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Production of an Extracellular Toxic Complex by Various Strains of *Klebsiella pneumoniae*

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Six isolates of *Klebsiella pneumoniae* (two serotype 1 isolates and a capsular variant of one of these, and two serotype 2 isolates and a capsular variant of one of these) possessing various degrees of virulence in rats and mice were examined for their in vitro production of an extracellular toxic complex (ETC). The ETC has been shown to be lethal for and produce extensive lung pathology in mice. This compound has been shown to be composed of capsular polysaccharide, lipopolysaccharide, and a small amount of protein. All six isolates produced the ETC. Immunization of experimental animals with sublethal doses of the ETC was protective against both homologous and heterologous strains, and this protection was due to antibody production. An examination of the various phases of growth of *K. pneumoniae* showed that there was extracellular release of the component parts of the ETC occurring during all phases of growth. The presence of the ETC in the supernatant fluids was due to actual release of this material as opposed to cell lysis. Antibodies to the lipopolysaccharide portion (which has been shown to possess the observed toxicity) of the ETC were protective against the homologous bacterium.

The importance of *Klebsiella* species in the ever-increasing number of gram-negative, aerobic bacillary nosocomial infections in the United States has been well documented (11, 15). Pneumonias caused by *Klebsiella* species (most notably *Klebsiella pneumoniae*) are particularly difficult to control (12, 15), and mortality rates can reach 50%, even after antibiotic therapy (11, 15). Also, *K. pneumoniae* pneumonias differ from most other bacterial pneumonias in that lung destruction seen in this disease state is often extensive (13).

We have shown that virulent strains of *K. pneumoniae* can produce a lobar pneumonia in normal animals that is characterized by extensive pulmonary tissue destruction (5). We also showed that there is a correlation between the production of extracellular polysaccharides (both lipopolysaccharide [LPS] and capsular polysaccharide [CPS]) and the virulence of the producing organism (4). However, only purified extracellular LPS (ELPS) enhanced the virulence of *K. pneumoniae* when coinjected into mice, and this virulence enhancement correlated with the content of ELPS but not the extracellular CPS in mixtures of these polysaccharides (4). Those studies, along with the work of Mizuta et al. (18), led us to hypothesize that there might be an extracellular toxic substance produced by these organisms that is responsible for the observed lethality and extensive lung pathology. Mizuta et al. (18) suggested that there is something other than the cell-associated capsular material that plays a role in the virulence of *K. pneumoniae*. These workers found avirulent strains of the O1:K2 serogroup of *K. pneumoniae* that were fully encapsulated.

We recently described the production of an extracellular toxic complex (ETC) that appeared to be responsible for lethality and the extensive lung pathology produced by *K. pneumoniae* in animal models (27). This ETC was made of 63% CPS, 30% LPS, and 7% protein. Saponification studies demonstrated that the toxicity of the ETC was associated with the LPS, but the LPS alone was decidedly less toxic than the entire complex. Since the ETC from only one strain of *K. pneumoniae* was employed in the above study, I decided to examine additional strains for the production of this compound.

MATERIALS AND METHODS

Bacteria. The *K. pneumoniae* serotype 1 (KP1) strains included ATCC 8047 (a lung isolate from the American Type Culture Collection, Rockville, Md.) and CDC 2-70 (Centers for Disease Control, Atlanta, Ga.). The *K. pneumoniae* serotype 2 (KP2) strains included ATCC 29011 and CDC 2-70. The KP1 8047 and the KP2 29011 strains have two variants with respect to capsule size (5). These subtypes bred true and were referred to as large capsule (LC) and small capsule (SC) variants. The four variants utilized in these studies were therefore designated KP1-LC, KP1-SC, KP2-LC, and KP2-SC. All strains belonged to the O antigen serogroup O1.

All strains were stored at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 20% glycerol. Serotype specificity was tested in capillary precipitin reactions against rabbit anticapsular antisera raised against Formalin-killed whole cells by the procedure of Edmondson and Cooke (6). The capsule size, rat lung infectivity, and virulence in mice of these organisms have already been established (4).

Media, growth conditions, purification, and quantitation of ETC from various *K. pneumoniae* strains and ELPS from KP2 2-70. Media, growth conditions, purification, and quantitation of ETC and ELPS were all as previously described (27). The ELPS from KP2 2-70 was obtained by electrodialysis and gel filtration in the presence of 0.1% sodium dodecyl sulfate as previously described (27).

The purified ETC and ELPS were assayed for protein by the procedure of Lowry et al. (16) with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Md.) as the standard. LPS was measured by the ketodeoxyoctonate assay of Osborn (19). Ketodeoxyoctonate assays utilized *Escherichia coli* O55:B5 LPS as the standard, and the LPS

TABLE 1. Production and LD_{50} values in mice of ETC from six isolates of *K. pneumoniae*

Isolate	LD_{50}[a]	ETC produced (mg)/ 2.5 liters[b]	ETC LD_{50} (μg)[c]	Composition of purified ETC[d]		
				% LPS	% CPS	% Protein
KP1-SC[e]	5.34×10^5	100.79	1,160 ± 40	20.8	74.9	4.3
KP1-LC[e]	4.99×10^1	79.93	410 ± 130	28.7	62.6	8.7
KP1 2-70	7.30×10^7	97.00	1,168 ± 80	14.3	79.5	6.2
KP2-SC	$>6.20 \times 10^7$	14.43	357 ± 99	33.5	56.3	10.2
KP2-LC	1.78×10^5	23.84	745 ± 80	24.7	71.3	4.0
KP2 2-70	$<1.00 \times 10^1$	17.54	393 ± 45	29.6	63.3	7.1

[a] Results from Domenico et al. (4).
[b] ETC was purified and quantitated as described in the Materials and Methods. The values reported are an average of two separate determinations.
[c] LD_{50} values determined by i.p. injection was described by Reed and Muench (21). Each determination was performed at least twice employing at least 30 animals each time.
[d] These chemical analyses were performed as described in the Materials and Methods and are an average of at least two separate determinations.
[e] Large capsule (LC) and small capsule (SC) variants.

units were expressed in micrograms of *E. coli* LPS equivalents. The amount of CPS in the ETC was assayed by the uronic acid assay of Blumenkrantz and Asboe-Hansen (1). This involved calculating the proportion of the polysaccharide structural repeat unit that is uronic acid for CPS from serotype 1 (30.98%) strains (7) and from serotype 2 (26.43%) strains (20).

Determination of rate of production of extracellular protein, ELPS, and extracellular CPS and percent lysis of *K. pneumoniae* at various points on the growth curve. Cultures of KP2 2-70 were grown in 1 liter of DW medium (4) for 24 h. Samples (200 ml) were removed at an optical density at 550 nm (OD_{550}) = 0.1 (lag phase), OD_{550} = 0.3 (early log phase), OD_{550} = 0.8 (late log phase), OD_{550} = 1.0 (early stationary phase), and OD_{550} = 1.2 (late stationary phase), immediately chilled on ice, and centrifuged at 17,700 × *g* for 30 min at 4°C. The supernatants were dialyzed with stirring at 4°C against 8 liters of deionized water for 3 days with daily changes and lyophilized. Samples were suspended in 5 ml of distilled water and assayed for CPS (1), LPS (19), and protein (16). The quantity of CPS was calculated from the proportion of the polysaccharide structural repeat unit that is uronic acid for CPS from serotype 2 (26.43%) strains (20). Ketodeoxyoctonate assays for LPS utilized *E. coli* O55:B5 LPS as the standard, and the LPS units were expressed in micrograms of *E. coli* LPS equivalents.

To determine the percent lysis of *K. pneumoniae* at various points in the growth curve, I used the following procedure. For each point in the growth curve, the amount of free DNA in the supernatant was calculated by the modified diphenylamine method described by Giles and Myers (10) with similarly hydrolyzed calf thymus DNA as a standard. To determine the amount of DNA in the cell pellets at each stage of growth, the cells were harvested by centrifugation and adjusted to a cell density of between 1×10^8 and 2×10^8 CFU/ml. Cell pellets were washed three times by centrifugation and suspended in 5 ml of ice-cold buffered saline containing 0.01% gelatin (usually at 10^9 cells per ml) with cell numbers being determined on a Coulter Counter. Concentrated perchloric acid was then added to a final concentration of 1.0 M, and the mixture was heated to 90°C for 20 min before stirring. The hydrolyzed samples were chilled in an ice bath and centrifuged to pellet the acid-insoluble cell components. The supernatant (acid-soluble cell contents) was assayed for DNA (10). The DNA in the various 200-ml supernatants plus the DNA released from the various cell pellets represented the total DNA. The amount of DNA found in the various 200-ml supernatants divided by the total amount of DNA represented the total lysis of the *K. pneumoniae* population.

LD_{50} determinations and immunizations with ETC. The 50% lethal dose (LD_{50}) value of each of the ETC preparations was determined by injecting the appropriate amount of ETC in 0.01 M Tris hydrochloride buffer (pH 8.0) intraperitoneally (i.p.) into Swiss-Webster mice (20 to 25 g). After 72 h of observation, dead mice were counted, and LD_{50} values were calculated by the method of Reed and Muench (21). Before injection, the various ETC preparations were sterilized by exposure to UV irradiation (100 $\mu W/cm^2$ at room temperature for 30 min). The final ETC preparation was streaked on Trypticase soy agar for confirmation of sterility.

For all animal immunizations, the appropriate extracellular *K. pneumoniae* product (either ETC or LPS) was injected i.p. into Swiss-Webster mice in 0.01 M Tris hydrochloride buffer (pH 8.0). The animals were then rested for 14 days before challenge with the appropriate organism or extracellular product. When the immunized animals were challenged with live bacteria, the organisms were grown to an OD_{550} of 0.1 (early log phase) in DW medium and diluted in sterile phosphate-buffered saline to the appropriate concentration. Volumes of 1 ml were always injected. In experiments in which the immunized mice were challenged with purified bacterial product, the following protocol was employed. The material was always dialyzed against 0.01 M Tris hydrochloride buffer (pH 8.0). Before i.p. injection of this material into mice, it was sterilized by exposure to UV irradiation (100 $\mu W/cm^2$) at room temperature for 30 min. The final ETC preparation was streaked on Trypticase soy agar to confirm sterility.

RESULTS

Production of ETC by various strains of *K. pneumoniae*. Table 1 shows the production and LD_{50} values in mice of ETC from two serotype 1 isolates and a capsular variant of one of these and two serotype 2 isolates and a capsular variant of one of these. As can be seen from this table, all six isolates examined produced the ETC with LD_{50} values ranging from 357 μg for KP2-SC to 1,168 μg for KP1 2-70. These data also indicate that, on the average, the serotype 1 strains produce a great deal more ETC in vitro than do the serotype 2 strains.

The compositions of the various ETCs also appear in Table 1. The most toxic ETC (that from KP2-SC with an LD_{50} value of 357 ± 99 μg) had the highest percentage of LPS (33.5%). The least toxic ETC (that from KP1-SC with

an LD_{50} value of 1,168 ± 40 μg) had the lowest percentage of LPS (14.3%). Indeed, there is an absolute correlation between the toxicity of the ETC preparation and its percentage of LPS. The LD_{50} of each ETC was inversely correlated to the ratio of LPS to ETC ($P < 0.01$), while the LD_{50} of each ETC was directly correlated to the ratio of CPS to ETC ($P < 0.01$).

Ability of ETC to provide protection against i.p. challenge with homologous and heterologous strains of *K. pneumoniae*. When mice were immunized with KP2 2-70 ETC, they were protected against a large challenge of the homologous organism (Table 2). To see whether immunization with the ETC protected against a heterologous strain with a different type-specific capsule, I performed the following experiment. Twenty mice were immunized i.p. with sublethal amounts of KP2 2-70 ETC and later challenged with 180 CFU of KP1-LC. As can be seen in Table 2, 80% of these animals survived the heterologous bacterial challenge, but 95% of control mice which received the same inoculum died as a result of their infection. This difference was shown to be statistically significant at the $P < 0.005$ level.

Ability of antibodies to ETC to neutralize ETC toxicity in vivo and passively protect against homologous *K. pneumoniae* challenge. Ten mice were injected i.p. with 150 μg of KP2 2-70 ETC. After 14 days, they were sacrificed by exsanguination, and the resulting serum constituted the immune mouse serum (IMS). Ten control mice were also exsanguinated to obtain normal mouse serum (NMS). A 2.5-mg sample of KP2 2-70 ETC was incubated for 30 min at room temperature with 0.5 ml of IMS, and a comparable amount of ETC was incubated under similar conditions with 0.5 ml of NMS. Then 500 μg of each ETC preparation was injected i.p. into 10 mice. Antibody to the ETC can neutralize its toxicity in vivo since antibody to the ETC protected 80% of the experimental animals from death, while 100% of animals that received ETC plus NMS died (Table 3). This difference was shown to be statistically significant at the $P < 0.005$ level.

In addition, I sought to determine whether antibodies to the ETC would protect experimental animals against infection with homologous *K. pneumoniae*. Five mice were injected with sublethal concentrations of KP2 2-70 ETC (150 μg). After 14 days, these animals were sacrificed by exsanguination. In addition, serum was taken from five control mice. Then five mice each received 0.1 ml of IMS intravenously (i.v.), while five additional mice each received 0.1 ml of NMS. i.v. All these animals then received 160 CFU of KP2 2-70 i.p. Five mice also received i.p. 160 CFU of KP2 2-70 that had been incubated for 30 min at room temperature in 0.5 ml of IMS, while five additional mice received i.p. 160 CFU of KP2 2-70 that had been incubated for 30 min at room temperature. The results of these studies can also be seen in Table 3. This table clearly shows that antibody to the ETC can passively protect mice against homologous challenge, whether the challenge organisms are delivered in combination with the antibody or separately after passive immunization of the animals with the antibody.

TABLE 2. Ability of ETC to induce protection against homologous and heterologous organisms

Source of ETC[a]	Challenge organism	No. of mice dead/ no. inoculated (%)
KP2 2-70	KP2 2-70[b]	1/21 (4.8)[c]
None (control)	KP2 2-70[b]	20/21 (95.2)
KP2 2-70	KP1-LC[d]	4/20 (20.0)[c]
None (control)	KP1-LC[d]	19/20 (95.0)

[a] ETC was purified as previously described (27). All immunized mice received 150 μg of KP2 2-70 ETC delivered i.p. in 0.01 M Tris hydrochloride buffer (pH 8.0).
[b] A 117-CFU sample of KP2 2-70 was injected i.p. into these mice 14 days after immunization.
[c] Significantly different from control values by chi-square analysis ($P < 0.005$).
[d] A 180-CFU sample of KP1-LC was injected i.p. into these mice 14 days after immunization.

TABLE 3. Ability of antibodies to ETC to neutralize ETC toxicity in vivo and passively protect against homologous *K. pneumoniae* challenge

Source of serum	Serum inoculation	Source of ETC (500 μg/mouse)[a]	No. of mice dead/ no. of mice injected (%)
KP2 2-70 ETC immune mice[b]	KP2 2-70 ETC incubated with 0.5 ml of IMS for 30 min at 25°C	KP2 2-70	2/10 (20)[c]
Normal mice	KP2 2-70 ETC incubated with 0.5 ml of NMS for 30 min at 25°C	KP2 2-70	10/10 (100)
KP2 2-70 ETC immune mice[b,d]	0.1 ml i.v.		0/5 (0)[c]
Normal mice[d]	0.1 ml i.v.		5/5 (100)
KP2 2-70 ETC immune mice[b,d]	KP2 2-70 (160 CFU) incubated with 0.5 ml of IMS for 30 min at 25°C		0/5 (0)[c]
Normal mice[d]	KP2 2-70 (160 CFU) incubated with 0.5 ml of NMS for 30 min at 25°C		5/5 (100)

[a] Purified as described by Straus et al. (27).
[b] Animals were immunized with 150 μg of KP2 2-70 ETC i.p. and rested for 14 days before serum was obtained.
[c] Significantly different from control values by chi-square analysis ($P < 0.005$).
[d] Immediately after injection of serum, these mice received 160 CFU of *K. pneumoniae* serotype 2-70.

Ability of antibody to purified LPS to protect experimental animals against lethal *K. pneumoniae* challenge. Ten mice were injected i.p. with 100 μg of purified LPS. None of these animals died as a result of this injection. After 14 days, all 10 mice were sacrificed by exsanguination, and their sera were obtained. Six mice received 160 CFU of KP2 2-70 i.p. along with 0.1 ml of NMS i.v. Six mice received 160 CFU of KP2 2-70 i.p. along with 0.1 ml of mouse anti-LPS serum i.v. The results showed that antibody to the LPS protected approximately 67% (four of six survived) of the animals against a lethal KP2 2-70 challenge. All (100%) of the control animals which received 160 CFU of KP2 2-70 succumbed to their infection (data not shown). This difference was found to be statistically significant with a P value of <0.025.

Production of ETC at various points in the growth curve of KP2 2-70. Table 4 shows the production of certain extracellular materials by KP2 2-70 during different phases of the growth curve. This table shows that there is constant in vitro release, by the entire population, of the materials that form the ETC (LPS, CPS, and protein). A determination of the total DNA (cell pellet plus cell supernatant) and the DNA found in the supernatant allowed for an examination of the relationship between cell lysis and the extracellular release of the materials found in the ETC. The only detectable cell lysis (9.9%) occurred in the late stationary phase.

TABLE 4. Production of extracellular material by KP2 2-70 during different phases of growth[a]

Growth phase	Total cells/200 ml[b]	Total extracellular LPS, CPS, and protein (mg)	LPS, CPS, and protein produced/cell (pg)	Total supernatant DNA (μg)	Total DNA (supernatant plus pellet) (μg)	% Cell lysis (total supernatant DNA/total DNA)
Lag	4.72×10^8	4.52	9.50	ND[c]	<10	ND
Early log	3.65×10^9	7.09	1.94	ND	40	ND
Late log	1.44×10^{10}	9.31	0.64	ND	200	ND
Early stationary	3.96×10^{10}	12.94	0.32	ND	420	ND
Late stationary	4.29×10^{10}	19.65	0.45	55	555	9.9

[a] *K. pneumoniae* (1 liter) was grown to various phases of growth in DW medium. Each sample represents 200 ml of culture supernatant.
[b] Determined with a Coulter Counter.
[c] ND, Not detected (less than 5 μg of DNA/500 μl).

DISCUSSION

We have recently described the production of an ETC by a strain of *K. pneumoniae* which appeared to be responsible for the lethality and extensive pulmonary tissue necrosis associated with pneumonia produced by this organism in an animal model (27). In this paper, I show that two serotype 1 isolates and a capsular variant of one of these and two serotype 2 isolates and a capsular varient of one of these also produce this ETC (Table 1). The *K. pneumoniae* serotype 1 strains appear to produce a great deal more ETC in vitro than do the *K. pneumoniae* serotype 2 strains (four- to fivefold), but the significance of this is unclear, as the LD_{50} values of the two groups (serotypes 1 and 2) of organisms are quite similar. In addition, isogenic strains have not yet been examined, and no correlation between virulence and the toxicity of the ETC can be inferred from the data of either of the *K. pneumoniae* serotypes examined.

Immunization with the ETC of KP2 2-70 induced protection in mice against homologous KP2 2-70 organisms (Table 2). This was not surprising in light of the studies which have shown that purified CPS of *K. pneumoniae* can elicit the production of protective antibody (3, 14, 24). What was surprising was the finding that immunization with the KP2 2-70 ETC would protect experimental animals against a heterologous organism (Table 2). Protection produced by *Klebsiella* capsular vaccines has, for the most part, been thought to be type specific (2), although Roe and Jones (14, 24) have reported heterologous protection using *Klebsiella* CPS vaccines. Because all the *K. pneumoniae* strains that I utilized in this study possessed the serotype 1 O antigen, it is most probable that the protective antibody was directed against the O (LPS) antigen.

The induced protection appears to be antibody mediated since antibody to the ETC passively protected experimental animals against homologous ETC and homologous *K. pneumoniae* (Table 3). Although it seems fairly well established that immunization of experimental animals with *Klebsiella* CPS can induce protection against homologous and heterologous *K. pneumoniae* (2, 3, 14, 24), the effect(s) of immunization with *K. pneumoniae* LPS seems less clear. Riottot et al. (22) and Fournier et al. (8) felt that antibody to LPS appeared to play no role in protection in experiments in which immunization of mice with ribosomal vaccines provided good serotype-specific protection. Robert et al. (23) demonstrated, however, that although LPS may play a part in the protective activity of O fractions isolated from *K. pneumoniae* ribosomes, their purified LPS preparations failed to protect mice even at doses 1,600 times greater than the amount present in a protective dose of rRNA. Finally, Williams et al. (28) examined the role of the O and K antigens in determining the resistance of *Klebsiella aerogenes* to serum killing and phagocytosis. They demonstrated that both the K and O antigens of *K. aerogenes* were necessary to protect the organism from either complement-mediated serum killing or phagocytosis in the absence of specific antiserum. In addition, they showed that optimal phagocytic ingestion of a *K. aerogenes* strain could be achieved in the presence of either anti-K or anti-O serum.

The components that form the ETC (LPS, CPS, and protein) appear to be released by *K. pneumoniae* KP2 2-70 during all phases of growth (Table 4). This table demonstrates that the ETC is probably released as a natural product of cell growth and not due to cell lysis. This is not surprising because as the members of the family *Enterobacteriaceae* grow in vitro, they shed outer membrane LPS into liquid media (17). These LPS molecules are usually physically associated with bacterial outer membranes and phospholipids (9, 25), and it is thought that they are shed into the media in fragments of bacterial outer membranes (26). We have previously shown that these LPS-containing complexes are produced in vivo by at least one strain of *K. pneumoniae* (27).

In conclusion, we envision the normal course of an infection by *K. pneumoniae* as follows. When *K. pneumoniae* is introduced into either the lungs or peritoneal cavity of an experimental animal, it is essentially the size of the cell-associated capsule (5) and the immunological status (2) of the animal that determines the outcome of the challenge. Once the infection has been established, it appears that the release of ELPS enhances the virulence of the invading organisms by producing a transient depression of the reticuloendothelial system, thus allowing for unrestrained growth of the organism in the early stages of the infection (4). Finally, when the invading bacterium has reached sufficiently high levels to produce enough ETC to cause lethality, the animal dies (27). All strains of *K. pneumoniae* that I have examined to date are capable of producing the ETC, but if they do not possess sufficient cell-associated capsule, they are cleared by the phagocytic mechanisms of the animal and never get the opportunity to release extracellular toxic products.

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