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Importance of a Lipopolysaccharide-Containing Extracellular Toxic Complex in Infections Produced by *Klebsiella pneumoniae*

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A *Klebsiella pneumoniae* serotype 2 strain was examined for its ability to produce extracellular toxic material. The organism was grown to the stationary phase in a defined medium, and the toxic material was isolated by ultrafiltration–ion-exchange chromatography on DEAE-Sephacel and gel filtration chromatography on Sepharose 4B or 2B. It was found to be comprised of 63% capsular polysaccharide, 30% lipopolysaccharide, and 7% protein and possessed a 50% lethal dose (when injected intraperitoneally into mice) of 393 ± 45 μg. The toxicity appeared to be associated with the endotoxin portion of the compound, because boiling for 15 min and exposure to proteolytic enzymes had no effect on the toxicity. However, saponification destroyed the toxicity of the compound. Studies employing radial immunodiffusion examining the sera of mice infected with this organism demonstrated in vivo production of the complex at levels sufficiently high to produce death. When sublethal amounts of this complex were placed in the lungs of specific-pathogen-free mice, the lung pathology observed after 24, 48, and 72 h was similar to the damage caused by an active *K. pneumoniae* lobar pneumonia. These data indicate that this extracellular toxic compound produced by *K. pneumoniae* may be responsible for the lethality and lung tissue destruction normally associated with an active lobar pneumonia caused by this organism.

Gram-negative, aerobic bacilli currently account for an ever-increasing number of hospital-acquired infections in the United States (1, 7). Of this group of bacteria, species of *Klebsiella* are an important cause of morbidity and mortality (13, 25). Those hospital populations at increased risk for *Klebsiella* spp. infections (most notably *Klebsiella pneumoniae*) include neonates (15, 33), patients undergoing respiratory therapy (18, 27), urology patients (6), and burn wound patients (19, 29). Pneumonias caused by *K. pneumoniae* are particularly dangerous, because once established they are difficult to control (16, 25), and mortality rates may reach 50% even in treated cases (13, 25). In addition, *K. pneumoniae* pneumonias differ from many other bacterial pneumonias in that the lung tissue destruction in this disease process is often extensive (17).

We have been able to show that *K. pneumoniae* can cause a lobar pneumonia with extensive pulmonary tissue destruction in normal animals (9). In those studies, transtracheal instillation of a clinical isolate of a serotype 1 *K. pneumoniae* strain into the lungs of normal rats resulted in the production of a characteristic, chronic lobar pneumonia (9). Those studies led us to hypothesize that perhaps there is an extracellular toxic substance produced by *K. pneumoniae* that is responsible for the lethality and extensive lung pathology. However, relatively little is known about the production of extracellular virulence factors by *K. pneumoniae*. Probably the best known of the virulence factors of *K. pneumoniae* is its capsular polysaccharide (CPS) (20). There is even a report in the literature that this material is toxic for mice (2). The immunological and biological properties of the CPS may have great significance in light of the work of Pollack (32). In that study, the author demonstrated (by counterimmunoelectrophoresis) detectable levels of circulating *K. pneumoniae* CPS in patients with *K. pneumoniae* infections and suggested that a direct correlation existed between antigenemia, severity of infection, and unfavorable prognosis. Other potential virulence factors produced by *K. pneumoniae* include the heat-labile and heat-stable enterotoxins (23), adherence-mediating pili (11), and the lipopolysaccharide (LPS) commonly associated with gram-negative bacteria.

However, substances other than cell-associated virulence factors may play a role in the disease process associated with *K. pneumoniae*. Mizuta et al. (28), examining the virulence of *Klebsiella* strains for mice belonging to the O1:K2 serogroup and its relationship to capsular types, found avirulent strains of *Klebsiella* that were fully encapsulated. Of nine *Klebsiella* serogroup O1:K2 strains they examined, two were avirulent for mice, as opposed to seven other strains with 50% lethal dose (LD_{50}) values of less than 10 CFU. This study suggested that there may be something other than cell-associated capsular material that plays a role in the virulence of *K. pneumoniae*. We describe here an extracellular toxic complex (ETC) produced by *K. pneumoniae* that appears to be responsible for the lethality and extensive pulmonary necrosis associated with lung infections caused by this organism.

MATERIALS AND METHODS

Bacterium. The strain employed in these studies was *K. pneumoniae* CDC 2-70, serotype 2 (KP2 2-70). This strain was obtained from Difco Laboratories (Detroit, Mich.), where it was employed as a strain to prepare antisera reactive against the type 2 capsule. This strain was shown to belong to the serogroup O1.

The organisms were maintained, serotyped, biotyped, and used for antiserum production as previously described (8). The capsule size, rat lung infectivity, and mouse virulence of this organism have already been established (8). The chemically defined medium (DW) (10) and growth conditions have been described previously (8).

Purification of the ETC. Cultures were grown in 2.5-liter quantities of DW medium for 24 h at 37°C in a shaker adjusted to 200 rpm. The bacteria were pelleted at 17,700 ×

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g at 4°C for 60 min, and the supernatant fluids were concentrated by ultrafiltration to a volume of 100 ml on an Amicon DC-2 concentrator (Amicon Corp., Danvers, Miss.) with an H1P100-43 hollow fiber filter that retains substances with a molecular weight greater than 100,000. Supernatant fluids were then lyophilized. This material was dissolved in 50 ml of 0.01 M Tris hydrochloride buffer (pH 8.0) and dialyzed overnight against 8 liters of the same buffer at 4°C. It was then placed on a DEAE-Sephacel (Pharmacia Fine Chemicals, Inc., Pistcataway, N.J.) column (2.5 by 40 cm) which had been equilibrated overnight at 4°C with 0.01 M Tris hydrochloride buffer (pH 8.0). The column was washed with 2 bed volumes of starting buffer, and the adsorbing material was eluted with a linear NaCl (0 to 0.5 M) gradient in the starting buffer. The eluant was monitored for protein at 280 nm with a flow-through 2138 Uvicord S monitor (LKB Instruments, Inc., Rockville, Md.) and recorded by a 6520-5 Chopper Bar six-channel recorder (LKB Instruments). Fractions of 100 drops (approximately 4.2 ml) were collected. In addition, the column eluate was assayed for uronic acids and ketodeoxyoctonate (KDO) in the following way. Every fifth tube was concentrated to dryness by lyophilization, and 0.4 ml of deionized distilled water was added to each tube. A 0.2-ml sample was assayed for uronic acid by the procedure of Blumenkrantz and Asboe-Hansen (4) with glucuronic acid (sodium salt; Sigma Chemical Co., St. Louis, Mo.) as the standard. The other 0.2 ml was assayed for KDO by the method of Osborn (30). KDO assays utilized *Escherichia coli* O55:B5 LPS (Difco) as the standard. The various peaks were pooled, lyophilized, and dialyzed against 0.01 M Tris hydrochloride buffer (pH 8.0). Pools that were lethal for mice after intraperitoneal (i.p.) injection were further purified on Sepharose 4B or 2B in the following way. Less than 100 mg of the ETC in 6 ml of 0.01 M Tris hydrochloride buffer (pH 8.0) was applied to an ascending-flow column (2.5 by 90 cm) of either Sepharose 4B or Sepharose 2B at 4°C. Fractions (100 drops) were collected (approximately 4.5 ml), and protein peaks were monitored at 280 nm and recorded. Every fifth tube was assayed for uronic acid (4) and KDO (30). The appropriate fractions were pooled, lyophilized, and then dialyzed against the 0.01 M Tris hydrochloride buffer (pH 8.0). This material represented the purified ETC and was used for chemical characterization as well as LD_{50} and pulmonary necrosis studies.

Chemical characterization of ETC and LPS. The purified ETC was assayed for protein by the procedure of Lowry et al. (26) with bovine serum albumin (fraction V; Sigma) as the standard and for KDO by the procedure of Osborn (30). KDO assays utilized *E. coli* O55:B5 LPS as the standard, and the LPS units were expressed in micrograms of *E. coli* LPS equivalents. The amount of CPS in the ETC was assayed by employing the uronic acid assay of Blumenkrantz and Asboe-Hansen (4) and calculated from the proportion of the polysaccharide structural repeat unit made up of uronic acid for CPS from serotype 2 (26.43%) strains (31).

To determine which portion of the ETC was responsible for its toxicity, 10 mg of material was boiled for 15 min or exposed to trypsin (1 mg/ml from bovine pancreas, type 1, crystallized twice, ethanol precipitated, and substantially salt free; Sigma) for 30 min at 25°C. To saponify the ETC, approximately 1 mg of ETC per ml in deionized distilled water was brought to 1 N with 10 N NaOH in a screw-capped tube and heated at 66°C for 18 h. This material was then neutralized with concentrated HCl and examined for toxicity in mice. The fatty acid analysis on the ETC was performed after acid or base hydrolysis as described by Rietschel (35) with a Varian gas chromatograph (model 3300) equipped with a 12-ft. (ca. 3.6-m) SE-30 capillary column and a flame ionization detector. Standards were obtained from Supelco, Inc. Identifications were based on comparison of retention times with those of standards, plots of the log of the retention times versus chain length, and, for certain fatty acid methyl esters, comparison of retention times with those of standards with a 10% Silar 10C column (10 ft. [ca. 3.0 m], 1/8-in. [ca. 4.5-cm] outer diameter). On this column hydroxy fatty acids were not resolved from other components. The fatty acid analysis of the isolated LPS was done in the same manner, except 0.23 mg of LPS was employed.

Determination of LD_{50} value of ETC in mice. The LD_{50} values of the ETC were determined by injecting 1-ml amounts of ETC in 0.01 M Tris hydrochloride buffer (pH 8.0) i.p. into Swiss-Webster mice. After 72 h of observation, dead mice were counted, and LD_{50} values were calculated by the method of Reed and Muench (34). Before injection of the ETC preparation 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 (BBL Microbiology Systems, Cockeysville, Md.) plates for confirmation of sterility. An examination of the toxicity of purified LPS was performed in the same manner.

Lung inoculation of CF-1 mice with ETC or LPS. Six male CF-1 mice (specific pathogen free, 20 to 25 g in weight; Sasco Inc., Omaha, Neb.) were anesthetized with halothane, and 0.05 ml of a sterile suspension of ETC (167 μg) or 160 μg of LPS was placed transtracheally in a distal bronchus via a bead-tipped needle. After inoculation, the animals were housed in cages with free access to food and water. Six male control CF-1 mice were inoculated in the same manner with 0.05 ml of sterile phosphate-buffered saline. After 24, 48, and 72 h, mice were sacrificed by cervical dislocation, the thoracic cavity was opened, and the lungs were aseptically removed. Samples of lung tissue were excised from the affected areas (similar areas were taken from control lungs) and fixed immediately in 10% Formalin. Samples (4 μm) were cut and stained with hematoxylin and eosin. The sections were examined microscopically and photomicrographs were made from representative areas.

Purification of LPS from the ETC. The LPS portion of the ETC was obtained by a combination of electrodialysis (ED) and chromatography on Sepharose 4B containing sodium dodecyl sulfate (SDS). Fifty milligrams of ETC was electrodialyzed by a technique adapted from Galanos and Lüderitz (12) as described by Domenico et al. (8). The ED was continued for 2 h or until no further increase in milliamperes was observed over a 30-min period. The tank was then emptied, and this procedure was repeated two additional times. After the third ED period, sufficient SDS was added to the ETC to bring it to 2% saturation, and the complex was chromatographed on an ascending-flow Sepharose 4B column (90 by 2.5 cm) that was equilibrated with 0.01 M Tris hydrochloride buffer (pH 8.0) containing 0.1% SDS. Every fifth tube was monitored for KDO (30) and uronic acid (4) as previously described. The LPS-containing peaks were pooled separately, and the SDS was extracted by the procedure of Konigsberg and Henderson (24) before biological activity was tested.

Quantitation of in vivo production of the ETC. Radial immunodiffusion studies were performed to quantitate the ETC found in the serum of infected mice that had received 100 LD_{50} of KP2 2-70 i.p. Fifteen mice were injected with KP2 2-70, and they were observed for 3 to 5 days postchallenge. When the animals looked as if they were ready to

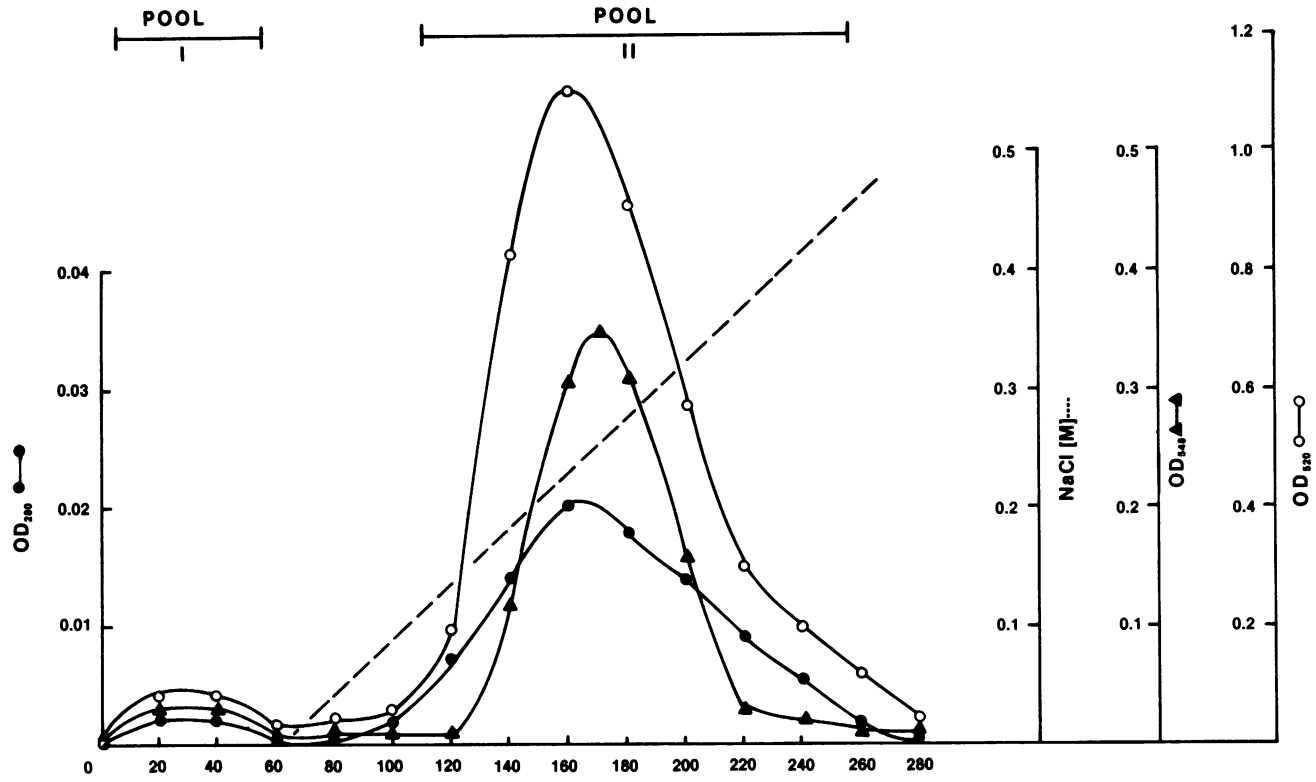


FIG. 1. Elution profile of the ultrafiltration concentrated extracellular material from KP2 2-70 (2.5 liters of stationary-phase culture) on DEAE-Sephacel. The peaks were eluted with an increasing NaCl gradient from 0 to 0.5 M in 0.01 M Tris hydrochloride buffer (pH 8.0). The eluate was continuously monitored for protein by absorbance (OD) at 280 nm, and every fifth tube was assayed for uronic acid (○) (4) and KDO (▲) (30).

expire, they were exsanguinated by cardiac puncture. Five of these animals died during the nights and therefore could not be tested. After the serum was obtained from the remaining 10 mice, the amount of ETC in their sera was quantitated by radial immunodiffusion in the following way. A 0.85% agarose solution in distilled water was heated to boiling and allowed to cool at 50°C. Seven hundred microliters of type-specific antiserum was then added to 12.7 ml of the agarose slurry and poured onto 3 1/4- by 4-in. (ca. 8.1- by 10-cm) glass plates and allowed to solidify. This serum was shown to be type specific in that it would only give lines of precipitin with extracts from serotype 2 *K. pneumoniae*, but not with the purified CPS from serotype 1 strains. It did, however, give a line of precipitin against purified LPS from KP2 2-70. The plates were incubated for 24 h at room temperature, and the zone diameters of the precipitin reaction within the gel were measured and compared with known quantities of ETC tested in the same manner.

RESULTS

Purification of the ETC and LPS. The extracellular material from the supernatant fluids of 2.5 liters of stationary-phase KP2 2-70 was concentrated by ultrafiltration and tested for toxicity in mice after sterilization by UV irradiation. One milliliter of this material was injected i.p. into 10 Swiss-Webster mice, and all 10 animals were dead within 48 h. Control animals (n = 10) that were injected with uninoculated media and treated in the same manner suffered no ill effects, indicating that some form of toxic material was elaborated by KP2 2-70.

The elution profile of the extracellular material produced by KP2 2-70 on DEAE-Sephacel can be seen in Fig. 1. The extracellular material could be separated into two distinct pools on DEAE-Sepharose. Pool I represented material that did not bind to the ion-exchange resin, whereas pool II eluted from DEAE-Sephacel at an NaCl concentration of between 0.15 and 0.35 M. Pools I and II both showed toxicity for Swiss-Webster mice; because there was much more material in pool II, it was further purified. When 10 Swiss-Webster mice were injected i.p. with 1 ml of pool II (300 μg of LPS, 400 μg of CPS, and 70 μg of protein per mouse), all 10 mice died within 48 h. Pool II was lyophilized, dissolved in 6 ml of 0.01 M Tris hydrochloride buffer (pH 8.0), and applied to an upward-flow Sepharose 4B column (2.5 by 90 cm). The eluate was monitored as was done for the DEAE-Sephacel column. The elution profile of DEAE-Sephacel pool II on Sepharose 4B can be seen in Fig. 2. This material could be fractionated by Sepharose 4B into two distinct pools on the basis of absorbance at 280 nm. The elution profile of this material on the Sepharose 2B column of the same dimensions was essentially identical. When material from these two pools was injected i.p. into Swiss-Webster mice, only pool I material (void volume pool) was toxic. The purified ETC was found to be composed of 29.6% LPS, 63.3% CPS, and 7% protein. The LD_{50} value for this material when injected i.p. into 20- to 25-g Swiss-Webster mice was calculated to be 393.5 ± 45 μg. The average time

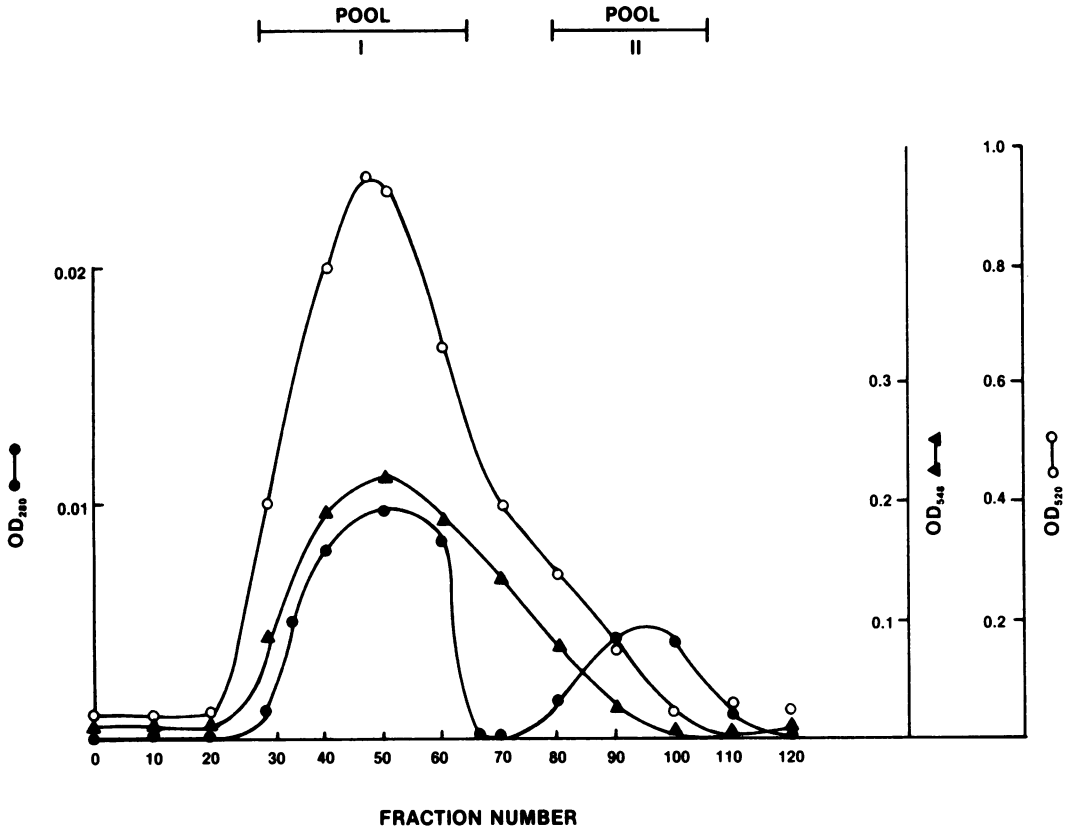


FIG. 2. Sepharose 4B elution profile of DEAE-Sephacel pool II obtained from the supernatant fluids (2.5 liters) of a stationary-phase culture of KP2 2-70. All material was eluted with 0.01 M Tris hydrochloride buffer (pH 8.0). The eluate was continuously monitored for protein by absorbance (OD) at 280 nm, and every fifth tube was assayed for uronic acid (○) (4) and KDO (▲) (30). The void volume of this column was 92 ml (tube 20), and the salt volume was 529 ml (tube 115).

to death for ETC injected animals was 48 h. This determination was performed three times.

It was found that the LPS moiety could be isolated from the ETC in the following way. Fifty milligrams of ETC that had been obtained by ultrafiltration, ion-exchange chromatography, and gel filtration chromatography was subjected to ED and gel filtration chromatography in the presence of SDS as described in Materials and Methods. The elution profile of this material on the Sepharose 4B column can be seen in Fig. 3. ED and gel filtration on Sepharose 4B containing 0.1% SDS allowed for the isolation of LPS relatively free of CPS. There was less than 1% CPS and protein in the purified LPS preparation. The molecular weight of the isolated LPS was decidedly smaller than that of the ETC which eluted from Sepharose 4B in the void volume.

Histology of ETC- and purified LPS-induced pulmonary necrosis. Since we had isolated an extracellular compound produced by *K. pneumoniae* that was capable of killing experimental animals in the absence of an infection, we were interested in seeing whether this material could produce lung pathology as well. Purified ETC (167 μg) was placed in a distal bronchus of the lungs of six CF-1 (specific-pathogen-free) mice in 50 μl of phosphate-buffered saline. This dose represented about 42% of the LD_{50} for mice. No mice died due to intoxication during the course of these experiments. Histological examination of the lung tissue showed extensive lung pathology in the presence of *K. pneumoniae* ETC (Fig. 4). This progressive destruction of lung tissue continued up to day 3 postinoculation, when the study was terminated. The lung tissue damage produced by the ETC alone was similar to the destruction produced by an active lobar pneumonia in the rodent lung (9).

Because the LD_{50} for the ETC of KP2 2-70 was 393 μg, a comparable amount of isolated LPS was examined for toxicity. Ten mice were injected i.p. with 210 μg of sterile, isolated LPS; although the mice looked sick (ruffled fur, lethargy, and diarrhea), there were no deaths after 72 h. Similar results were obtained when 10 additional mice were injected i.p. with 420 μg of purified LPS.

To examine the ability of the isolated LPS to produce lung pathology, six CF-1 mice each received 160 μg of this product transtracheally. No mice died as a result of intoxication during the course of these experiments. Histological examination demonstrated that an active process was occurring as a result of the introduction of the isolated LPS, but the tissue destruction was not as severe as that which occurred in the presence of the ETC. At 24 h, the process appeared to initially favor a subpleural localization. Most of the bronchioles were clear. There was, however, congestion evident in the alveolar septa. Extravasation of erythrocytes into the affected alveoli was evident, as was fibrin deposition. An outpouring of leukocytes was evident, with polymorphonuclear leukocytes predominating. At 48 h after LPS inoculation, the active process was equivalent to that produced by the ETC at 24 h. There were localized areas of an active process characterized by an outpouring of leukocytes

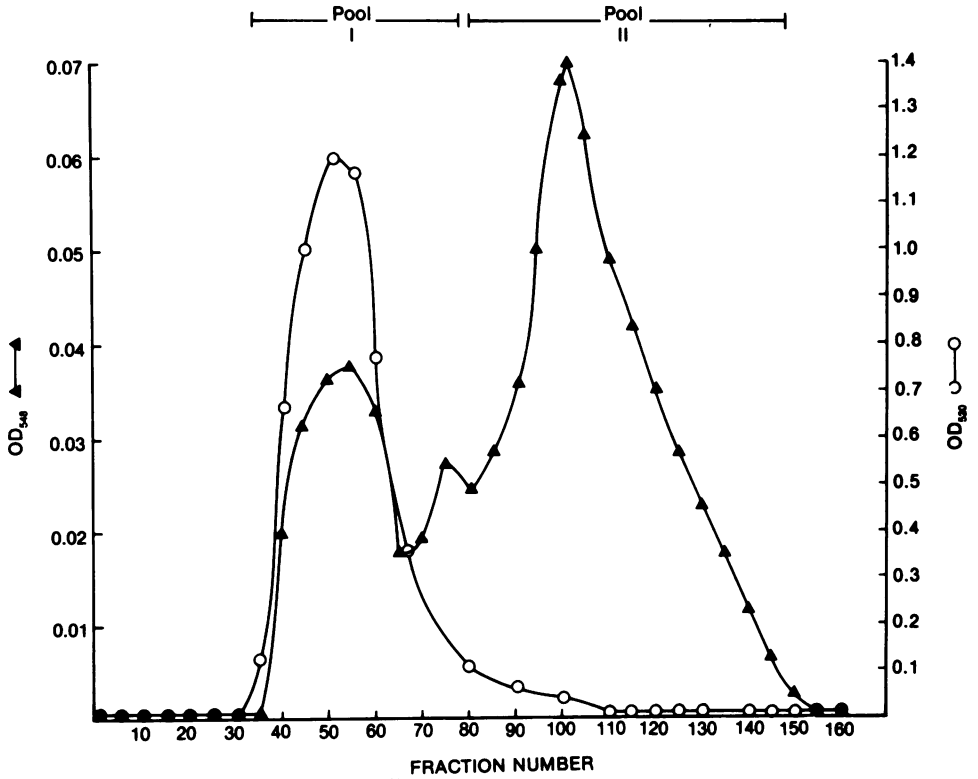


FIG. 3. Sepharose 4B elution profile of the partially purified ETC after ED. All material was eluted with 0.01 M Tris hydrochloride buffer (pH 8.0) plus 0.1% SDS. Every fifth tube was assayed for uronic acid (○) (4) and KDO (▲) (30). The void volume of this column was 112 ml (tube 35), and the salt volume was 512 ml (tube 160). OD, optical density (absorbance).

with some macrophages present. The terminal airways were observed to contain some purulent material. At 72 h postinoculation, there was a marked subpleural distribution of the active process. It still appeared as if the process was in the early stages of lesion formation. Fibrin was present, but only in small amounts. Areas of frank necrosis that were present in the lungs of animals that had been exposed to the ETC for 72 h were absent in animal lungs exposed to isolated LPS for the same period of time (data not shown).

Determination of the component of ETC responsible for toxicity and fatty acid analysis. As the next step in the characterization of the ETC, we performed experiments to determine which of the three components (LPS, CPS, or protein) that were present in the ETC was responsible for the observed animal toxicity. Boiling the ETC for 15 min in 0.01 M Tris hydrochloride (pH 8.0) or exposure to trypsin (1 mg/ml) did not change its LD_{50} in mice. After saponification, however, the ETC was no longer toxic after i.p. injection into experimental animals. Thus, it appeared that the LPS portion of the ETC was required for the toxicity in mice to be expressed.

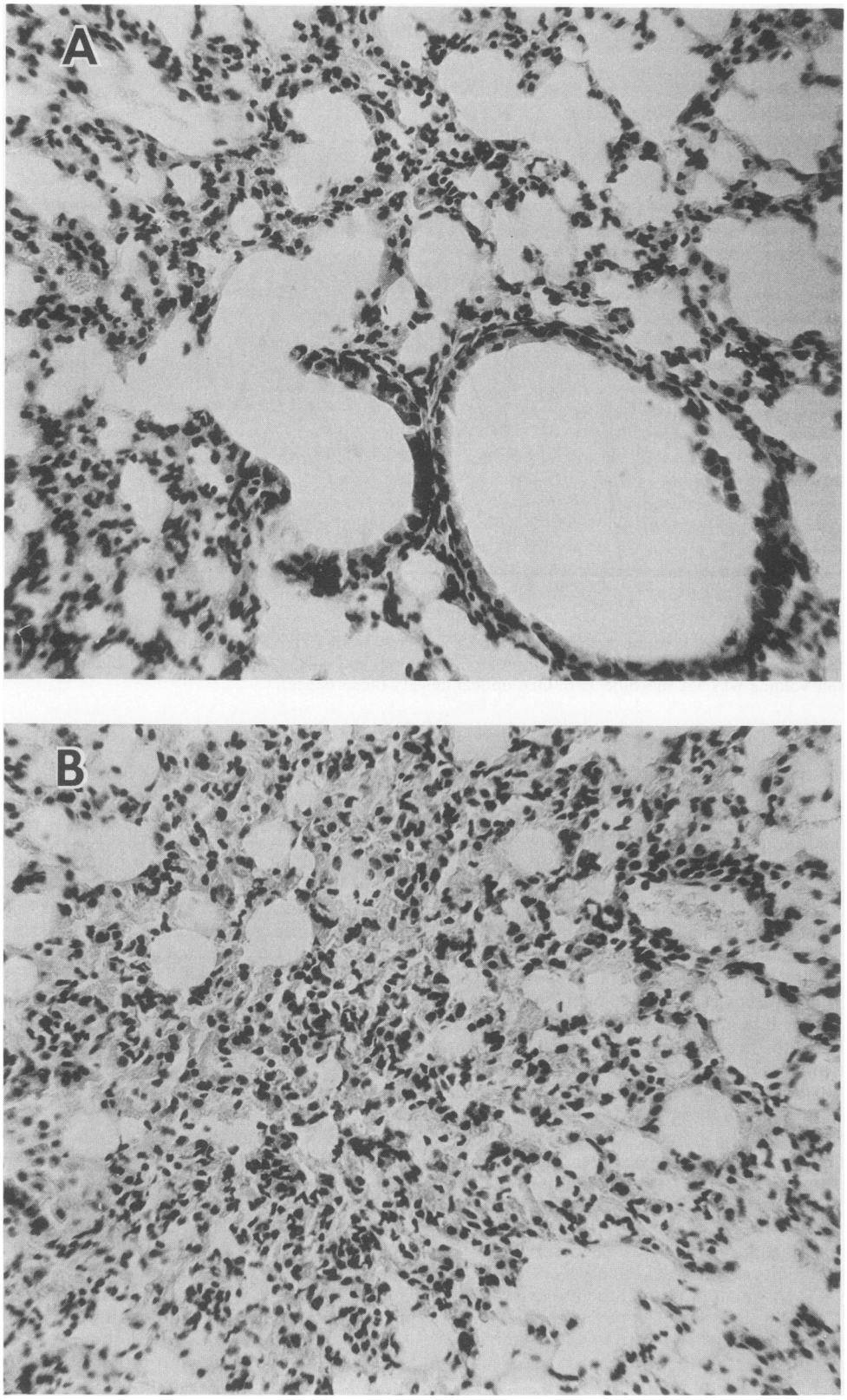
Because the lipid portion of LPS has been shown to be responsible for its toxicity, a fatty acid analysis of the ETC was attempted by gas chromatography of methyl esters. The ETC was found to be 9% fatty acid, whereas the LPS was about 18% fatty acid. Fatty acids were released by base hydrolysis under conditions which released these compounds from both ester and amide linkages (14). The ETC fatty acid composition data are shown in Table 1. The compositions of both the ETC and the purified LPS were found to be similar to that of the lipid A component of *Salmonella* spp. lipopolysaccharides (35). Especially prominent were the hydroxylated fatty acids, mainly 3-hydroxymyristic acid.

Quantitation of in vivo production of the ETC. Radial immunodiffusion was performed to quantitate the ETC found in the sera of mice infected with KP2 2-70. Table 2 shows the time period postinoculation that the serum was drawn and the amount of ETC found in that serum sample. Fifteen mice were injected i.p. with 100 LD_{50} of KP2 2-70. Of these 15 mice, 5 died. The remaining 10 mice all had detectable ETC in their blood when they were sacrificed. The average mouse at 70 h postinoculation had approximately 250 μg of ETC per ml of blood. Values ranged from 130 to 505 μg of ETC per ml.

DISCUSSION

The ETC produced by KP2 2-70 was composed of LPS, CPS, and protein that were not separable by ultrafiltration or ion-exchange (Fig. 1) or gel filtration (Fig. 2) chromatography. It therefore appears that the ETC is excreted as a complex by KP2 2-70 in the stationary phase. We have also found that the five clinical isolates of *K. pneumoniae* that we have examined so far produce an ETC that has approximately the same LD_{50} value for Swiss-Webster mice (D. C. Straus, manuscript in preparation).

The toxicity of the ETC was thought to be associated with the LPS, since boiling and protease treatment did not alter its toxicity, and only saponification rendered it nontoxic. However, the possibility that the toxicity may be associated with a protein has not yet been excluded. If, however, it is the LPS portion that is toxic, it is not entirely clear how LPS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B

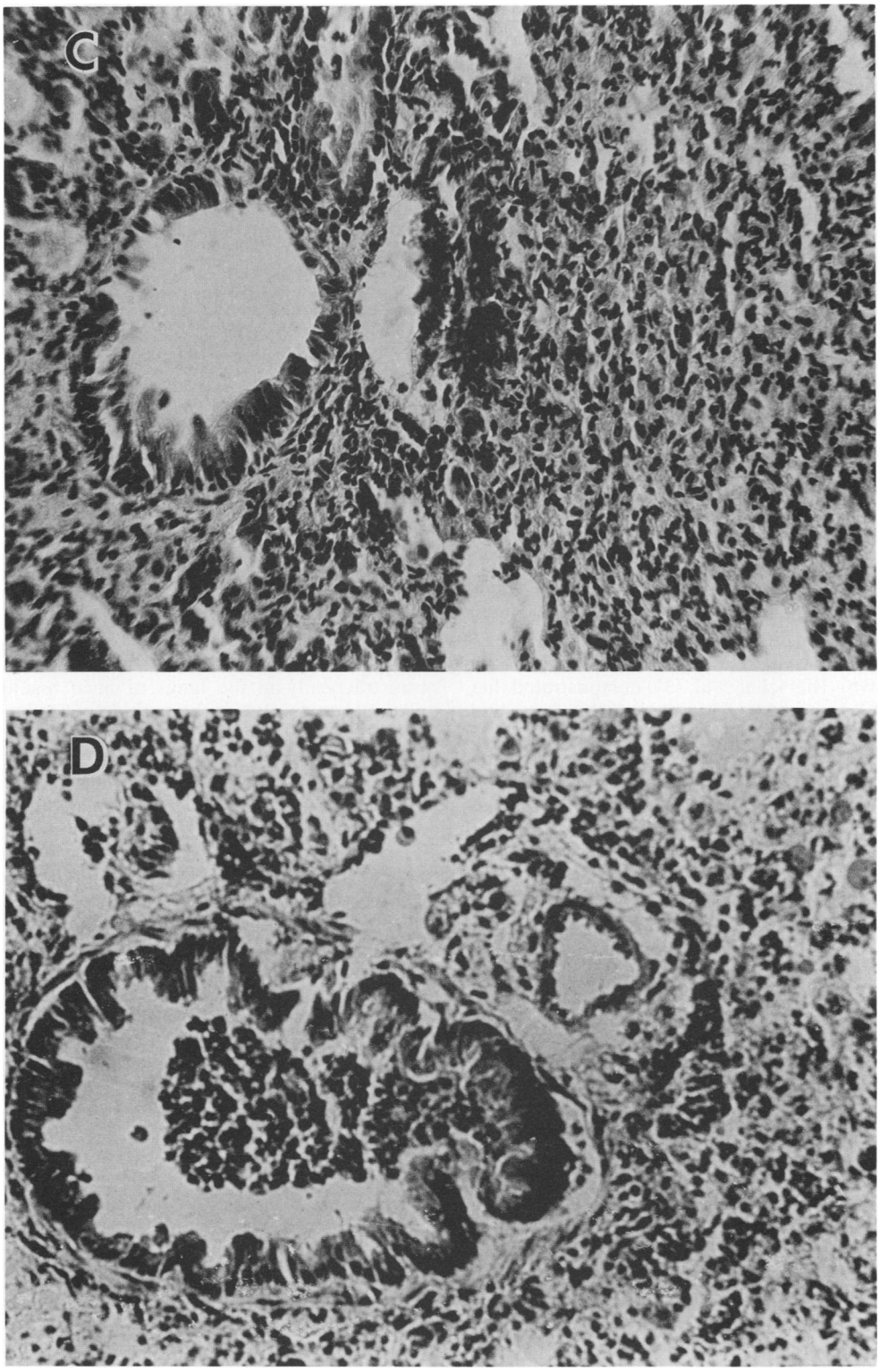

FIG. 4. Photomicrographs of hematoxylin- and eosin-stained sections of mouse lung (magnification, ×250). (A) Normal lung tissue representative of all controls can be seen. (B) Lung tissue typical of 24 h post-ETC inoculated mice shows mostly congestion. Bronchioles show purulent exudate, and fibrin was present. (C) By day 2 after ETC inoculation, the pathological process was quite widespread. There was a great deal of blood extravasation into the alveoli, and the terminal airways contained a purulent exudate. Also, areas of frank necrosis were present. (D) By day 3, a very active lobar process was observed. Fibrin was present as well as a great deal of proteinacious exudate. The alveoli were hyperinflated, the bronchioles contained a purulent exudate, and areas of frank necrosis were also evident.

TABLE 1. Fatty acid content of the ETC[a]

Fatty acid	μg of fatty acid/mg of ETC
Lauric	4
2-Hydroxydodecanoic	6
Myristic	14
Unknown	7
2-Hydroxytetradecanoic	6
3-Hydroxytetradecanoic	23
Palmitoleic	4
Palmitic	17
Oleic	5
Stearic	3

[a] These fatty acids released by base hydrolysis accounted for 97% of the total fatty acids present. No additional single fatty acid was found in quantities >1% of the total.

TABLE 2. Quantitation of KP2 2-70 ETC in mouse sera[a] by radial immunodiffusion and time postinoculation that serum was drawn

Mouse[b]	Time postinoculation (h)	ETC (μg/ml)
1	40	293
2	48	505
3	48	185
4	72	185
5	66	345
6	72	185
7	72	130
8	90	243
9	96	243
10	96	217

[a] Samples (10 μl) of serum were placed in each well, and plates were incubated for 24 h at room temperature.

[b] Mice received 100 LD_{50} doses of exponential-phase KP2 2-70 cells i.p. Five mice died during the course of this experiment.

is capable of killing experimental animals. When LPS is administered to animals intravenously, a prompt and transitory hypertensive state results, followed by a progressive, severe hypotension (3). Tissue perfusion decreases, and death is thought to occur as a result of circulatory collapse. The cause of the vascular changes which result in circulatory collapse have not been fully determined.

Lauric, myristic, palmitic, and 3-hydroxytetradecanoic acids accounted for the majority (64%) of the fatty acids found in the ETC. The unknown fatty acid was most likely delta-2-tetradecanoic acid, since hydrolysis of the ETC in 4 N HCl resulted in a 50% reduction in the amount of this fatty acid (data not shown). Rietschel et al. (35) demonstrated that the conversion of 3-hydroxymyristic acid to this fatty acid occurred twice as fast in base as in acid.

The role that the CPS portion of the ETC plays in the toxicity of the compound is not yet clear. The extracellular polysaccharides of *K. pneumoniae* have been proposed to have infection-promoting capabilities by inhibiting the release of acid phosphatase from the lysosomal fraction of peritoneal macrophages to the extralysosomal fraction (21, 22). Yokochi et al. (36, 37) showed that minute quantities of *K. pneumoniae* extracellular capsular polysaccharide inhibited macrophage maturation and function. They also demonstrated an increased virulence for a strain of *Salmonella* when coinjected i.p. into mice (22). The LPS portion of the ETC can be obtained free of CPS by ED and gel filtration in the presence of 0.1% SDS (Fig. 3). This results in an LPS molecule that is greatly reduced in toxicity for mice when compared with the intact ETC. Since the isolated LPS is so much smaller (approximately 12,000 to 20,000 daltons) than the intact ETC (in excess of 2×10^6 daltons), the presence of CPS may enhance the toxicity of the LPS moiety by making it more difficult to be excreted from the body, for example, in the urine. A compound with a molecular mass less than 20,000 daltons is excreted by the glomerular filtration system with relative ease. However, a compound with a molecular mass in excess of 2×10^6 daltons would tend to lodge in the glomerular filtration system, thus setting up a gradient in the blood stream, keeping the amount of the LPS containing ETC in the blood stream at very high levels.

The ETC could be lethal when injected i.p. into Swiss-Webster mice. The isolated LPS, although toxic for mice at approximately 400 μg, did not cause death in any of the animals. There is one other report in the literature of a toxic polysaccharide produced by *K. pneumoniae*. Baer et al. (2), while studying the effect of the dose of bacterial polysaccharide antigen on antibody production in mice, noted that the polysaccharide produced by *K. pneumoniae* type B was toxic for mice in high concentrations. These workers noted that the LD_{50} for a 20-g mouse was approximately 500 μg. This value is similar to but slightly higher than the LD_{50} for the KP2 2-70 ETC which we report here.

The ETC described here also could produce extensive pulmonary pathology in the lungs of specific-pathogen-free mice when placed there in the absence of an active bacterial infection (Fig. 4). Sublethal amounts of the ETC placed transtracheally in the lungs of mice resulted in a clinical picture reminiscent of the pathology produced by an active *K. pneumoniae* pneumonia (9). The introduction of isolated LPS into the lungs of these animals produced some pathology, but it was not as severe as that caused by comparable levels of ETC. Cash et al. (5), in similar experiments, instilled 500 μg of purified *Pseudomonas aeruginosa* LPS in a bead-buffer slurry into rat tracheas. The resultant pathology was relatively minor and resulted in a monocytic response in the parenchyma close to the airways. However, the damage produced by intratracheal inoculation of exotoxin A or proteases was considerably more extensive and developed a pulmonary histopathology similar to that produced by the experimental bacterial infection (5).

In conclusion, our data (Table 2) suggest that of 10 mice infected with KP2 2-70, all possessed sufficient circulating ETC to have resulted in their death if they had not been sacrificed. We therefore propose that the ETC produced by *K. pneumoniae* is responsible for the lethality and extensive pulmonary tissue necrosis associated with pneumonias produced by this organism in mice.

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LITERATURE CITED

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