THE ROLE OF DIFFERENT K ANTIGENS OF *ESCHERICHIA COLI* IN PHAGOCYTOSIS BY POLYMORPHONUCLEAR LEUKOCYTES

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SUMMARY. The importance of K antigens from six strains of *Escherichia coli* for the interaction with polymorphonuclear leukocytes (PMNL) was studied. The major factor influencing this interaction was the ability of strains to activate complement by the classical route during opsonisation, this process being reduced for most K-positive strains. Interference of K antigens with the functioning of common pili as adhesins of eukaryotic cells was not observed nor a toxic effect of K antigens on PMNL.

INTRODUCTION

K antigens of *Escherichia coli* have been described as virulence factors because of their higher incidence in strains causing invasive infections (McCabe *et al.*, 1975; Weinstein and Young, 1978; van Dijk *et al.*, 1979a). Moreover, some K types are more often involved than others (Robbins *et al.*, 1974; Weinstein and Young, 1978). The greater virulence of K⁺ strains may be explained by interference of the K antigen with phagocytosis by polymorphonuclear leukocytes (PMNL) (Howard and Glynn, 1971; Stevens, Chu and Young, 1980; Van Dijk *et al.*, 1979a; Verweij-van Vught *et al.*, 1983).

The interaction between PMNL and bacteria is a complex process, which can be influenced by K antigens in different ways. Most studies have concentrated upon the inability of many K⁺ strains to activate complement via the alternative pathway, so that antibodies are required for effective opsonisation (Welch and Stevens, 1979; Van Dijk *et al.*, 1981). K antigens may also influence the expression of other surface antigens involved in bacterial adherence to PMNL. The adherence of bacteria to PMNL by a specific receptor interaction mediated by common pili has been described (Bar-Shavit *et al.*, 1977; Mangan and Snyder, 1979; Silverblatt, Dreyer and Schauer, 1979); interaction between capsules and pili might influence this receptor activity.

Previously we described the relationship between K antigens, virulence of *E. coli* and susceptibility to phagocytosis (Verweij-van Vught *et al.*, 1983), using six strains of

E. coli and their isogenic K^- derivatives, obtained by spontaneous single step mutation. In this paper we describe mechanisms that could be responsible for the observed difference in phagocytosis of these strains.

MATERIALS AND METHODS

Bacterial strains. The origin of the strains of *E. coli* and the isolation of the K^- mutants were described in a previous paper (Verweij-van Vught *et al.*, 1983).

Growth of *E. coli* strains. Unless otherwise stated, bacteria were grown in Brain Heart Infusion broth (BHI, Difco) until in log-phase. For phagocytosis, the cells were harvested by centrifugation and washed in phosphate-buffered saline (PBS) pH 7.4. For haemagglutination experiments the bacteria were grown in nutrient broth for 24–48 h to improve pili production (Duguid, Clegg and Wilson, 1979). Broth supplemented with glucose 0.05% was used to enhance the production of K antigens (Orskov *et al.*, 1971).

Preparation of leukocytes. PMNL were separated from heparinised venous blood from healthy volunteers as described before (Verweij-van Vught *et al.*, 1983). The PMNL were resuspended in Hanks's balanced salt solution (HBSS) to a concentration of 5×10^6 cells/ml.

Opsonisation. Bacteria were opsonised in normal pooled human serum (NHS) for total opsonisation. Inactivation of complement was achieved by heating serum for 30 min at 56°C (HIS). Activation of complement via the classical pathway was inhibited by chelation of calcium in the serum with MgEGTA (MgEGTA-S). Undiluted serum and MgEGTA (10 mM final concentration) were mixed 15 min before the phagocytosis experiments to ensure complete chelation of calcium before the addition of the bacteria. Preopsonisation of zymosan was performed by incubation of equal volumes of zymosan (8 µg/ml) and NHS (20%) or MgEGTA-S for 60 min at 37°C. The serum was removed by centrifugation and the opsonised zymosan was resuspended in PBS.

Chemiluminescence (CL). The induction of CL of PMNL was measured by incubation of 1×10^6 PMNL with 1×10^7 bacteria, 10% serum, luminol (2×10^5 M) and HBSS in a total volume of 0.5 ml in a Lumacuvette P. CL was measured every 10 min in a Lumacounter TMM2080 (Lumac Systems A.G., Basel, Switzerland). The total amount of CL was calculated by adding the counts at 10, 20, 30, 40, 50, 60 and 70 min together. Zymosan was used for CL studies at a final concentration of 1.6 µg in 0.5 ml.

Complement consumption. Tests for consumption of serum complement by strains of *E. coli* were performed as described by Van Dijk *et al.* (1979b).

Serum agglutinins. After incubation overnight on an agar plate (Blood Agar Base No. 2, Oxoid) bacteria were suspended in PBS pH 7.4 to a density of $c. 8 \times 10^8$ cells/ml. Serial two-fold dilutions of NHS were made in 0.1 ml of PBS in wells of a Multiwell Disposotray with a well capacity of 1 ml (Flow Laboratories, Irvine, Scotland). To each dilution, 0.1 ml of the bacterial suspension was added and agglutination was read after incubation overnight at room temperature.

Haemagglutination test. The ability of the strains of *E. coli* to agglutinate guinea pig erythrocytes was tested as previously described (Verweij-van Vught *et al.*, 1983).

Preparation of cell extracts. Bacteria, grown for 18 h on agar plates (Blood Agar Base No. 2, Oxoid) at 37°C, were suspended in PBS pH 7.4 to a concentration of 0.3 mg (wet weight)/ml. The suspensions were incubated for 1 h at 60°C and centrifuged. The supernates were filter sterilised and used as crude extracts.

RESULTS

Chemiluminescence of K^+ and K^- strains

All K^+ and K^- strains induced CL. In the presence of NHS, at a bacteria/PMNL ratio of 10/1, K^- strains were better inducers of PMNL CL than K^+ strains, resulting in a K^+/K^- ratio of CL less than unity for all six K antigens tested (table 1). There were, however, considerable differences in K^+/K^- ratios with the different K antigens,

TABLE I
Properties of strains of E. coli

Strain	Serotype	Ratio K ⁺ /K ⁻ in NHS	CL of PMNL		Complement consumption (°)*		Titres of agglutinating antibodies in NHS†
			with HIS (percentage of CL in NHS)	with MgEGTA-S (percentage of CL in NHS)	With NHS	with MgEGTA-S	
AD35K ⁺ K ⁻	O6K ² H1 O6K ⁻ H1	0.76	14 12	28 33	34 100	0 22	<4 64
AD36K ⁺ K ⁻	O6K2H1 O6K ⁻ H1	0.61	6 5	16 40	23 100	0 12	8 64
AD121K ⁺ K ⁻	O6K23H1 O6K ⁻ H1	0.79	11 15	17 42	18 100	0 0	<4 64
AD307K ⁺ K ⁻	O8K(A) ² H19 O8K ⁻ H19	0.30	9 35	7 46	4.9 37	0 4.2	<4 512
AD311K ⁺ K ⁻	O8K(A)28H ⁻ O8K ⁻ H ⁻	0.10	6 43	2 48	3.8 38	0 13	<4 512
AD412K ⁺ K ⁻	O7K1H ⁻ O7K ⁻ H ⁻	0.29	6 39	5 33	7.4 47	0 11	<4 256

^{*} The consumption of complement was measured after incubation of the bacteria for 30 min in serum and is expressed as the percentage of the haemolytic complement remaining in a control sample of serum incubated with buffer alone.

† Titres of agglutinating antibodies were measured against whole bacteria.

being lower for the two K(A) antigens and for K1. Without serum, no induction of CL could be measured even at bacteria/PMNL ratios of 100×1 and $300/1$.

Inactivation of K antigens by heat treatment

The induction of CL by K^+ strains before and after inactivation of the K antigen by heat treatment seemed another possible way to study the importance of K antigens in phagocytosis. We, therefore, compared the induction of CL by bacterial suspensions before and after heat treatment. K(L) and K(A) antigens were inactivated by heating for 30 min at 70°C and 10 min at 120°C respectively. The results (fig. 1) show that heat-treated K(L) strains were much better inducers than the K^- mutant strains. This would suggest that more thermolabile surface antigens are involved in inhibition of phagocytosis. Surprisingly, heated K(A) cells were as poor inducers of CL as the untreated K^+ cells. In view of the purpose of this paper we decided not to try to elucidate the reason for the observed differences between K^- cells and the heat-treated K^+ cells. All further experiments were performed with K^- mutant cells, thus avoiding undesired side effects of heat treatment.

Effect of K antigens on common pili

Common pili adhere to specific receptors on PMNL and their presence can be shown by mannose sensitive haemagglutination (MSHA) of guinea pig erythrocytes. We tested the MSHA properties of K^+ and K^- cells. Broth cultures incubated for 24 and 48 h were used to enhance pili production (Duguid *et al.*, 1979). The difference in haemagglutination titre was a factor of two at the most (table II). Growth in the presence of glucose stimulates capsule production but inhibits pili production (Ofek *et al.*, 1979). Cells grown in the presence of glucose showed much lower haemagglutination titres, which may be explained by the effect of glucose on production of pili. We conclude that even in conditions that promote capsule production, no substantial difference could be detected between the haemagglutinating capacities of K^+ and K^- cells. These results do not indicate an important effect of K antigens on the function of pili.

TABLE II
*Haemagglutination of guinea pig erythrocytes by K^+ and K^- strains of *E. coli**

Strain	Haemagglutination titres of					
	24-h broth cultures		48-h broth cultures		24-h glucose-broth cultures	
	K^+	K^-	K^+	K^-	K^+	K^-
AD35	64	64	32	64	<2	<2
AD36	64	64	64	64	16	16
AD121	64	128	64	128	8	8
AD307	16	16	16	16	<2	<2
AD311	<2	<2	2	2	<2	<2
AD412	2	<2	32	16	2	<2

TABLE III

Effect of extracts of K⁺ and K⁻ E. coli cells on zymosan induced CL of PMNL

Strain	CL of PMNL in the presence of cell extracts of		Ratio K ⁺ /K ⁻
	K ⁺ strains	K ⁻ strains	
AD35	60	57	1.05
AD36	55	53	1.03
AD121	50	50	1.00
AD307	25	27	0.91
AD311	30	27	1.12
AD412	64	67	0.95

* CL is expressed as the percentage of CL without extracts.

Toxic effects of K⁺ and K⁻ strains on PMNL

To determine any toxic effect of K antigens on PMNL, we tested the influence of extracts of K⁺ and K⁻ strains on CL of PMNL induced by preopsonised zymosan. Extracts of both types of strains inhibited the CL to a similar extent (table III) with a K⁺/K⁻ ratio of about unity. The inhibiting effect was most pronounced for the two strains of *E. coli* O8.

Role of antibodies and complement in opsonisation

The role of antibodies and complement in the opsonisation of K⁺ and K⁻ strains was studied. The induction of CL by K⁺ and K⁻ strains was measured in the presence of NHS, HIS and MgEGTA-S. The results of these experiments are given in figs 2 and 3 and in table I. Total inactivation of complement resulted in a considerable loss

FIG. 1.—Chemiluminescence induced by K⁺ strains (▨), K⁻ mutant strains (□) and K⁺ strains after heat-treatment (▧). CL is expressed as the percentage of CL induced by K⁻ strains.

of PMNL CL. The remaining activity, which was only 10% for most of the strains, can most probably be ascribed to opsonisation by antibodies alone. Three strains (AD307K⁻, AD311K⁻ and AD412K⁻) showed considerable activity in HIS, which varied from 35 to 43% of that in NHS. This high activity was in agreement with the high titre of antibodies against these strains in the serum (table I). The kinetics of CL production with these three strains in the presence of NHS and HIS were quite different (figs 2 and 3). CL in HIS only rose after an initial period of 30 min and reached a maximum after 50 min. In the presence of MgEGTA-S this late rise in CL was observed with all K⁻ strains and with one K⁺ strain (AD35). The other K⁺ strains showed very low activities in MgEGTA-S with a maximum at about 20 min. The slow rise in CL could be the result of ineffective opsonisation. It has already been shown that opsonisation via the alternative pathway of complement activation is a relatively slow process (Tofte *et al.*, 1980; Roberts and Phillips, 1983). The low total activity of PMNL could be the result of this inefficient opsonisation, but may also be explained by an impairment of the PMNL by the time opsonisation is completed. Preopsonisation of the strains to test this hypothesis was not possible because of the extreme serum sensitivity of the K⁻ strains. Therefore, we used zymosan to elucidate this problem. CL with zymosan was depressed in the presence of MgEGTA-S (fig. 4a); preopsonisation of zymosan with NHS and MgEGTA-S resulted in a similar activity (fig. 4b). This suggests that the low counts with MgEGTA-S are based on slow opsonisation via the alternative pathway of complement activation combined with an

FIG. 2.—Chemiluminescence with strain AD35K⁻ (O6K⁻H1) in the presence of NHS (●—●), HIS (▲—▲) and MgEGTA serum (*—*).

FIG. 3.—Chemiluminescence with strain AD412K⁻ (O7K⁻H⁻) in the presence of NHS (●—●), HIS (▲—▲) and MgEGTA serum (*—*).

FIG. 4a.—Chemiluminescence with zymosan in the presence of NHS (●—●) and MgEGTA serum (*—*).

FIG. 4b.—Chemiluminescence with zymosan, preopsonised with NHS (●—●) and MgEGTA serum (*—*).

impairment of PMNL function after incubation for 30 min at 37°C. To exclude a possible direct effect of MgEGTA on PMNL, a control experiment was performed. Zymosan was preopsonised with NHS and the induction of CL of PMNL by this zymosan suspension was measured in the presence and absence of MgEGTA. No difference was observed.

Complement consumption by K⁺ and K⁻ cells

Because the results of CL in the presence of MgEGTA-S are difficult to interpret, we tested the role of complement activation via the classical and alternative pathways by measuring complement consumption in NHS and MgEGTA-S. The results (table I) show that complement consumption was lower for all K⁺ strains compared with their K⁻ variants in the presence of NHS. In the presence of MgEGTA-S, complement consumption was greatly reduced in all K⁻ strains and was negative for all K⁺ strains.

DISCUSSION

K antigens of *E. coli* may influence the interaction of bacteria with PMNL in several ways. Three possible mechanisms are evaluated in this paper. Our results showed that strains of *E. coli* with heat-inactivated K antigens were not the same as K⁻ strains with respect to CL induction; all experiments were, therefore, performed with naturally derived K⁻ mutants of *E. coli*. The first mechanism for interference of K antigens with phagocytosis that we studied was the effect on common pili. Common pili adhere to red blood cells and PMNL via specific receptor interaction (Bar-Shavit *et al.*, 1977; Duguid *et al.*, 1979; Mangan and Snyder, 1979; Silverblatt *et al.*, 1979). Mangan and Snyder (1979) using a bacteria/PMNL ratio of 300/1, showed that the interaction of bacteria with PMNL via pili in the absence of serum resulted in CL production. Their pili-negative strains did not induce CL under these circumstances. We doubt if common pili are of any importance for induction of CL in our experiments, because even at a ratio of 300/1 none of our bacterial strains, in the absence of serum, was able to induce CL. The reason for this could be that the bacterial culture conditions for our phagocytosis experiments (log-phase cells) do not favour production of pili.

A second possibility considered was that the K antigen has a toxic effect on PMNL as described for *Bacteroides melaninogenicus* (Okuda and Takazoe, 1973). Heat-extracted K antigens, which always contain certain amounts of other surface antigens, were toxic for PMNL. Extracts of K⁻ cells were equally toxic and this toxicity was probably due to endotoxin in cell extracts rather than K antigen.

Thirdly we studied differences in opsonophagocytosis of K⁺ and K⁻ strains. The phagocytosis of *E. coli* is impaired by the presence of K antigens. This conclusion can be drawn from our experiments and is described by many authors (Howard and Glynn, 1971; Van Dijk *et al.*, 1979a; Stevens *et al.*, 1980). Most conclusions, however, are based on studies where a set of K⁺ strains is compared with a set of completely different K⁻ strains. This always leaves the possibility of interference of other surface antigens.

With all strains we studied, whether K antigens were present or not, optimal

opsonisation occurred in the presence of antibodies and complement. Opsonisation by antibodies alone is possible albeit at a reduced rate as can be concluded from the results of CL production in heat-inactivated serum. This opsonisation depended upon relatively high titres of agglutinating antibodies in the serum. Van Dijk *et al.* (1981) also showed that antibodies alone, when present in substantial amounts, had opsonic capacities, although they did not observe the delay in opsonisation which we observed. Possibly, differences in antibody titres may be responsible for this discrepancy. The CL of PMNL in the presence of MgEGTA confirmed observations of other authors that K⁻ strains are more able to activate complement via the alternative pathway than K⁺ strains (Roberts and Phillips, 1983). The differences between the CL induction by the K⁺ and the K⁻ strains in the presence of MgEGTA were in good agreement with the results of the complement consumption studies. Although the general conclusion about K-antigen interference with complement activation is confirmed by our experiments, the differences between the individual strains clearly indicate that different K antigens do not influence the interaction of *E. coli* with complement in the same way.

The kinetics of CL production strongly depended on the route of opsonisation, being delayed when the classical pathway of complement activation was blocked. The relatively slow opsonisation when serum is inactivated or when the alternative pathway alone is available for complement activation has been described by Tofte *et al.* (1980) and by Roberts and Phillips (1983). Our results with zymosan suggested that a slow opsonisation resulted in a lower total CL response as a result of declining PMNL function by the time opsonisation was completed. These observations made it very difficult to compare quantitatively the CL results in the presence of NHS with those in the presence of HIS or MgEGTA-S. Preopsonisation of the bacteria would solve this problem, but this was not possible because of the extreme serum sensitivity of some of our K⁻ mutants. Our conclusions therefore relate to the qualitative aspects of opsonophagocytosis.

In general we conclude that all six K types interfered with phagocytosis by an effect on opsonisation.

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