antigen varied from 1280 to 2560. In normal rabbit serum antibody titres were all low.

Opsonic capacity of rabbit sera

Uptake was not observed when bacteria were incubated in HBSS instead of serum. *E. coli* strains J5, O111B4, F11 and O75K were effectively opsonised by 1 in 100, 1 in 10, 1 in 20 and 1 in 5 dilutions of normal rabbit serum respectively (table II). All immune sera except the O75 antiserum, possessed a significantly better opsonic capacity for the corresponding strain than normal rabbit serum. The specific opsonins were heat stable. Heating

TABLE II
Opsonisation titres of rabbit sera

Test strain	Opsonisation titre* of					
	NRS	anti J5	anti O111B4	anti F11	anti O75	anti O75K
<i>E. coli</i> J5	100	1000	500	500	500	500
<i>E. coli</i> O111B4	10	10	50	10	10	10
<i>E. coli</i> F11	20	20	20	1000	20	20
<i>E. coli</i> O75K	5	5	5	5	5	20

NRS = normal rabbit serum.

* Given as the serum dilution that resulted in more than 50% phagocytosis.

of the sera at 56°C for 30 min did not significantly alter their opsonisation titres, whereas heating of normal rabbit serum abolished opsonic activity. Cross-reacting opsonising antibodies could not be demonstrated in the rabbit antisera (table II).

The kinetics of opsonisation of *E. coli* O75K are shown in fig. 1. After 1-min incubation of *E. coli* O75K in 1 in 5 diluted O75 antiserum, 60% of the

FIG. 1.—Kinetics of opsonisation of *E. coli* O75K in rabbit antiserum raised against the heat-treated strain (O75), in rabbit antiserum raised against the formalin-treated strain (O75K) and in normal rabbit serum (NRS). ●—● Represents phagocytosis by normal donor polymorphonuclear leucocytes after opsonisation for various times in unheated serum; x—x represents phagocytosis after opsonisation for various times in heated serum that had been heated at 56°C for 30 min.

pre-opsonised bacteria had been taken up by the PMNs. Effective opsonisation in O75 antiserum occurred only after 30 min. The efficient opsonisation of *E. coli* O75K in O75K antiserum suggested that K antibodies were necessary for effective phagocytosis. To test the importance of these antibodies for opsonisation, O75K antiserum was absorbed with heat-killed *E. coli* and with live *E. coli* O75K. Only absorption with live bacteria abolished the opsonic capacity of the serum (fig. 2).

Protective activity of rabbit antisera

Antisera against strains O111B4, F11 and O75K protected against

FIG. 2.—Phagocytosis of *E. coli* O75K after opsonisation in O75K antiserum unabsorbed (O75K), after absorption with heated *E. coli* O75K (O75 abs) and after absorption with live *E. coli* O75K (O75K abs).

challenge with the homologous strains but antisera against O75 and J5 did not protect against O75K and J5 respectively. Cross-protective sera were not detected (table III).

TABLE III

*Survival of mice passively immunised with rabbit sera after challenge with live *E. coli**

Challenge strain	Number of cells in $3 \times \text{LD}_{50}$ challenge dose	Percentage survival at 48 h after immunisation with					
		NRS	anti J5	anti O111B4	anti F11	anti O75	anti O75K
<i>E. coli</i> J5	2.7×10^{10}	0	14	0	0	NT	0
<i>E. coli</i> O111B4	3.0×10^8	14	0	100	NT	NT	15
<i>E. coli</i> F11	2.1×10^8	0	0	0	100	14	0
<i>E. coli</i> O75K	3.0×10^7	0	19	14	NT	14	100

NRS = normal rabbit serum; NT = not tested.

DISCUSSION

In a previous study (van Dijk *et al.*, 1979a) the amount of K antigen present could be correlated with resistance of the micro-organism to phago-

cytosis. In the present study we found that in noncapsulate strains of *E. coli* the length of the O-antigenic side chains determined opsonic requirements in normal rabbit serum. *E. coli* J5, a mutant of *E. coli* O111B4 lacking O-antigenic side chains was efficiently opsonised in 1 in 100 diluted serum, whereas 1 in 5 diluted serum was required to opsonise the parent strain O111B4.

After intravenous immunisation of rabbits with heat-killed bacteria, high-titre antisera, as measured by ELISA and agglutination, could be raised. These contained heat-stable opsonins specific for the corresponding non-K-antigen-bearing strain. Recent studies have shown that only antisera with specific antibodies against K antigens were opsonic for K1-containing and K92-containing *E. coli* strains (Welch *et al.*, 1979; Welch and Stevens, 1979). These results were confirmed in the present study in which a strain with an untypable K antigen was used. This strain was used because it was isolated from a patient with *E. coli* septicaemia and because it was known that many strains isolated from the blood have untypable K antigens (McCabe *et al.*, 1978). Only antisera containing antibodies against the K antigen were opsonic for the capsulate strain. When rabbits were immunised with heat-killed *E. coli* O75K lacking the heat-labile capsule, antibodies against the somatic O-antigen were raised as was determined by the ELISA method, but this serum was no more efficient at opsonising than normal rabbit serum. It is possible that anti-O antibodies are not opsonic for capsulate strains either because of an inability to penetrate the capsule or, if bound, because of an inability to interact with PMNs due to interference by surrounding capsular components.

The range of susceptibility to in-vitro phagocytosis of *E. coli* strains reflected differences in virulence. This confirms previous studies (Wolberg and DeWitt, 1969; Howard and Glynn, 1971). *E. coli* J5, a mutant lacking O-antigenic side chains, was the least virulent. This strain is susceptible to the lytic action of serum (van Dijk *et al.*, 1979a) and is effectively opsonised in 1 in 100 diluted serum. Therefore, after intraperitoneal injection, this strain may be cleared rapidly from the mouse peritoneal cavity by phagocytosis or lysis or both. Because *E. coli* J5 has a relatively high content of lipid A, the toxic substance of lipopolysaccharide, the lethal dose of *E. coli* J5 may be determined by its endotoxic activity rather than by its ability to escape phagocytosis.

The in-vivo protective ability of antiserum directed against outer-membrane and capsular components was examined in mice challenged with noncapsulate strains and *E. coli* O75K. The anti-O sera raised against the noncapsulate *E. coli* strains proved to be protective against a challenge with the corresponding live *E. coli* strains. As with in-vitro phagocytosis studies, only antiserum raised against the K-antigen-bearing strain was protective against a challenge with live *E. coli* O75K. The lack of protection observed with O75 antiserum and with core-glycolipid antiserum (J5 antiserum) is in agreement with the results of others (Mullan *et al.*, 1974; Ng *et al.*, 1976; Greisman, Du Buy and Woodward, 1978; Welch *et al.*, 1979). However, in

addition to capsular antibodies it has been reported that O antisera, core-glycolipid antisera and lipid A antiserum protect animals against challenge with *E. coli* (Wolberg and DeWitt, 1969; Galanos, Lüderitz and Westphal, 1971; Kaijser *et al.*, 1972; McCabe, 1972). In contrast to studies by Braude *et al.*, (1973) and Ziegler *et al.*, (1973), antiserum raised against *E. coli* J5 was not protective in mice challenged with capsulate and noncapsulate strains of *E. coli*. Moreover, protection was not observed against challenge with three times the LD₅₀ of *E. coli* J5. Also Ng *et al.* (1976) and Greisman *et al.* (1978) were unable to demonstrate protection by antisera against rough mutants. Recently Peter *et al.* (1980) suggested that the challenge dose given to mice after passive immunisation with J5 antiserum affects the outcome of infection. The difference in the results might also be due to the mice that were used. We used Swiss mice whereas McCabe (1972) used CF1 mice and Young *et al.*, (1975) used CB mice. Greisman *et al.*, (1978) suggested that differences in protection could partly be explained by failure to use pre-immune serum from the same rabbit as the control. They found that normal rabbit and horse sera protected mice against various gram-negative challenge strains. However, we did not observe protection by serum pooled from five normal rabbits.

Another possible explanation for the controversy regarding the protective value of anti-core-glycolipid sera (J5 antiserum) is the use of different strains of *E. coli*. It is possible that the protective capacity of the sera is conferred only against a variety of noncapsulate strains, and that when more virulent challenge strains are used, protection is not observed (Welch *et al.*, 1979). Data on protection against *E. coli* infections by antibodies are still inconclusive; careful dose-related protection and opsonisation studies with a wide variety of *E. coli* strains are required.

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