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The Virulence for mice of Strains of *Escherichia coli* related to the Effects of K Antigens on their Resistance to Phagocytosis and Killing by Complement

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Summary. Strains of *Escherichia coli* with sufficient K antigen to resist killing by complement were poorly phagocytosed when injected intravenously into mice. Phagocytosis was markedly increased by anti-OK but not by anti-O sera. In contrast anti-K sera had little or no effect on the bactericidal reaction. This was not because K antigenic sites were scarce but may have been because their position was such that complement was activated at a distance from its substrate. Red cells coated with K antigen were poorly lysed by complement and anti-K serum, suggesting that the K antibody did not activate complement very effectively although again the sites may have been too superficial. The effect of K antigens on phagocytosis and complement killing or lysis could all be explained by their ability to impair protein binding.

Strains of *E. coli* rich in K antigen were resistant to phagocytosis and complement killing and were virulent for mice on intracerebral injection. The significance of K antigens in animal and human infections is discussed.

INTRODUCTION

Complement resistance in *Escherichia coli* is due to the presence of adequate amounts of K antigen (Muschel, 1960). We have recently confirmed this and shown that some K antigens are more effective, weight for weight, than others. The K antigens appear to inhibit haemolysis and by analogy bacteriolysis and bactericidal activity, by impeding the attachment of antibody to the target cell. Such antibody as is bound is less effective at inducing complement activity (Glynn and Howard, 1970).

If K antigens reduce antibody binding then O antibodies should not readily opsonize strains of *E. coli* rich in K, however, K antibodies might be effective. A series of strains of *E. coli* of known complement sensitivity and K antigen content have been examined for their virulence for mice, their susceptibility to phagocytosis and their ability to be opsonized by specific anti-O and anti-K sera. The effect of anti-K sera on the bactericidal reaction and their ability to lyse red cells coated with K antigen were also studied.

MATERIALS AND METHODS

Bacteria

Eight strains of *E. coli* were examined. Strains WF98, WF86, WF8 and WF26 are all of serotype 06:K13. The other strains were WF41 (017:K16:H18), WF96 (07:K1:H6),

WF82 (0:117 K? : H27) and WF95 (0:117 K? : H32). Bacteria used for estimating complement sensitivity, phagocytic indices or virulence were always in the logarithmic growth phase.

Complement sensitivity

Complement sensitivity is expressed as the number of CH_{50} units required to kill 50 per cent of a standard inoculum in 15 minutes at 37° (Glynn and Howard, 1970).

Susceptibility to phagocytosis

Strains of *E. coli* were labelled with ^{51}Cr (Howard, Biozzi, Halpern, Stiffel and Mouton, 1959). The phagocytic index K was calculated for each strain from its rate of clearance following intravenous injection of 5×10^8 organisms per 20 g mouse (Biozzi, Benacerraf, Stiffel and Halpern, 1954).

To test the effect of anti-O and anti-K antibodies 0·2 ml of the appropriate antiserum was injected 15 minutes before the bacteria.

TABLE 1

AGGLUTINATION TITRES OF ANTISERA BEFORE AND AFTER ABSORPTION

Immunizing agent (*E. coli*) strains	Absorbent	Antibodies present	Agglutination titre against	
			Live bacteria	Heated bacteria
WF82 0:117K? live	—	OK	640	2560
WF82 0:117K? live	Alkali treated phenol extract of WF82	K	640	<20
WF82 0:117K? heated	—	O	<10	2560
WF98 06:K13 live	—	OK	2560	2560
WF96 07:K1 live	—	OK	5120	1280
WF96 07:K1 live	Heated WF96	K	5120	<20
WF96 07:K1 live	live WF96	(K)	40	<20
WF96 07:K1 heated	—	O	<10	2000

Preparation of antisera

Anti-O and anti-OK sera were raised in rabbits against heated and live bacteria respectively (Edwards and Ewing, 1962). Anti-WF96 K serum was prepared by absorbing the specific anti-OK serum with heated (100°, 1 hour) *E. coli* WF96. Anti-WF82 K serum was prepared by absorbing an anti-WF82 OK serum with a phenol water extract of *E. coli* WF82 which had first been treated with 0·05 N NaOH for 18 hours at 37° to destroy any K antigen present.

The antigens used and the agglutination titres of the antisera before and after absorption are summarized in Table 1.

Estimations of K antigens in bacterial extracts

Bacteria were grown overnight at 37° on nutrient agar plates, scraped off and dried with acetone. Suspensions containing 20 mg/ml of dried bacteria in 0·15 M saline were homogenized on ice for 3 minutes and centrifuged at 1200 *g* for 30 minutes. The K antigen content of the clear supernatant was measured by radial immunodiffusion (Mancini, Carbonara and Heremans, 1965) in agar plates containing a suitable concentration of

anti-K13 serum. Purified K13 antigen isolated from *E. coli* WF26 was prepared by cetavlon fractionation according to the method of Hungerer, Jann, Jann, Ørskov and Ørskov (1967) and used as a standard. The plates were incubated for 24 hours and the diameter of the rings measured from photographs.

Measurement of virulence

Groups of six white female mice weighing 15–20 g were injected intracerebrally with 0·025 ml of saline suspensions of known numbers of bacteria.

The times of death were recorded and the LD_{50}'s were calculated by the method of Reed and Muench (1938).

Effect of K antibody on the bactericidal activity of serum

For strains WF26 and WF98, tubes were set up containing approximately 1×10^5 bacteria in 0·1 ml saline, 0·1 ml of saline dilutions of anti-K serum or a saline control, and 1·8 ml of fresh normal human serum. The final complement concentration was 32·5 CH_{50} units/ml. For the sensitive strain WF96, the amount of fresh human serum was reduced to 0·2 ml and the volume made up with 1·6 ml heated human serum, i.e. the final concentration of complement was reduced to 3·6 CH_{50} units/ml. The mixtures were incubated at 37° and 0·1 ml aliquots taken at intervals for viable counts.

The experiments with precolostral piglet serum used 20 per cent serum in tris buffer at a final ionic strength of 0·06 (Glynn and Milne, 1967).

In some experiments the bacteria were first sensitized with antibody and then washed before testing with complement.

RESULTS

K ANTIGENS, COMPLEMENT SENSITIVITY AND PHAGOCYTOSIS

Comparison of the four strains of *E. coli* of serotype 06:K13 shows that an increase in K antigen content was associated with increased resistance to both killing by complement and to phagocytosis (Table 2). In a similar comparison of strains of other serotypes the more complement resistant strains were again less readily phagocytosed (Fig. 1).

OPSONIC ACTIVITY OF ANTI-O AND ANTI-K ANTIBODIES

The complement sensitive strain WF96 had a high phagocytic index ($K = 0{\cdot}150$) and the rate of phagocytosis was not increased by specific anti-O or anti-OK sera. The three complement resistant strains WF82, WF8 and WF26 were all cleared more slowly. Their clearance was slightly faster after the injection of specific anti-O serum, but was very much faster after the injection of specific anti-OK serum (Table 3).

THE INFLUENCE OF ANTI-K SERUM ON THE UPTAKE OF O ANTIBODIES

From anti-O serum (agglutination titre 2560) specific for strain WF82, the γ-globulin fraction was precipitated by sodium sulphate (Kekwick, 1940) and labelled with ^{125}I (McFarlane, 1958).

TABLE 2

COMPLEMENT SENSITIVITY, PHAGOCYTIC INDEX AND LD_{50} ON INTRACEREBRAL INJECTION IN MICE, RELATED TO THE K ANTIGEN CONTENT OF FOUR STRAINS OF *E. coli* SEROTYPE 06:K13

Strain	K antigen content (μg/mg dry weight bacteria)	Complement sensitivity (CH_{50}/LD_{50})	Phagocytic index	LD_{50} (no. of bacteria)
WF98	8·0	8·0	0·144	1000
WF86	14·7	27·0	0·110	100
WF8	27·5	>32·4*	0·063	100
WF26	51·0	>32·4†	0·051	10

* 20 per cent killed in 15 minutes.
† None killed in 15 minutes.

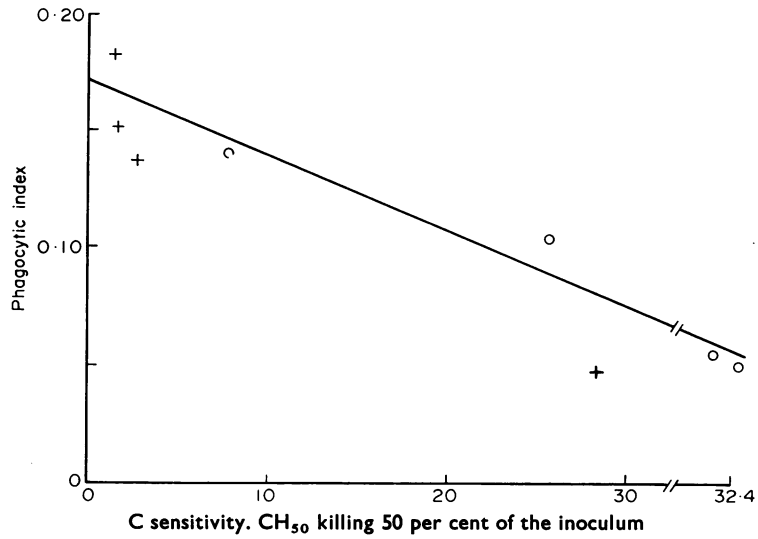

FIG. 1. Phagocytosis of eight strains of *Escherichia coli* related to their complement sensitivity. ○, 06:K13 strains; +, other strains.

TABLE 3

THE EFFECT OF ANTI-O AND ANTI-OK ANTIBODIES ON THE PHAGOCYTOSIS OF STRAINS OF *E. coli*

Strain	Serotype	Phagocytic index		
		No previous antibody	After anti-O	After anti-OK
WF26	06:K13	0·051	0·063	0·166
WF8	06:K13	0·063	0·069	0·103
WF82	0:117K?	0·044	0·058	0·140
WF96	07:K1	0·150	0·130	0·159

Suspensions of live WF82 were treated with mixtures of anti-K serum at dilutions of 1 : 100–1 : 2000 and labelled anti-O serum at dilutions of 1 : 10–1 : 100, washed and the uptake of O antibody measured. No effect of anti-K on the uptake of anti-O could be detected.

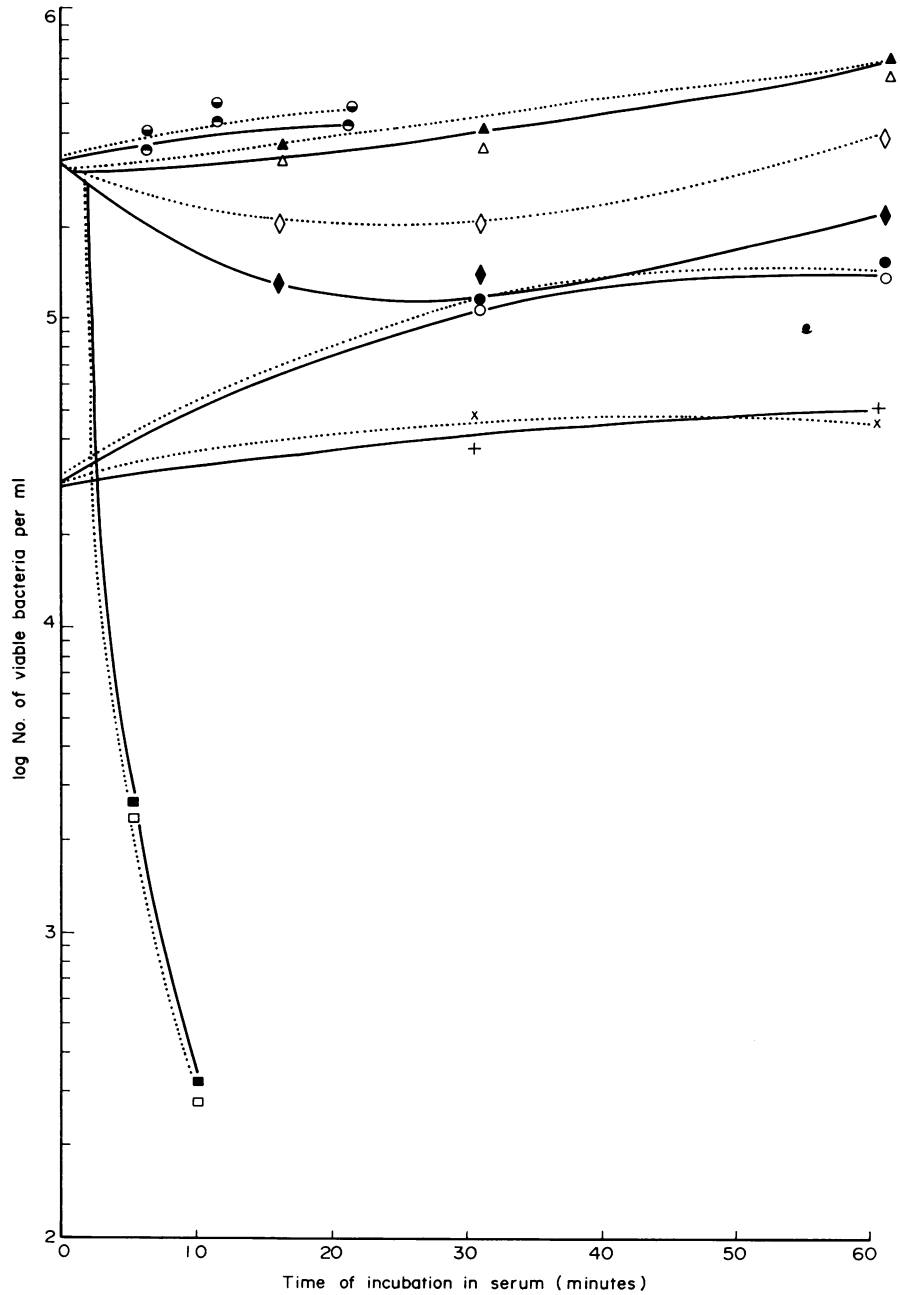

FIG. 2. Bactericidal action on *E. coli* of normal human serum with and without added anti-OK serum. □, WF96 with normal human serum (NHS); ■, WF96 with NHS and anti-OK antibody; ◒, WF96 with heated serum (HS); ◓, WF96 with HS and anti-OK antibody; ◇, WF98 with NHS; ◆, WF98 with NHS and anti-OK antibody: ▲, WF98 with HS; △, WF98 with HS and anti-OK antibody; ×, WF26 with NHS; +, WF26 with NHS and anti-OK antibody; ●, WF26 with HS; ○, WF26 with HS and anti-OK antibody.

THE BACTERICIDAL ACTION OF ANTI-K SERA

The complement sensitive strain WF96 was killed very rapidly by normal human serum alone, and no enhancement was detected on the addition of specific anti-K serum. K antibodies did not affect the killing of the resistant strain WF26 but at the highest concentration used (1 : 500) may have caused a marginal increase in the killing of strain WF98 (Fig. 2).

Further experiments with strain WF96 were carried out using precolostral piglet serum as a source of complement free from antibodies. Piglet complement alone was inactive but

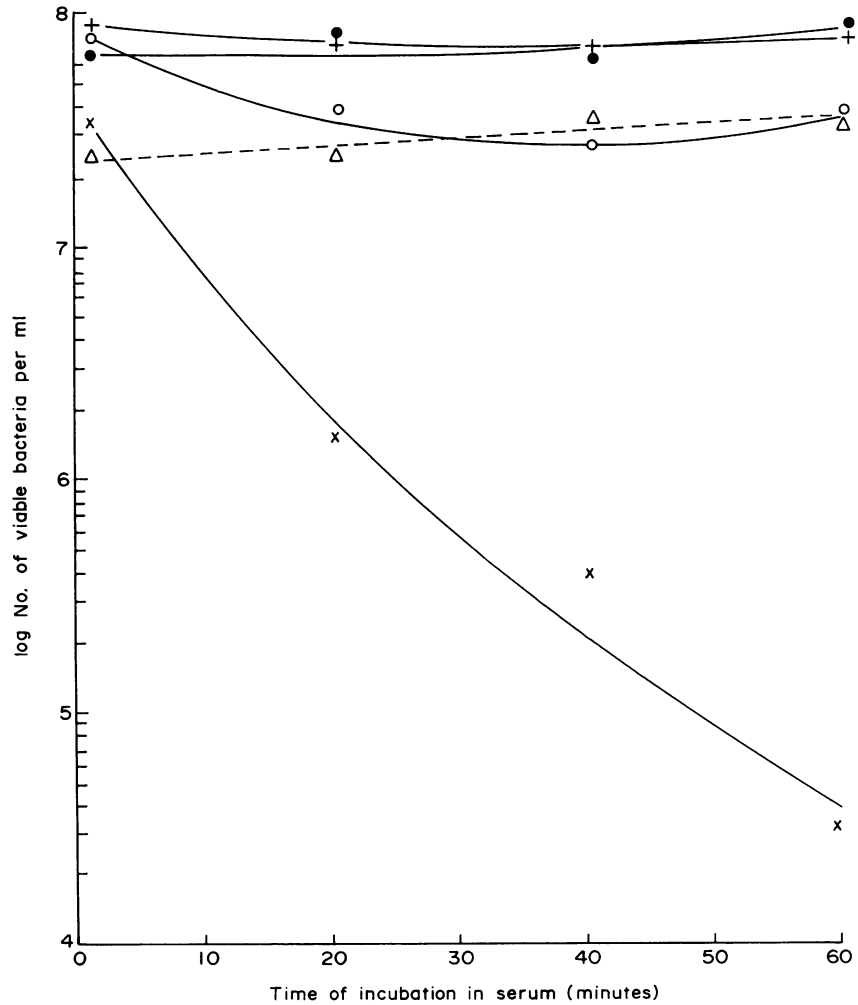

FIG. 3. Bactericidal action of pre-colostral piglet serum on *E. coli* strain WF96 sensitized with anti-OK and anti-K sera. +, Precolostral piglet serum and unsensitized WF96. ×, precolostral piglet serum and WF96 sensitized with anti-OK antibody; ○, precolostral piglet serum and WF96 sensitized with anti-K antibody; ●, precolostral piglet serum and WF96 sensitized with anti-OK antibody previously absorbed with live WF96; △, heated precolostral piglet serum and WF96 sensitized with anti-OK antibody.

a marked bactericidal effect was produced when anti-OK serum was added. If the anti-OK serum was first absorbed with heated bacteria to remove O antibody the percentage killed fell from 99·9 to 40–50. Absorption with live cells removed the remaining bactericidal activity. Similar results were obtained when the bacteria were first sensitized with antibody before being added to the complement (Fig. 3).

The most likely explanation of the difference between absorption with live or dead cells is the small bactericidal activity of anti-K. Although it is impossible to be sure that absorption in each case was complete there is no reason to suppose that live cells would absorb anti-O more efficiently than dead cells.

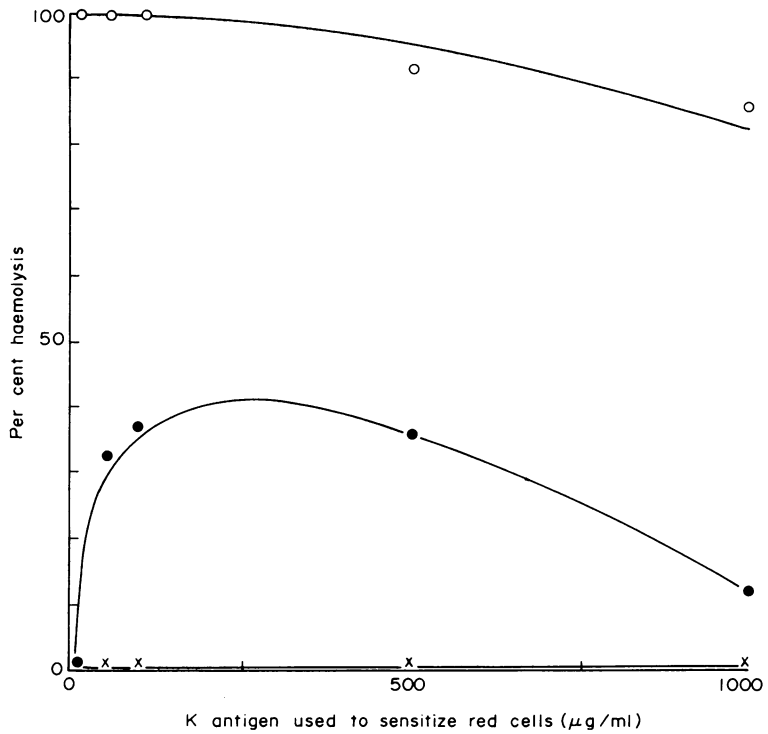

FIG. 4. Lysis by anti-K antibody and complement of sheep red cells sensitized with K antigen. ●, 1/100 anti-K antibody; ×, 1/400 anti-K antibody and no antibody; ○, 1/500 rabbit anti-sheep red cell antibody.

COMPLEMENT FIXATION AND PASSIVE HAEMOLYSIS BY ANTI-K SERUM

Sheep red cells treated with solutions of K antigen from strain WF82 in concentrations of up to 1 mg/ml were readily agglutinated by anti-OK serum to a dilution of 1 : 1000. However, to get haemolysis in the presence of excess human complement the concentration of anti-OK serum had to be increased to 1 : 100 (Fig. 4), i.e. to ten times the minimal agglutinating dose. Even so only 40 per cent haemolysis was obtained compared with nearly 100 per cent reached when rabbit anti-sheep red cell serum was added to the system. In these experiments the anti-OK serum had previously been absorbed with sheep red cells to remove natural antibodies.

THE COMPLEMENT SENSITIVITY OF STRAINS OF *E. coli* AND THEIR VIRULENCE FOR MICE

When mice were injected intracerebrally with different strains of *E. coli* the LD_{50}'s covered a range of 7 $\log_{10}$ units. Increased resistance to complement was associated with increased virulence for mice in the four strains of serotype 06:K13 tested and in three other strains (Fig. 5). An exception was strain WF95 which was more virulent (LD_{50} for mice 6×10^2) than would have been expected from its complement sensitivity (50 per cent killed by 1·2 CH_{50} units).

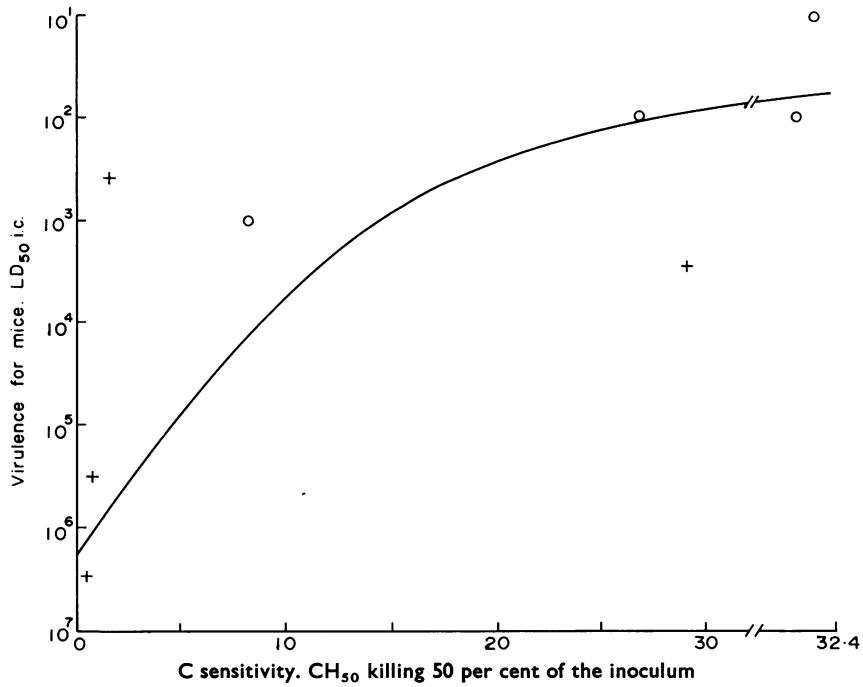

FIG. 5. LD_{50} on intracerebral injection into mice of strains of *E. coli* related to their complement sensitivity. ○, 06:K13 strains; +, other strains.

DISCUSSION

The results confirm (Table 2) that in *E. coli* as in *Salmonella typhi*, strains which are rich in K antigen (Vi in *S. typhi*) are not only resistant to serum killing but are also difficult to phagocytose. One reason why serum fails to kill such strains is that 'O' antibodies have difficulty in attaching themselves to the bacterial surface (Glynn and Howard, 1970). Under such circumstances, as expected, O antibodies were not good opsonins (Table 3). Phagocytosis was speeded up by K antibodies, which apparently acted as opsonins in their own right and not by neutralizing K antigen thus allowing greater uptake of O antibodies.

In contrast K antibodies had very little effect on the killing of either sensitive or resistant strains of *E. coli* by normal human serum. Nagington (1956) found that an 'anti-Vi' serum increased the killing of *S. typhi*, particularly of strains with only a moderate amount

of Vi, but did not kill *Paracolobactrum ballerup*. Muschel and Treffers (1956) also showed some effect of anti-Vi on *S. typhi* but not on *P. ballerup*.

In order to explain why K antibodies were opsonic but not, or only slightly, bactericidal, one needs to consider the relative sensitivity of the two tests, the number of antigenic sites involved, where these sites may be, how effective complexes of K antigen and K antibody are at fixing complement, and the effect of K antigen on the whole sequence of complement reactions.

Rowley and Turner (1966) calculated that 8 IgM or 2200 IgG molecules of specific anti-O antibody were required to opsonize one bacillus (*S. adelaide*). It is possible that the number of antibody molecules required for killing is ten times this (Robbins, Kenny and Suter, 1965). In the present experiments several agglutinating doses of K antibody were used so that there was probably no shortage of antibody. What killing effect there was with anti-K was with the most concentrated sera so that a prozone or Neiser-Wechsberg effect is unlikely to have been involved. There was clearly no shortage of K antigen.

It is easy to imagine that a more superficial attachment of antibody is less likely to affect opsonization than killing. Activation of complement at a distance from its substrate has been used to explain poor killing mediated by antibody to Vi antigen (Nagington, 1956) and to artificially attached protein antigens (Rowley and Turner, 1969). O antigens themselves may project as much as 150 mμ from the surface (Shands, 1965). Presumably the anti-O effective in the bactericidal reaction is that which reacts with the deeper layers of O but there is no proof of this. K antigens are probably more superficial than O but more information on the distribution and disposition of K antigens in bacterial cell walls is badly needed.

Although H antibodies are not opsonic (Felix and Olitzki, 1926) antibodies to very superficial antigens may be (Rowley, Thoni and Isliker, 1965).

Complement is not absolutely necessary for phagocytosis (Ward and Enders, 1933) but certainly accelerates it. Since so few antibody molecules are necessary there is no question of them covering the bacterial surface (Rowley *et al.*, 1965) but they must affect it in some way and the effect is presumably greater if each site of attached antibody activates complement. The experiments with passively coated red cells show that anti-K is not very effective haemolytically. Again it is unlikely that this is because the sites are too few but may be because they are too superficial. Lysis of red cells coated with pneumococcal polysaccharide type III, another acid polysaccharide, and treated with anti-S3 and complement, needed large amounts of antibody Electron microscopy showed that the polysaccharide and antibody were in uneven masses not closely applied to the cell membrane (Humphrey and Dourmashkin, 1969). In addition it is known (Glynn and Howard, 1970) that K antigens impede the action of complement activated by Forssman antibody so they may well impede the action of complement activated by K antibody.

Bacterial killing involves all nine complement components while phagocytosis is generally thought to require only C1423 (Nelson 1965, Johnson, Klemperer, Alper and Rosen, 1969) and perhaps C5 (Smith, Shin and Wood, 1969; Miller and Nilsson, 1970). There are therefore more stages at risk in the bactericidal reaction. It is also possible that K antigen might interfere with the later reactions more than the earlier. In any case the action of K antigen on individual complement reactions needs investigating.

Since K antigens inhibit both the serum bactericidal reaction and phagocytosis it is not surprising that strains of *E. coli* rich in K antigen should be more virulent for mice than strains with little or no K antigen. Rowley (1954) found that the complement resistance of

strains of *E. coli* was related to their virulence for mice. It has been suggested (Glynn and Medhurst, 1967; Medhurst and Glynn, 1970) that complement may also play a role in killing *E. coli* intracellularly so that there is possibly yet another site where K antigens may influence the fate of bacteria *in vivo*.

We challenged mice intracerebrally, and similar results with intracerebral inoculation were found by Wolberg and De Witt (1969) who compared K deficient (K−) mutants with their parent (K+) strains. Their results also emphasize the point made here and previously (Glynn and Howard, 1970) that sufficient K antigen to be detected serologically may not be sufficient to affect virulence. Complement resistant strains of *E. coli* were dealt with less effectively than sensitive strains on intravenous injection in rabbits (Roantree and Pappas, 1960).

The properties of K antigens correspond very closely with those of the Vi antigen of *S. typhi* in relation to virulence (Felix and Pitt, 1934; Landy, Gaines and Sprinz, 1957), O inagglutinability (Felix, Bhatnagar and Pitt, 1934), resistance to complement (Felix and Olitzki, 1926; Muschel, Chamberlin and Osawa, 1958), and decreased susceptibility to phagocytosis (Bhatnagar, 1935). Anti-Vi serum is opsonic for strains of *S. typhi* rich in Vi (Felix and Bhatnagar, 1935).

Although extrapolation of results from mouse to man must be made with caution there are indications that K antigens have a role in the pathogenesis of human infections. K antigens were relatively more frequent in strains of *E. coli* isolated from peritonitis following appendicitis (Vahlne, 1945) and from urinary tract infections (Sjöstedt, 1946) than in random strains from faeces. Vahlne and Sjöstedt's figures were suggestive rather than conclusive but again they were only considering the presence or absence of K antigen not the amount in each strain. Roantree and Rantz (1960) found that strains of *E. coli* isolated from the blood of patients with septicaemia were more often resistent to complement than strains from other sources. Current investigations on urinary tract infections in pregnant women (Glynn, Howard and Brumfitt, to be published) suggest that K antigens may be related to renal involvement. The importance of Vi antigens in man has been discussed previously (Glynn and Howard, 1970).

In view of current controversy about the merits of intraperitoneal and intravenous routes of experimental infection when the natural route is oral (Ornellas, Roantree and Steward, 1970), it is interesting that K antibodies in colostrum have been found to be important in protecting calves against *E. coli* infections (Ingram, 1964).

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