INFECTION AND IMMUNITY, Nov. 1987, p. 2741–2746
0019-9567/87/112741-06$02.00/0
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Vol. 55, No. 11

Role of Lipopolysaccharide and Complement in Susceptibility of *Klebsiella pneumoniae* to Nonimmune Serum

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Received 3 April 1987/Accepted 14 August 1987

The role of lipopolysaccharide (LPS) in the susceptibility of *Klebsiella pneumoniae* to serum and the mechanism of complement activation by serum-susceptible (Ser^s^) strains were investigated. The classical and alternative complement pathways are involved in serum killing of susceptible *K. pneumoniae* strains. The LPS composition seems to play a very important role in the serum bactericidal reaction, while capsular polysaccharide from this bacterium does not play any role. High-molecular-weight LPS from serum-resistant (Ser^r) *K. pneumoniae* strains was able to inhibit completely the serum bactericidal activity. LPS from Ser^s *K. pneumoniae* strains was not able to inhibit completely the serum bactericidal activity; low-molecular-weight LPS from Ser^r *K. pneumoniae* strains could not either. All these findings suggested that LPS composition, especially the O-antigen polysaccharide chains, contributes to the susceptibility of *K. pneumoniae* strains to complement-mediated serum bactericidal activity.

The bactericidal effect of nonimmune serum plays an important role in the host defense against bacterial infections. This phenomenon has been widely noted and studied since the late 1800s (4, 26) and has been shown to be complement mediated (31). Although the serum resistance of gram-negative bacteria probably has a multifactorial basis in vivo, the outer membrane clearly is involved as the most peripheral component of the bacterial cell envelope. Several studies have implicated outer membrane components, such as lipopolysaccharide (LPS) (8, 23–25, 34) and outer membrane proteins (11, 13, 21, 28), and other components of the bacterial surface, such as capsular polysaccharide (7, 19, 36), in the resistance of bacterial strains to the bactericidal activity of serum.

Complement activation by gram-negative bacteria can occur via the classical pathway (CPC) or the alternative pathway (APC). The CPC can be activated by the interaction of antibody with bacterial surface antigens or directly by the lipid A moiety of LPS (22). The APC can be activated by bacterial surface polysaccharides independent of antibody (22).

In the present study, we have investigated the mechanism of complement activation by *Klebsiella pneumoniae* strains and the role of LPS and the capsular polysaccharide in the susceptibility of these strains to the bactericidal activity of serum. Furthermore, we also have investigated the role of the high-molecular-weight LPS (HMW-LPS) (O-antigen enriched) and the low-molecular-weight LPS (LMW-LPS) (core and lipid A enriched) isolated by LPS fractionation. The results suggest that both complement pathways are involved in the serum killing of serum-susceptible (Ser^s) *K. pneumoniae* strains and that the HMW-LPS of serum-resistant (Ser^r) *K. pneumoniae* strains plays a critical role in their susceptibility to nonimmune serum. Capsular polysaccharide seems not to be involved in the serum resistance of *K. pneumoniae* strains.

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MATERIALS AND METHODS

Bacterial strains and media. The strains used are listed in Table 1. The basal medium used for bacterial growth was Luria broth (20) or Luria broth with 1.5% agar.

Bacterial survival in fresh nonimmune serum. The survival of logarithmic-phase bacteria in serum was measured as previously described (40). Controls, bacteria in phosphate-buffered saline (PBS), showed no significant changes in viable counts over the incubation period. Rabbit serum was usually used on the day of collection or stored at −80°C.

Treatment of serum. CPC activity in serum was selectively inhibited by chelation with 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) plus 2 mM $MgCl_2$ as previously described (6). Serum was treated with inulin (2 mg/ml) by the method of Gotze and Muller-Eberhad (10) or heated at 50°C for 20 min to inactivate factor B, which is required for APC activity in serum (5). After each serum treatment, we assayed the anticomplement activity of the treated serum by the method of Morrison and Kline (22) as modified in a microtiter assay by Vukajlovich (42) to be sure that the treatment was correct. Both pathways were inhibited by the treatment of serum with 20 mM EDTA or by heating at 56°C for 30 min. EGTA, EDTA, and inulin at the concentrations mentioned above had no effect on the survival of *K. pneumoniae* strains in PBS during 2 h of incubation.

LPS and capsule isolation. LPS from *K. pneumoniae* strains was purified by the method of Westphal and Jann (44) as modified by Osborn (27). Capsular polysaccharide was purified by the method of Wilkinson and Sutherland (45).

Subfractionation of LPS by column chromatography. Lyophilized LPS from *K. pneumoniae* C3 (Ser^r) was solubilized at a final concentration of 7.5 mg/ml in buffer containing 3% (wt/vol) sodium deoxycholate, 0.2 M NaCl, 5 mM EDTA, and 20 mM Tris hydrochloride (pH 8.3) and applied at room temperature to a column of Sephacryl S-300 (Pharmacia Fine Chemicals) equilibrated in buffer containing 0.25% (wt/vol) sodium deoxycholate, 0.2 M NaCl, 5 mM EDTA, and 10 mM Tris hydrochloride (pH 8.0). Fractions (2.5 ml) were col-

TABLE 1. *K. pneumoniae* used in this work[a]

Strain	Relevant characteristics
C3	Wild type; O1:K66; Ser^r
KT701	Ser^s; contains capsular polysaccharide but not HMW-LPS
KT703	Ser^s; contains capsular polysaccharide but not HMW-LPS
KT707	Ser^s; contains capsular polysaccharide but not HMW-LPS
KT723	Ser^r mutant derived from KT707; contains capsular polysaccharide and HMW-LPS

[a] From reference 40.

lected at a flow rate of 12 ml/h and analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Before chemical analyses and serum inhibition studies, fractions were extensively dialyzed against distilled water, first at room temperature and then at 4°C.

Electrophoretic techniques. SDS-PAGE was performed by the procedure of Laemmli (16) as modified by Ames et al. (2). Samples were mixed 1:1 with sample buffer (containing 4% SDS) and boiled for 5 min, and 10-μl portions were applied to the gel. LPS bands were detected by the silver stain method of Tsai and Frasch (41).

Analytical procedures. The amount of organic phosphate was measured by the method of Ames and Dubin (1) with $NaH_2PO_4 \cdot 2H_2O$ as the standard, and total carbohydrates were measured by the phenol reaction procedure (12) with glucose as the standard. 2-Keto-3-deoxyoctulosonic acid (KDO) was measured by the thiobarbituric acid method after hydrolysis of samples in 0.1 N sulfuric acid for 30 min (15).

Inhibition of serum bactericidal activity by complete or fractionated LPS and capsular polysaccharide. LPS was suspended in PBS (pH 7.2) to a final concentration of 1 to 5 mg/ml and sonicated briefly at 4°C until the solution cleared. LPS solution in the concentration range of 0.01 to 0.2 mg/ml was added to 0.5 ml of serum in a tube. The volume was adjusted to 0.9 ml with PBS, and the solution was incubated at 37°C with shaking (200 rpm) for 30 min. Then, 0.1 ml of bacterial suspension (5×10^7 CFU) in the logarithmic phase was added to the tube and incubated at 37°C for 1 h before dilution plating. Similar experiments were performed with LPS fractions lyophilized and suspended in PBS and added at a final concentration of 0.05 mg/ml to 0.5 ml of serum. Experiments with capsular polysaccharide in the concentration range of 0.01 to 0.2 mg/ml were performed as described above for the LPS experiments. Controls (without LPS, LPS fractions, or capsular polysaccharide) showed no inhibition of serum bactericidal activity.

Measurement of the anticomplement activity of *K. pneumoniae* LPS and capsular polysaccharide. The anticomplement activity of LPS and capsular polysaccharide was measured by the method of Shafer et al. (35) with slight modifications. Serum (0.1 ml) was mixed with LPS or capsular polysaccharide (final concentration, 0.025 to 0.4 mg/ml) suspended in PBS or with PBS alone to a final volume of 0.2 ml and incubated with shaking at 37°C for 30 min. Antibody-sensitized sheep erythrocytes in 0.2 ml of PBS were added to a fourfold dilution of treated rabbit serum and incubated for 30 min in a 37°C water bath. Ice-cold saline (3 ml) was added to the mixture, the cells were pelleted by centrifugation, and the A_{412} of the supernatant was measured. The positive control was sensitized sheep erythrocytes plus serum without added LPS, and the negative control was LPS or capsular polysaccharide without added serum.

RESULTS

Mechanism of complement activation by *K. pneumoniae* strains. The mechanism of complement activation by Ser^s *K. pneumoniae* strains was examined. Serum treated with Mg^{2+}-EGTA (which selectively inhibits CPC) and serum pretreated with inulin or serum heated at 50°C for 20 min (which depletes APC) were bactericidal for Ser^s *K. pneumoniae* KT707 (Fig. 1). This strain was also rapidly killed in untreated serum. However, serum treated with EDTA or heated at 56°C for 30 min (which inhibits both CPC and APC) was nonbactericidal for Ser^s *K. pneumoniae* KT707 (Fig. 1).

Similar results were obtained with other Ser^s strains of *K. pneumoniae*, such as KT701 or KT703 (data not shown). These results suggested that serum killing of Ser^s *K. pneumoniae* strains is mediated by both CPC and APC.

Inhibition of serum bactericidal activity by *K. pneumoniae* LPS and capsular polysaccharide. Capsular polysaccharide from *K. pneumoniae* C3 (wild type, Ser^r) in the concentration range of 0.025 to 0.4 mg/ml was unable to inhibit the bactericidal activity of serum against Ser^s strain KT707.

Purified LPS from *K. pneumoniae* C3 at various concentrations completely inhibited the bactericidal activity of serum against Ser^s strain KT707. The percentages of survival of strain KT707 cells in serum after 60 min of incubation at 37°C were 0, 48, 89, 120, 108, and 115% at LPS concentrations of 0, 0.025, 0.05, 0.1, 0.2, and 0.4 mg/ml, respectively.

Purified LPS from *K. pneumoniae* KT707 (Ser^s) at various concentrations was unable to inhibit completely the bactericidal activity of serum against the same strain. The percentages of survival of strain KT707 cells in treated serum after 60 min of incubation at 37°C were 0, 0, 0, 6, and 20% at LPS concentrations of 0, 0.025, 0.05, 0.1, and 0.2 mg/ml, respectively.

Table 2 shows the percentage of survival of *K. pneumoniae* strains in nonimmune serum treated with 0.1 mg of LPS from Ser^r and Ser^s strains per ml. Ser^s strains (KT707, KT701, and KT703) showed a percentage of survival in control serum of less than 1% after 60 min of incubation, whereas these strains survived well in serum pretreated with LPS obtained from either of the two Ser^r *K. pneumoniae* strains. LPS from Ser^s strains (KT707, KT701, and KT703) was unable to inhibit the bactericidal activity of the control serum, but a small increase in the percentage of survival of the Ser^s strains with respect to control serum was noticed (Table 2).

Subfractionation of LPS. LPS from *K. pneumoniae* C3 was fractionated as described in Materials and Methods. The presence of LPS in column fractions was monitored by the determination of KDO and total hexose and by SDS-PAGE (Fig. 2 and 3, respectively).

Two major peaks of KDO were observed in the column profile. The first peak, centered at fraction 27 (Fig. 2), corresponded to the main peak of hexose and to HMW-LPS (Fig. 3). In fractions 25 to 29, the apparent hexose/KDO ratio was approximately 10 to 12. This value decreased in the following fractions until the second peak of KDO, centered at fraction 39 (Fig. 2), in which the apparent hexose/KDO ratio was approximately 1 to 2, a feature consistent with LPS fractionation on the basis of a decreasing number of repeating units in the O-antigen side chains. Fractions 35 to 47 contained LMW-LPS (Fig. 3).

Inhibition of serum bactericidal activity by *K. pneumoniae* LPS fractions. Table 3 shows the percentage of survival of *K. pneumoniae* KT707 (Ser^s) in nonimmune serum treated with

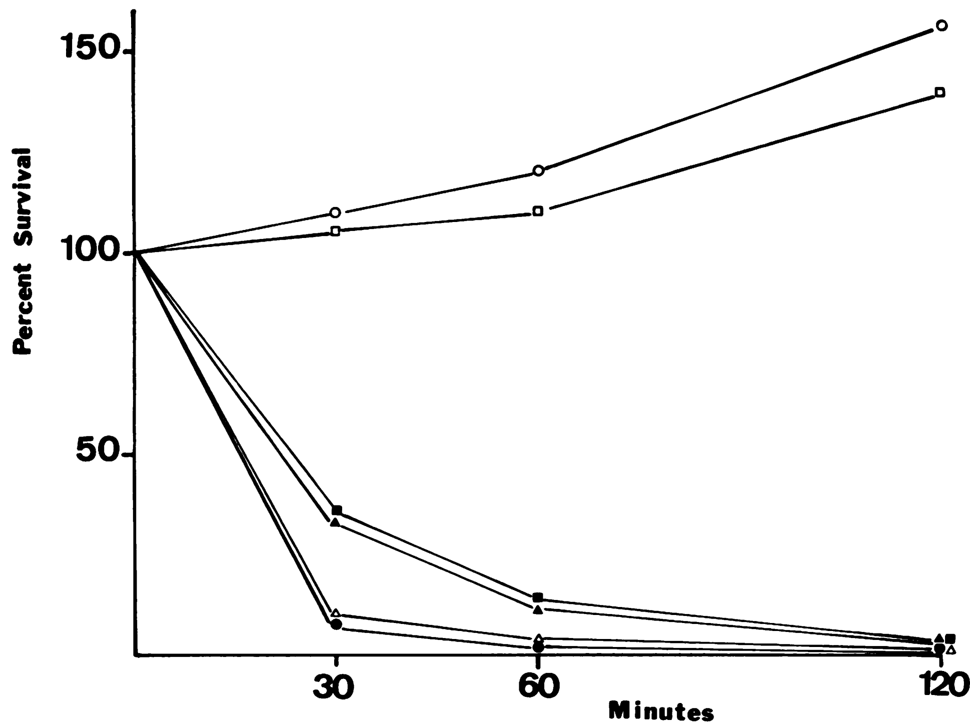

FIG. 1. Kinetics of killing of *K. pneumoniae* KT707 (Ser^s) by nonimmune serum. Symbols: ●, serum control; ○, serum heated at 56°C for 30 min; □, serum plus 20 mM EDTA; △, serum plus 20 mM EGTA and 2 mM $MgCl_2$; ▲, serum plus inulin (2 mg/ml); and ■, serum heated at 50°C for 20 min.

0.05 mg of different LPS fractions per ml. Strain KT707 showed a high percentage of survival in serum treated with LPS fractions containing a high hexose/KDO ratio (HMW-LPS), such as fraction 26 or 28. When the hexose/KDO ratio decreased in the LPS fractions, there was also a decrease in the percentage of survival of KT707 cells in serum treated with these LPS fractions.

When nonimmune serum was treated with LPS fractions containing a low hexose/KDO ratio (LMW-LPS), the percentage of survival of KT707 cells in this treated serum was always less than 10%, but some residual effect on the serum bactericidal activity was found.

Measurement of the anticomplement activity of *K. pneumoniae* LPS. The anticomplement activity of *K. pneumoniae* C3 LPS was measured to determine whether its inhibitory effect on serum bactericidal activity was due to its ability to activate and deplete complement. The anticomplement activity of *K. pneumoniae* C3 LPS was dose dependent. The LPS of *K. pneumoniae* KT707 (Ser^s) had low anticomplement activity, even at high concentrations (0.4 mg/ml), while the LPS of *K. pneumoniae* C3 (Ser^r) had anticomplement activity even at a concentration of 0.025 mg/ml and afforded good protection against hemolysis at 0.1 mg/ml (Fig. 4).

TABLE 2. Inhibition of serum bactericidal activity against *K. pneumoniae* strains by homologous LPS and heterologous LPS

Strain	% Survival[a] of strains after 60 min of incubation in:					
	Control serum	Serum plus LPS from strain:				
		KT707	KT701	KT703	C3	KT723
KT707	<1	6	3	2	120	117
KT701	<1	2	7	3	128	121
KT703	<1	2	4	6	118	122

[a] Averages of three independent experiments. LPS was used at 0.1 mg/ml.

DISCUSSION

The bactericidal effects of immune and nonimmune sera are mediated by activated components of the CPC or APC (28, 38). Activation of either can lead to membrane damage, culminating in cell death (38). Our study of the mechanism of complement activation by *K. pneumoniae* strains indicates that both the CPC and the APC are involved in the serum killing of Ser^s strains. Selective inhibition of CPC activation by the treatment of serum with Mg^{2+}-EGTA or the treatment of serum with inulin or heating at 50°C for 20 min, which depletes it of APC, did not abolish serum bactericidal activity. In all cases there was a slight decrease in serum bactericidal activity, as compared with untreated serum. These results suggested that the serum killing of Ser^s *K. pneumoniae* strains is mediated by both the CPC and the APC. Other enterobacteria, such as *Escherichia coli* (37) and *Salmonella* spp. (14), are also known to activate both complement pathways.

Several studies have implicated capsular polysaccharide in bacterial resistance to serum killing (7, 19). These studies were usually performed in *E. coli*, a nonconstitutive encapsulated bacterium. Previous results obtained by us (3, 40) plus the present results indicated that in *K. pneumoniae*, a naturally encapsulated bacterium, capsular polysaccharide does not play any important role in serum resistance. Also, isogenic nonencapsulated mutants (O^+ K^-) had the same serum resistance as *K. pneumoniae* C3 (O^+ K^+) (data not shown).

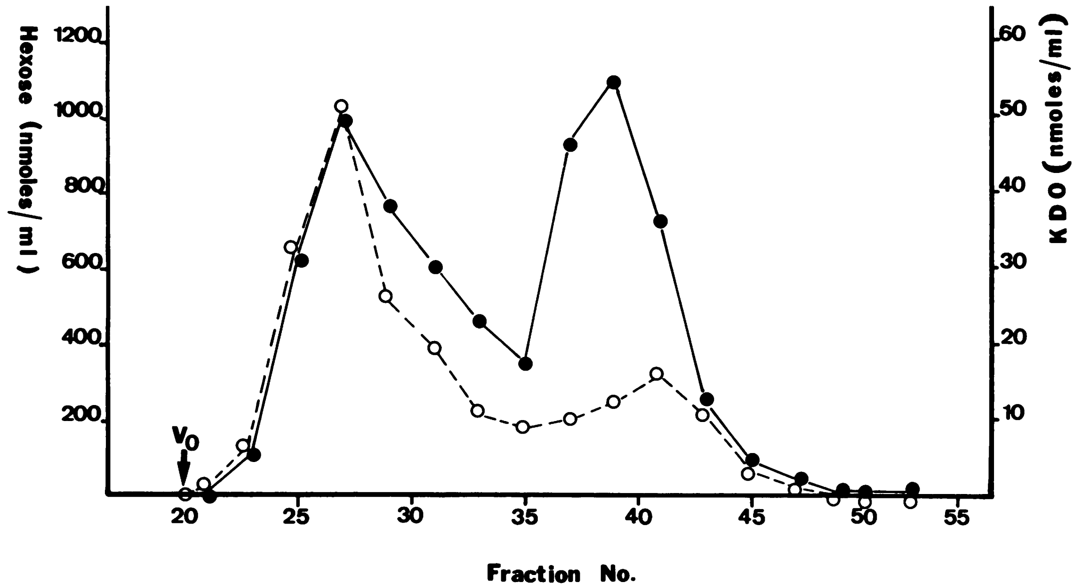

FIG. 2. Subfractionation of LPS from *K. pneumoniae* C3 (Ser^r) by column chromatography. LPS was applied to a column of Sephacryl S-300 and eluted with 0.25% buffered deoxycholate as described in Materials and Methods. Eluted fractions were analyzed for KDO and total hexose after extensive dialysis and by SDS-PAGE in conjunction with silver staining for carbohydrate (see Fig. 3). Symbols: ●, KDO; ○, total hexose; V_0, voided volume.

The susceptibility of a number of gram-negative bacteria to the bactericidal activity of immune and nonimmune sera has been attributed to their LPS composition (23, 30, 32, 33, 35, 37). We found that LPS from Ser^r *K. pneumoniae* strains inhibited serum bactericidal activity against Ser^s strains, whereas LPS from Ser^s strains was not inhibitory even at high concentrations. Furthermore, we showed that there was a correlation between the anticomplement activity of LPS, measured as the percent inhibition of hemolysis with sensitized erythrocytes, and its inhibitory effect on serum bactericidal activity.

As described previously (18, 29), the fractions containing HMW-LPS contained a large number of O-antigen side-chain repetitions. This feature was consistent with the apparent hexose/KDO ratio in the HMW-LPS fractions (10 to 12 O-antigen side-chain repetitions). The fractions containing HMW-LPS were able to inhibit serum bactericidal activity, while the fractions containing LMW-LPS (lipid A and core) were not able to do so completely. LMW-LPS fractions had a low apparent hexose/KDO ratio (1 to 2), indicat-

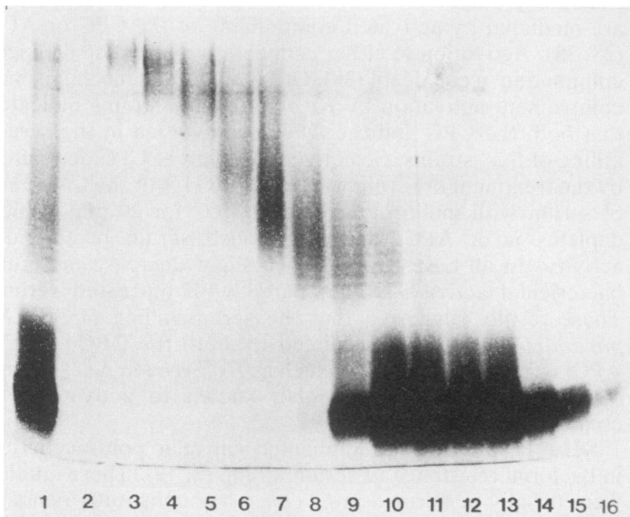

FIG. 3. Analysis of subfractionated LPS from *K. pneumoniae* C3 (Ser^r) by SDS-PAGE. LPS was fractionated by gel filtration as described in Materials and Methods (see Fig. 2). Eluted fractions were analyzed and stained with silver stain (41) for carbohydrate. Lanes: 1, purified LPS from *K. pneumoniae* C3; 2 to 16, fractions 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, and 49, respectively. The fraction numbers are the same as those in Fig. 2.

TABLE 3. Inhibition of serum bactericidal activity against *K. pneumoniae* KT707 (Ser^s) by complete LPS and fractionated LPS from *K. pneumoniae* C3 (Ser^r)

LPS	Hexose/KDO ratio[a]	% Survival[b] of KT707 cells after 60 min of incubation in serum plus LPS or LPS fraction
Fraction no.[c]		
24	12–15	38
26	10–12	87
28	10–12	85
30	8–10	65
32	4–6	42
34	3–4	12
36	1–3	<10
38	1–3	<10
40	1–2	<10
42	1–2	<10
44	1	<10
46	1	<10
Complete		89

[a] Estimated from the LPS subfractionation shown in Fig. 2.

[b] Averages of three independent experiments. The concentration of complete LPS or LPS fractions used was 0.05 mg/ml. No cells survived when incubated in control serum.

[c] Same as in Fig. 2.

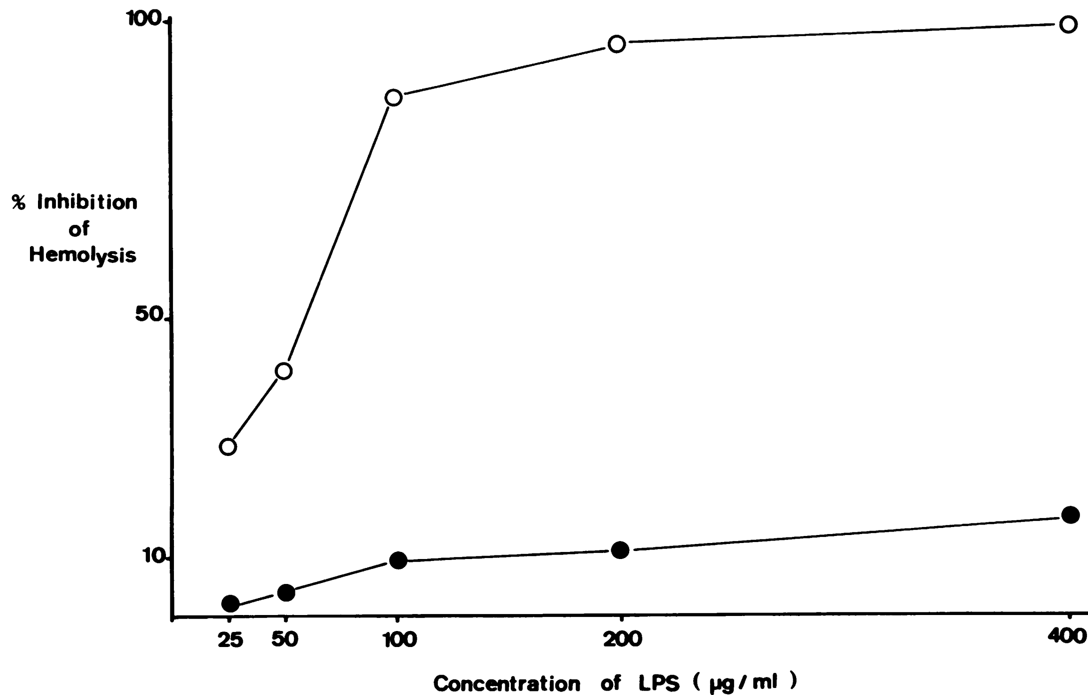

FIG. 4. Inhibition of complement-mediated hemolysis of sensitized sheep erythrocytes after 30 min of incubation in nonimmune rabbit serum with purified LPS from *K. pneumoniae* C3 (Ser^r) (○) and KT707 (Ser^s) (●).

ing a small number of O-antigen side chains per lipid A-KDO molecule. All these facts suggested that the O-antigen side chains are the part of the LPS molecules involved in serum resistance through their anticomplement activity. This point seems to correlate with the results from other laboratories indicating that C3b preferentially attaches to the longest O-antigen side chains in the LPS (9, 17).

All the results seem to indicate that HMW-LPS is able to activate the APC by a mechanism dependent upon the presence of specific O antigens, which have been previously demonstrated to restrict the inactivation of C3b by the regulatory proteins H and I (28). Also, as indicated by Vukajlovich et al. (43), the O antigens may interact with C1 and activate the CPC. This last point should be investigated further. Nevertheless, the LPS from Ser^s *K. pneumoniae* strains seems to be unable to activate either the APC or the CPC, even though strain KT707 has chemotype Rd with L-glycero-D-*manno*-heptose, and it is well established that LPS from rough mutants of *E. coli* or *Salmonella minnesota* (43) is able to activate the APC by a second mechanism different from the one dependent on the presence of specific O antigens and to activate the CPC. At the present time we do not have any explanation for this abnormal behavior, just the suggestion that maybe this monosaccharide (L-glycero-D-*manno*-heptose) in *K. pneumoniae* is not accessible. Also, we have some preliminary evidence that the capsular polysaccharide is linked to this part of the inner core LPS in *K. pneumoniae* (data not shown) and that this inner core LPS is not accessible.

In conclusion, the data presented in this report suggest that both complement pathways are involved in the serum killing of *K. pneumoniae* strains. Furthermore, the LPS composition (mainly the HMW-LPS) of these strains may play an important role in the serum bactericidal reaction, while capsular polysaccharide does not seem to play any important role in the serum killing of *K. pneumoniae* strains.

ACKNOWLEDGMENTS

B. Ciurana was supported by a fellowship from Fondo de Investigaciones Sanitarias.

We thank V. J. Benedí for his technical assistance.

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