The Role of the O and K Antigens in Determining the Resistance of *Klebsiella aerogenes* to Serum Killing and Phagocytosis

By PAUL WILLIAMS,¹ PETER A. LAMBERT,¹
MICHAEL R. W. BROWN¹* AND RODERICK J. JONES²

¹ Microbiology Research Group, Department of Pharmacy, University of Aston in Birmingham, Birmingham B4 7ET, U.K.

² MRC Vaccine Research Laboratories, Clinical Research Block, Medical School, University of Birmingham, Birmingham B15 2TJ, U.K.

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The presence of both K and O antigens of *Klebsiella aerogenes* was found necessary to protect the organism from either complement-mediated serum killing or phagocytosis in the absence of specific antisera. Optimal phagocytic ingestion of *K. aerogenes* NCTC 5055 could be achieved in the presence of either anti-K or anti-O sera or to a much smaller extent in antisera raised against a rough unencapsulated mutant (M10B) derived from NCTC 5055. Anti-O sera failed to opsonize a clinical klebsiella isolate (DL1) possessing immunologically identical lipopolysaccharide, but did so when the amount of capsule was physically reduced. The serum sensitivity of the encapsulated strains was unaffected by the addition of specific antisera. Fresh serum was bacteriostatic for an unencapsulated smooth mutant (M10) derived from NCTC 5055. This bacteriostatic effect was reduced by heat-inactivation of the serum or by the addition of anti-O serum. M10 was rendered sensitive to the bactericidal action of serum in the presence of antisera raised against M10B or after chelation with MgEGTA to isolate alternative complement pathway activity. The rough unencapsulated mutant (M10B) was rapidly killed by fresh serum, an effect which could be delayed by chelation with MgEGTA. The serum sensitivity of M10B was unaffected by the presence of anti-M10B sera. Thus, the O antigen, unlike the K antigen, of these klebsiella strains is not antiphagocytic but it does confer some protection against the rapid bactericidal activity of serum complement.

INTRODUCTION

Klebsiella species are opportunistic pathogens which can infect debilitated patients with urinary or respiratory tract complications and are an important cause of bacteraemia in patients compromised by neutropenia, immunosuppression, thermal injury or surgery (Rennie & Duncan, 1974; S. E. J. Young, 1982; Montgomerie & Ota, 1980).

The virulence of many *Enterobacteriaceae* has been closely linked to their O antigens (Stendahl *et al.*, 1979), which in *Escherichia coli* and *Salmonella* spp. have been shown to protect these organisms from the bactericidal effects of serum (Rowley, 1968) and from phagocytic ingestion and killing (Stendahl & Edebo, 1972). In encapsulated Gram-negative bacteria the polysaccharide K antigen may also contribute to the virulence of the organism by enabling it to resist complement activation and phagocytosis (Howard & Glyn, 1971). Most encapsulated bacteria appear to require specific anticapsular antibodies for effective opsonization to occur (Robbins *et al.*, 1980). However, much controversy still surrounds the ability of anti-O and anti-core glycolipid antibodies to opsonize encapsulated Gram-negative bacteria (Van Dijk *et al.*,

Abbreviations: LPS, lipopolysaccharide; NHS, normal human serum; PMN, polymorphonuclear leukocyte; WHB, whole human blood.

1981). In the present investigation the role of the O and K antigens of *Klebsiella aerogenes* in enabling this species to resist serum killing and phagocytosis was studied by means of isogenic mutants lacking either the K or both K and O antigens.

METHODS

Bacteria. *Klebsiella aerogenes* NCTC 5055 (capsular type K2) was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, U.K. Mutants M10 (K-O⁺) and M10B (K-O⁻), isolated after nitrosoguanidine mutagenesis of NCTC 5055, were kindly donated by Drs I. R. Poxton and I. W. Sutherland (Poxton & Sutherland, 1976). *Klebsiella aerogenes* DL1 was a clinical isolate of capsular type K1 but possessing lipopolysaccharide (LPS) immunologically identical to that of NCTC 5055 and M10.

Bacteria were grown for 18 h on nutrient agar (Oxoid) plates at 37 °C to optimize capsular production in the encapsulated strains. Cells were resuspended in 0.85% NaCl before addition to blood or serum. Resuspension directly from nutrient agar plates reduced the loss of capsular material that resulted from growth in liquid media and subsequent centrifugation at the high speed (30000 g) required to sediment these encapsulated bacteria.

Antiserum. Antiserum against *K. aerogenes* (whole bacterial cells) was raised in male New Zealand White rabbits (4.5 kg). Suspensions of *K. aerogenes* containing 10⁸ bacteria ml⁻¹ were prepared from overnight nutrient agar (Oxoid) cultures in 0.85% saline containing 0.5% formaldehyde. Intravenous injections of suspensions of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.0 ml were given on days 1, 2, 3, 9, 10, 11, 17, 18 and 19, respectively. Blood (10 ml) was taken from the marginal ear vein before immunization, before the final injection, and 10 d after the last injection. Serum was separated and filtered through a 0.45 µm Millipore membrane filter into sterile bottles and stored at 4 °C.

Absorption studies. Antiserum to *K. aerogenes* NCTC 5055 was absorbed with whole live cells of mutant M10, grown overnight on nutrient agar (Oxoid) and resuspended in 0.85% saline. To 1.0 ml of antiserum, 1.0 ml of a suspension of M10 containing 10¹⁰ cells ml⁻¹ was added and the mixture allowed to stand at 4 °C for 1 h. The suspension was then filtered through a 0.45 µm Millipore membrane filter and the procedure repeated until no agglutination could be observed by slide agglutination against saline suspensions (10¹⁰ cells ml⁻¹) of *K. aerogenes* M10.

Preparation of LPS and capsular polysaccharide. LPS was prepared from M10 and DL1 by the hot phenol extraction method (Westphal & Jann, 1965). A crude preparation of O and K antigens was obtained by ethanol precipitation of the cell supernatant of NCTC 5055 grown in a simple salts medium. The precipitate was collected by centrifugation, dialysed for 24 h against running tap water and freeze-dried.

Preparation and inactivation of serum. Fresh venous blood was obtained from a single healthy volunteer. The blood was allowed to clot for 1 h at room temperature and the serum was separated by centrifugation at 3000 g for 5 min. Serum was heat-inactivated at 55 °C for 30 min. To assay for functional alternative complement activity, samples of serum were treated with 10 mM-EGTA and 10 mM-Mg²⁺ in 0.85% saline prepared according to the method of Fine *et al.* (1972).

Serum bactericidal assays. To 0.6 ml of human serum containing the appropriate rabbit antiserum or pre-immune serum together with 0.85% saline or MgEGTA in a capped polypropylene tube, 0.6 ml of a bacterial suspension (*A*₄₇₀ 0.2) was added. The serum/bacteria mixtures were incubated at 37 °C in a reciprocal shaking water bath shaking at 120 r.p.m. At intervals 100 µl samples were taken from the mixture and diluted with sterile water, and viable counts were made according to the methods of Miles & Misra (1938) after overnight growth for 18 h at 37 °C. Each experiment was carried out in triplicate.

Phagocytosis of *Klebsiella aerogenes* in whole human blood. Phagocytosis was carried out in heparinized whole blood by the method described by Jones *et al.* (1979). Fresh venous blood (10 ml) drawn from a single volunteer was placed in a heparinized sterile plastic tube (New Brunswick LH/10) and used within 1 h of withdrawal. To 0.5 ml of blood in a capped polypropylene tube, 40 µl of the appropriate rabbit antiserum or pre-immune rabbit serum was added followed by 0.5 ml of a bacterial suspension (*A*₄₇₀ 0.2) prepared as above. The volume of antiserum added was chosen to give optimum opsonization without detectable agglutination of the bacteria. A direct microscopic count showed that the bacteria: polymorphonuclear leukocyte (PMN) ratio in this incubation mixture was 15:1. This ratio was chosen to optimize the level of ingestion; in some experiments the ratio was reduced to 1.5:1 by reducing the number of bacteria. Pre-opsonization of the bacteria before addition to the blood did not affect phagocytosis. The blood/bacteria mixtures were incubated in a shaking water bath at 120 r.p.m. and 37 °C. Samples (100 µl) were removed at intervals, added to 0.9 ml of sterile water at room temperature, and allowed to stand for 5 min before further dilution to lyse the blood cells without killing the bacteria before making a viable count.

At 30 min after the start of each experiment a small sample was removed from the blood/bacteria mixture and spread on a glass microscope slide. The blood smear was dried in air, fixed with methanol and stained with Giemsa's stain. The number of bacteria associated with 50 PMNs randomly counted under the microscope was

Fig. 1

Fig. 1. Kinetics of killing of *K. aerogenes* by normal human serum (NHS): ●, DL1 (K1+O+); ▲, NCTC 5055 (K2+O+); ■, M10 (K-O+); □, M10B (K-O-). The survival values indicate viable counts relative to time zero (100).

Fig. 2

Fig. 2. Kinetics of killing of *K. aerogenes* M10B by NHS: □, NHS alone; ○, NHS chelated with 10 mM-MgEGTA. The survival values are expressed as for Fig. 1.

recorded to provide an estimate of the number of bacteria ingested per PMN. Each experiment was repeated in duplicate twice. To retain some continuity a single blood donor was used. However, the results obtained were confirmed by repeating some of the experiments with blood from three other volunteers.

RESULTS

Interpretation of the results of this study assumes that the mutations affected only the O and K antigens, other outer envelope constituents remaining unchanged. Outer membrane protein profiles of *K. aerogenes* NCTC 5055, M10 and M10B above 18 kDal, revealed by sodium dodecyl sulphate polyacrylamide gel electrophoresis of Sarkosyl-insoluble membranes, failed to show differences.

Effect of serum on encapsulated Klebsiella aerogenes

Klebsiella aerogenes NCTC 5055 and DL1 were found to be resistant to serum (Fig. 1), their viable counts increasing by some 600–800% over the 3 h incubation period. Their viability was unaffected by the addition of a range of homologous and heterologous antisera raised against each of the klebsiella strains used in the study or by chelation of the serum with MgEGTA to prevent activation of the classical complement pathway whilst leaving the alternative pathway functional. Both strains grew in heat-inactivated serum.

Effect of serum on non-encapsulated Klebsiella aerogenes

Normal human serum was bacteriostatic for M10 whilst the rough mutant M10B was rapidly killed (Fig. 1). Both strains grew in heat-inactivated serum.

The results of further investigation of the effect of serum on M10B are shown in Fig. 2, which

Fig. 3. Kinetics of killing of *K. aerogenes* M10 by NHS: ■, NHS alone; ○, NHS plus antiserum to M10 (1 in 20 dilution); □, heat-inactivated serum chelated with 10 mM-MgEGTA; △, NHS chelated with 10 mM-MgEGTA; ●, NHS plus antiserum to M10B (1 in 20 dilution). The survival values are expressed as for Fig. 1.

reveals that the viable count was reduced by some 3 log scales in 20 min in fresh serum whilst chelation with MgEGTA delayed the rapid kill by some 15 min. Thus both classical and alternative complement pathways are rapidly bactericidal for M10B. The kinetics of the alternative pathway are such that its activation and subsequent effect are slower than the more efficient classical complement pathway (Root *et al.*, 1972).

During the 3 h incubation period in fresh serum at 37 °C no increase in the viable count of M10 was observed. Addition of a range of concentrations of anti-M10 serum (to final concentrations of between 1 in 100 000 and 1 in 20) had no effect on the bacteriostasis at the higher dilutions, but at a concentration of 1 in 20, the antiserum appeared to antagonize the bacteriostatic effect thus allowing the organism to proliferate (Fig. 3). Chelation of the serum to allow assay of alternative pathway activity resulted in a fall in viable count over the 3 h incubation period whilst a similar effect was observed upon addition of anti-10B serum (which lacks anti-O antibodies) to the assay system (Fig. 3). However, the organism multiplied in the presence of MgEGTA in heat-inactivated serum and therefore the effect was not due to the MgEGTA alone (Fig. 3). Addition of homologous anti-M10B serum to M10B did not antagonize the bactericidal effect of normal human serum for this strain.

Phagocytosis of Klebsiella aerogenes

A similar bactericidal response to that obtained in serum in the absence of added specific antibody was found to occur in blood (Fig. 4). Cell association rather than ingestion is the preferred term as the assay system employed does not differentiate completely between those bacteria adhering to the neutrophil outer surface and those enveloped by the phagosome. Analysis of the bacteria/PMN association profile revealed that whilst M10 was phagocytosed to the extent of 4.2 ± 0.5 bacteria per PMN (mean $\pm$ S.D.), the encapsulated strains NCTC 5055 and DL1 effectively resisted phagocytosis (Fig. 5). Thus the O antigens of these klebsiella strains are not in themselves antiphagocytic. The effects of specific antisera on phagocytic ingestion in terms of cell association of *K. aerogenes* NCTC 5055, DL1 and M10 are summarized in Fig. 6. Antisera to M10, M10B, 5055 and DL1 each increased the number of M10 associated with PMN more than the control pre-immune rabbit serum (Fig. 6c).

Fig. 4

Fig. 4. Kinetics of killing of *K. aerogenes* by whole human blood (WHB): ▲, NCTC 5055; ●, DL1; ■, M10; □, M10B. The survival values are expressed as for Fig. 1.

Fig. 5

Fig. 5. Bacteria/PMN cell association profiles. Ingestion of *K. aerogenes* M10, NCTC 5055 and DL1 by WHB in the absence of specific antisera. The bars represent s.d.

Uptake of the encapsulated strain NCTC 5055 occurred not only in the presence of anti-5055 serum but also in the presence of anti-M10 serum. As anti-M10 serum did not contain anticapsular antibodies it appeared that antibodies directed against components other than the polysaccharide capsule were responsible for opsonization. By contrast, anti-M10B serum gave only a small increase in the number of PMN-associated NCTC 5055. These findings indicate that the capsule and O antigens were the main surface structures involved in opsonization and that the presence of K antigen does not completely mask access of antibody directed to O antigen. Confirmation that anti-K antibodies alone could opsonize NCTC 5055 was obtained by absorbing anti-5055 serum with whole live M10 organisms. The immunodiffusion plate shown in Fig. 7 shows the absence of anti-O antibodies in the absorbed antiserum.

Klebsiella aerogenes DL1 has a type K1 polysaccharide capsule but possesses an O antigen that immunologically cross-reacts with M10 LPS (see Fig. 8). DL1 resisted phagocytosis in the absence of homologous antiserum (0.3 ± 0.05 bacteria per PMN as opposed to 12 ± 0.4 bacteria per PMN in the presence of anti-DL1) (Fig. 6*b*). In this case, addition of anti-M10 or anti-M10B to the blood/bacteria mixture did not result in opsonization of DL1. On the basis of colonial morphology, DL1 is much more mucoid than NCTC 5055; a greater covering of acidic polysaccharide may shield the O antigen or prevent access of the anti-O antibodies to their target receptor. Physical reduction of the amount of capsule surrounding DL1 was achieved by gently homogenizing the organism before centrifuging at 18000 r.p.m. and resuspension in fresh saline. After this treatment, DL1, as Fig. 6*b* demonstrates, was found to be opsonized to a greater extent by anti-10 antiserum (1.64 ± 0.08 as compared to 0.3 ± 0.05 bacteria per PMN).

The phagocytic system employed was optimized to study the effect of various antisera in promoting PMN association and ingestion. The ratio of bacteria to PMNs was approximately 15:1. Little of the bactericidal capability of the PMNs was apparent at this bacterial cell density. Figure 9 shows the result of reducing the ratio 10-fold, i.e. to 1.5:1, which makes clearly apparent the killing ability of the PMNs in the presence of added antisera.

Fig. 6. Bacteria/PMN cell association profiles. Ingestion of *K. aerogenes* NCTC 5055 (a), DL1 (b) and M10 (c) in the presence or absence of antisera prepared against whole cells of *K. aerogenes* NCTC 5055, M10, M10B and DL1 as indicated. 5055 abs. indicates addition of NCTC 5055 antiserum pre-absorbed with whole live cells of M10. M10* (panel b) represents the association profile obtained after homogenizing and washing the cells (DL1) before subjecting them to the WHB ingestion assay in the presence of antiserum to M10. The bars represent s.d.

DISCUSSION

The O and K antigens of *K. aerogenes* were both found to be involved in determining the resistance of *K. aerogenes* to complement-mediated serum killing and to phagocytic ingestion by human PMNs. The O antigen of M10 was not antiphagocytic but it was found to protect M10 from the rapid bactericidal action of serum. This protection was not complete since, unlike the encapsulated parent strain, M10 could not grow in fresh serum, which exerted a bacteriostatic effect. This could, however, be reversed since the strain grew upon addition of anti-M10 serum. As anti-M10B serum failed to reverse bacteriostasis, it is most likely that IgG antibodies directed against the O antigen of M10 exerted a blocking effect. A reversal from serum sensitivity to complete resistance and growth has been reported for other Gram-negative bacteria (Taylor, 1972; Traub, 1981; Guttman & Waibron, 1975) as a result of the presence of, or following the addition of immunoglobulins. This was interpreted in terms of the prevention of access of complement components to cell surface receptors by anti-O IgG binding to the O polysaccharide chain of LPS; IgM alone was found to enhance the bactericidal activity of serum (Traub, 1981).

The prompt killing of M10B (K⁻O⁻) in fresh human serum was unaffected by a range of added concentrations of anti-M10B serum. Therefore, the blocking effect of antibody is only manifested in the presence of the O antigenic determinants.

Fig. 7. Double diffusion precipitation in 1% agarose, Tris/barbital buffer, pH 8.6. The centre well contained antiserum to NCTC 5055 pre-absorbed with whole live cells of M10. The outer wells contained purified LPS from M10 prepared by phenol extraction of whole cells (wells 3, 5) or a crude mixture of O and K antigens from NCTC 5055 prepared by ethanol precipitation of cell culture supernatant (wells 1, 2, 4, 6).

The loss of the O antigen of *Klebsiella* rendered the organism very sensitive to rapid complement-mediated serum killing. This effect was delayed upon chelation of the serum with MgEGTA to assay alternative complement pathway activity. This may reflect the slower kinetics of activation of the alternative pathway (Root *et al.*, 1972), although the rapid lethal effect may involve other serum bactericidal factors such as those reported by Chedid *et al.* (1968). Another factor might be the heat-labile, calcium-dependent, complement-requiring factor specific for the Ra mutant of *Salmonella* (Ihara *et al.*, 1982). This mutant is equivalent to M10B inasmuch as both lack the O antigen whilst retaining the core polysaccharide and lipid A moieties of LPS (Poxton & Sutherland, 1976). M10 was resistant to the bactericidal capacity of fresh serum but was sensitive, like M10B, to serum chelated with MgEGTA. This effect on M10 was not due to a toxic effect of MgEGTA alone, as the organism multiplied in heat-inactivated serum chelated with MgEGTA. A synergistic effect between complement and EGTA cannot, however, be completely ruled out.

In the absence of specific antisera, the encapsulated *K. aerogenes* strains NCTC 5055 and DL1 were not ingested to any appreciable extent by PMNs in whole human blood. As expected, addition of homologous antisera to the bacteria resulted in a dramatic increase in phagocytic ingestion. Anti-M10 serum (lacking anti-capsular antibodies) opsonized NCTC 5055 as effectively as the homologous antiserum, but failed to opsonize DL1 unless the amount of capsule was reduced by gentle homogenization of the cells followed by resuspension in fresh 0.85% saline.

Ehrenworth & Baer (1956) studied the effect of the amount of capsule on phagocytosis of *klebsiella* capsular type 2 mutants and found that this did not affect phagocytic ingestion to any

Fig. 8. Double diffusion precipitation: (a) the centre well contained antiserum to DL1, the outer wells contained purified LPS from M10; (b) the centre well contained antiserum to M10, the outer wells contained purified LPS from DL1 (1, 3, 5) or purified LPS from M10 (2, 4, 6).

Fig. 9. Kinetics of killing of *K. aerogenes* NCTC 5055 by WHB with bacteria:PMN ratio 15:1 (▲) or 1:5:1 (△); and by WHB plus antiserum to NCTC 5055 with bacteria:PMN ratio 15:1 (▼) or 1:5:1 (▽). The survival values are expressed as for Fig. 1.

great extent. Our findings showed that, whilst physical reduction of the amount of DL1's capsule did not itself enhance phagocytosis, addition of antisera containing anti-O antibodies rendered the organism susceptible to opsonization and subsequent phagocytic engulfment.

More than 80 distinct *K. aerogenes* capsular types have been recognized (Powell, 1979) but they appear to share just 12 different O antigens (Nimmich & Korten, 1970). Since we have shown that under certain conditions it is possible to opsonize encapsulated strains of klebsiella with antibody to the O antigen, this opens the possibility that a vaccine conferring protection against a wide range of klebsiella capsular types might be composed of the 12 O antigens together with K antigens from those strains in which the capsule masks the O antigen.

Several investigators (McCabe, 1972; Ziegler *et al.*, 1978; Young *et al.*, 1975) have reported that antiserum raised against rough mutants of the *Enterobacteriaceae* contain antibodies to the core glycolipid of LPS and could passively transfer broad-spectrum protection against parenteral challenge with smooth strains of the *Enterobacteriaceae*. Whilst this effect is thought by some to be mainly antitoxic (Braude *et al.*, 1973), others, e.g. Crowley *et al.* (1982), have shown that opsonization of smooth *E. coli* with antiserum to Re *Salmonella minnesota* could be observed. There was, however, some doubt as to whether antibodies to surface components other than the core glycolipid were also involved, since similar findings were obtained when both *E. coli* and klebsiella strains were tested in opsonization studies with antiserum to Re *S. minnesota* in which the core glycolipid antibodies had been absorbed.

Anti-M10B serum, presumably containing anti-core glycolipid antibodies, opsonized not only M10 but also, though to a much lesser extent, the encapsulated parent. However, in terms of formulation of an effective anti-klebsiella vaccine, the opsonization due to the anti-core glycolipid is not as significant as that conferred by the anti-O and anti-K sera.

The conditions used to grow the organisms for the preparation of antisera and for serum and phagocytosis assays were not the same as those encountered *in vivo*. The nutritional environment will greatly affect the composition of the outer envelope of Gram-negative bacteria and therefore the response of the organism to serum (Taylor *et al.*, 1981) and phagocytosis (Finch & Brown, 1978). Sensitivity of Gram-negative bacteria to serum has been shown to vary with a number of factors, including growth rate and the availability of magnesium and carbon (Taylor *et al.*, 1981). The effects of these parameters on phagocytosis have not been extensively explored.

We believe that future studies of this nature should use bacteria grown under conditions which more closely mimic those occurring *in vivo*, for example, at a slow growth rate in chemically defined media, or in normal serum (Brown, 1977). Similar consideration should be given to conditions employed to grow organisms for antigen and vaccine production.

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