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Smooth Lipopolysaccharide Is the Major Protective Antigen for Mice in the Surface Extract from IATS Serotype 6 Contributing to the Polyvalent *Pseudomonas aeruginosa* Vaccine PEV

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The nature of the protective antigen in one of the sixteen monovalent extracts (viz., extract-6) contributing to the pseudomonas polyvalent extract vaccine (PEV) was studied in a mouse challenge assay. Selective removal, by filtration through Sep-Pak C_{18} cartridges, of two major protein antigens with molecular weights of 16,200 and 21,000 had no effect on the protection afforded by extract-6. When analyzed on the basis of 2-keto-3-deoxyoctonate, lipopolysaccharide (LPS) purified by hot phenol extraction (LPS-A) from *Pseudomonas aeruginosa* (International Antigenic Typing System serotype 6) could account in full for the protective capacity of extract-6. Comparative analysis of LPS heterogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining indicated that both extract-6 and LPS-A possessed similar spectra of smooth LPS molecules, containing between 10 and ≈50 O-antigen repeating units. Differences in the profiles of heterogeneity displayed by LPS in LPS-A and extract-6 were restricted to molecular species with short O-antigen chains. Subfractionation of LPS molecules on the basis of number of O-antigen repeating units was achieved by gel filtration in the presence of deoxycholate. Protection experiments performed on the subfractionated species of LPS-A revealed a relationship between O-antigen chain length and protective capacity; molecules with over 18 O-antigen repeating units being 50 to 100 times more protective than those with zero-two repeating units. The results indicate that most of the protection afforded by LPS-A and extract-6 can be accounted for by LPS molecules possessing extended (10 or more) O-antigen repeating units.

Pseudomonas aeruginosa is a common nosocomial pathogen of particular groups of patients, e.g., immunocompromised patients and those suffering from severe burns (3, 30). Prophylactic treatment by active immunization or by passive transfer of immune globulin is a possible option for reducing infection in such high-risk patients. For this reason, interest has naturally focused on the development of a vaccine which is capable of inducing high levels of protective (opsonizing) antibody directed against pathogenic strains of *P. aeruginosa* (for a recent review see reference 6).

One such candidate vaccine is polyvalent extract vaccine ([PEV] Wellcome Biotechnology Ltd., Beckenham, United Kingdom). This vaccine is prepared by extracting surface antigens from strains belonging to 16 of the 17 internationally recognized serotypes (27) of *P. aeruginosa* (31). High levels of anti-PEV antibody have been detected in the sera of rabbits (28) and also of humans (22, 28) immunized with the vaccine, and the results of preliminary clinical trials with PEV indicate that active immunization as well as passive immunotherapy protect patients at high risk to infection by *P. aeruginosa* (23, 43).

Recently, some progress has been made in determining the antigenic composition of this pseudomonas vaccine (28). Thus, it has been demonstrated that lipopolysaccharide (LPS) is a major constituent of at least 15 of the 16 cell surface extracts comprising PEV. The major resolvable protein component of each monovalent extract and of the vaccine is a 16.2-kilodalton surface protein which cannot be detected in isolated outer membranes and which appears to be unrelated to pili. Antiserum raised against PEV, in addition to containing high levels of anti-LPS antibody and significant titers of antibody directed against the 16.2-kilodalton polypeptide, also possesses detectable levels of antibody directed against several minor protein components of PEV, such as flagella and outer membrane proteins F and H1/H2 (28). (The nomenclature used for outer membrane proteins follows that of Hancock and Carey [18].)

The question naturally arises as to which antigen or which combination of antigens in the PEV vaccine represent(s) the major protective species. Certainly, several recent studies from other laboratories have documented the ability of LPS to protect mice against infection by *P. aeruginosa* (6-9). However, other surface components of *P. aeruginosa* have also been shown to elicit protective immune responses in the same host. These include flagella (21) and outer membrane protein F (15, 19) as well as a number of different carbohydrate-protein preparations isolated from the slime layer. Notable amongst this last class of molecules is a glycoprotein complex, the carbohydrate moiety of which confers protection (2, 12) and a high-molecular-weight polysaccharide (PS), which contains neither the core sugars nor the lipid A moiety of LPS, but which is serologically similar to (although possibly chemically distinct from) the O-antigen side chains of LPS (8, 38, 42). Data, albeit of a somewhat equivocal nature, has also been presented concerning the ability of certain detoxified extracellular products, notably exotoxin A toxoid and elastoid, to confer protection against infection by *P. aeruginosa* (reviewed in reference 6).

In the present communication we studied the nature of the protective antigen in one of the component monovalent extracts of PEV (viz extract-6). This extract was chosen for study since International Antigenic Typing System (IATS)

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serotype 6 is one of the serotypes most frequently associated with nosocomial infection (6). We showed that LPS can account for the full protective capacity of this extract and that the major 16.2-kilodalton protein has little, if any, role in protection. Furthermore, by subfractionating the LPS species, we demonstrated that LPS molecules with extended O-antigen side chains are the dominant immunogens in this vaccine extract.

(Preliminary accounts of this work have been previously presented at the 103rd Ordinary Meeting, Society for General Microbiology, Warwick, United Kingdom, 1985, P33, and at the Lunturen Lectures on Molecular Genetics, The Netherlands, 1984, p. 30.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* CN6675 of IATS serotype 6 was obtained from Wellcome Biotechnology Ltd. and was grown on minimal medium and harvested as previously described (28, 31). For mouse protection studies, the challenge strain was grown on nutrient agar slopes for 24 h at 37°C, harvested, washed once in 0.85% (wt/vol) NaCl, and stored in samples (1.0 ml) over liquid nitrogen.

Preparation of extract-6. The monovalent vaccine component, extract-6 was prepared from viable cells of *P. aeruginosa* (IATS serotype 6) by EDTA-glycine extraction according to published procedures (31). The routine treatment of the extract with formaldehyde was only omitted in some experiments designed to assess the adsorptive properties of Sep-Pak C_{18} cartridges. Unless otherwise indicated, extract-6 was dialyzed against 20 mM Tris hydrochloride buffer (pH 7.4) before use.

Isolation of LPS and phospholipid. Two different procedures were adopted for the preparation of pseudomonas LPS. LPS-A was extracted from lyophilized whole cells by the hot aqueous phenol procedure (47) and was isolated by alcohol precipitation after removal of nucleic acids by fractional precipitation with cetyltrimethylammonium bromide as previously described (28). LPS-B was isolated by the procedure recently described by Darveau and Hancock (10). Briefly, this involved solubilization of cell envelopes with buffer (pH 9.5) containing sodium dodecyl sulfate (SDS) and EDTA, followed by removal of peptidoglycan and proteins by centrifugation. After enzymatic degradation of remaining proteins and nucleic acids, LPS was precipitated with ethanol in the presence of Mg^{2+} and, after resuspension, was finally recovered by centrifugation (200,000 × *g*, 2 h, 15°C). Both LPS-A and LPS-B were extensively dialyzed against distilled water and were estimated to represent between 3.8 and 6.0% of the cellular dry weight. As determined from measurements of A_{260} (11), both preparations of LPS contained less than 0.5% nucleic acid. LPS-A and LPS-B contained <1.0% and about 2.3% (wt/wt) protein, respectively.

Phospholipids were isolated from packed cells of *P. aeruginosa* by the Folch procedure as detailed by Gmeiner and Martin (16). Approximately 75 μmol of lipid phosphate were obtained from 4.5 g (wet weight) of cells. For the preparation of liposome dispersions, phospholipids were dried under nitrogen and were dispersed at 0°C in buffer (0.1 M NaCl, 20 mM Tris hydrochloride, [pH 7.2]) in the presence or absence of LPS (20 nmol of 2-keto-3-deoxyoctonic acid [KDO] per μmol of lipid phosphate), using five 1-min bursts from an MSE sonicator (Measuring and Scientific Equipment, Ltd., London, United Kingdom) set on low power.

Subfractionation of LPS by column chromatography. Lyophilized LPS-A 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, 20 mM Tris hydrochloride (pH 8.3) and was applied at room temperature to a column (1.6 by 62.5 cm) of Sephacryl S-300 (Pharmacia Fine Chemicals) equilibrated in buffer containing 0.25% (wt/vol) sodium deoxycholate, 0.2 M NaCl, 5 mM EDTA, 10 mM Tris hydrochloride (pH 8.0). Fractions (2.5 ml), collected at a flow rate of 12 ml/h, were analyzed directly by fused rocket immunoelectrophoresis (RIE) and SDS-polyacrylamide gel electrophoresis. Before KDO estimations and protection studies, fractions were dialyzed extensively against distilled water, first at room temperature and then at 4°C.

Sep-Pak filtration of monovalent extract-6. Sep-Pak C_{18} hydrophobic cartridges (Waters Associates, Milford, Mass.) were prewetted according to the manufacturer's instructions by the passage through each cartridge of 2 ml of methanol, followed by 5 ml of distilled water. To prevent subsequent dilution of sample, excess water was expelled from the cartidges which were then flushed with 0.5 ml of extract-6, the filtrates being discarded. A further 1.5 ml of extract-6 was then cycled three times through each Sep-Pak C_{18} filter, and the resultant effluents were retained for analysis. Before examination of polypeptide profiles by SDS-polyacrylamide gel electrophoresis, samples were concentrated by lyophilization.

Electrophoretic techniques. Crossed immunoelectrophoresis, rocket immunoelectrophoresis, and fused RIE were performed at 20°C in 1% (wt/vol) agarose (Seakem LE, relative mobility $[-m_r] = 0.10$ to 0.15; Miles Laboratories, Stoke Poges, United Kingdom) in barbital-hydrochloride buffer (pH 8.6) containing 1% (vol/vol) Triton X-100 (35). For crossed immunoelectrophoresis and (fused) RIE, the antibody-containing gels routinely contained 4.7 and 9.5 μl of rabbit anti-PEV immunoglobulins (28) per ml, respectively (equivalent to 0.78 and 1.56 mg of protein per ml, respectively). Gels were washed, pressed, and dried, and the immunoprecipitates were stained with Coomassie brilliant blue as described previously (35). Quantitative measurements of antigen content were based on measurement of peak height after RIE (35).

SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (26), using 12.5% (wt/vol) acrylamide separating gels, 4% (wt/vol) acrylamide stacking gels, and up to 16 molecular weight standards (5a). Proteins were stained with Coomassie brilliant blue, and LPSs were stained with silver nitrate (14).

Protection studies. Groups of 10, inbred, female NIH mice (haplotype *H-2*^{q}; Olac '76 Ltd., Bicester, United Kingdom), weighing 17 to 20 g were immunized subcutaneously on day 1 with 0.5-ml doses of dilutions of the appropriate test sample in physiological saline. Booster injections containing identical doses of test sample were given on day 8. If required, blood samples (0.2 ml) were collected on day 13. In all cases, mice were challenged intravenously on day 14 with one 95% lethal dose of *P. aeruginosa* serotype 6 (equivalent to 4×10^6 to 5.3×10^6 viable cells in 0.2 ml of saline). Mortality was recorded over five days, and the 50% protective dose (PD_{50}) values were calculated by probit analysis. To directly compare the protective capacity of similar numbers of different LPS molecules, test samples were analyzed and compared on an equivalent KDO basis. This reasoning assumes naturally that all species of LPS contain similar numbers of KDO residues (viz two; reference 44).

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay, using both purified LPS and extract-6 as

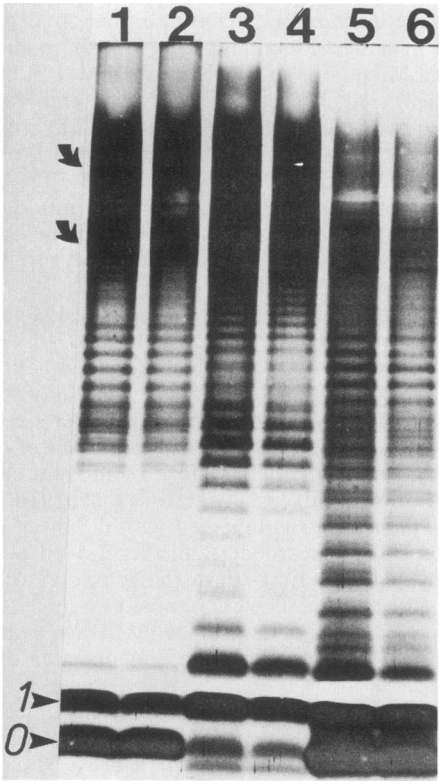

FIG. 1. Analysis of LPS-A, LPS-B, and extract-6 by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2, monovalent extract-6 (0.90 and 0.63 nmol of KDO, respectively); lanes 3 and 4, LPS-A (1.06 and 0.63 nmol of KDO, respectively); lanes 5 and 6, LPS-B (2.39 and 1.45 nmol of KDO, respectively. The arrows at the bottom of the gel and labelled *0* and *1* indicate bands thought to correspond to lipid A-core and lipid A-core-$(O\text{-antigen})_1$, respectively. Unresolved smooth LPS is indicated by curved arrows towards the top of the gel which has been silver-stained for carbohydrate. Note that to facilitate qualitative comparison of LPS subspecies, the loadings of KDO used to analyze LPS-B are about 2.4-fold greater than those used to analyze LPS-A or extract-6.

test antigens was performed in the standard manner with antigen-coated polyvinyl chloride microtitration plates and rabbit anti-mouse immunoglobulin conjugated to horse radish peroxidase.

Anti-PEV immunoglobulins. Rabbit anti-PEV immunoglobulins were raised, purified, and concentrated as previously described (28).

Analytical procedures. The protein level was estimated by a modification of the Lowry procedure (29) using bovine serum albumin as standard, the amount of organic phosphate was measured by the method of Ames and Dubin (1) using $NaH_2PO_4 \cdot 2H_2O$ as standard, and total hexose was measured by the anthrone procedure (33) with glucose as standard. Nucleic acid was determined from A_{260} values (11), and KDO level was measured by the thiobarbituric acid method after hydrolysis of samples in 0.1 N sulfuric acid for 30 min (24). Neutral sugars were analyzed as their alditol acetates and separated by gas-liquid chromatography on both a 3% ECNSS-M column at 200°C and a 3% OV-225 column at 210°C.

RESULTS

Properties of purified LPS. LPSs isolated from members of the family *Enterobacteriaceae* exhibit a high degree of size heterogeneity attributable to the number of O-antigen repeat units (17, 36, 37). In outer membranes from smooth strains of *Salmonella typhimurium*, for example, 50 to 70% of the LPS molecules contain one or no O-antigen repeats, the balance consisting of a range of smooth LPS molecules with side chains containing up to 70 repeat units (36, 37). Of additional significance is the fact that the apparent ratio of rough to smooth LPS molecules extracted from gram-negative bacteria can vary dramatically depending on the isolation procedure used (10, 14, 47). For this reason, we used two different procedures to isolate LPS from *P. aeruginosa* serotype 6. LPS-A was obtained by the classic Westphal extraction procedure (47), which selects largely for smooth-type LPS. It was found to contain about 1.4 to 2.1% (wt/wt) KDO. LPS-B was prepared by a method recently described by Darveau and Hancock (10), which appears to yield an LPS preparation more closely resembling that of the cell wall. This latter LPS preparation (LPS-B) contained about 4.9% (wt/wt) KDO. When analyzed by crossed immunoelectrophoresis against rabbit anti-PEV serum, both preparations gave a single immunoprecipitate which showed reactions of complete identity with the major antigen (antigen no. 80) observed for extract-6 of PEV (for nomenclature of antigens see reference 28).

Analysis of LPS-A and LPS-B by gas-liquid chromatography revealed that rhamnose and glucose were the sole neutral sugars in both preparations and that the ratio of rhamnose to glucose was 3.5 times higher for LPS-A than for LPS-B. These observations are of significance on two counts. Firstly, the immunologically related, high-molecular-weight PS isolated from *P. aeruginosa* (IATS serotype 6) by Pier and his colleagues was reported to contain arabinose and galactose (24 and 30% by weight of total carbohydrate, respectively) in addition to the other neutral sugars, viz., rhamnose and glucose found for LPS (38, 39). Thus, the inability to detect either arabinose or galactose in either LPS-A or LPS-B would argue against the presence of PS in our preparations. Secondly, recent structural studies (25) indicate that the O-antigen of IATS serotype 6 is a repeating linear tetrasaccharide with the following structure: →2[L-rhamnose α(1→4)2-acetamido-2-deoxy-D-galacturonamide α(1→4)2-deoxy-2-formamido-D-galacturoni acid α(1→3)2-acetamido-2,6-dideoxy-D-glucose]α1→. Accordingly, rhamnose but not glucose is a constituent of the O-antigen repeat of this serotype LPS. Both sugars are likely constituents of the core region (44). Thus, one obvious explanation for both the higher rhamnose/glucose ratio and the lower KDO content of LPS-A in comparison to LPS-B is that the latter preparation contains more rough LPS species. This was confirmed by analysis of samples on SDS-polyacrylamide gels (Fig. 1). LPS-B contained a range of LPS species, most dominant amongst which were two of low-molecular-weight (Fig. 1, lanes 5 and 6). By analogy with profiles of enterobacterial LPS (17, 36, 37), we assume that these species represent LPS with zero and one O-antigen repeat. In contrast, LPS-A contained relatively few core-lipid A molecules and had a higher content of species with extended O-antigen chains (Fig. 1, lanes 3 and 4). The corresponding carbohydrate profile of extract-6 (Fig. 1, lanes 1 and 2) suggested that the vaccine constituent possesses levels of rough LPS intermediate between those of LPS-A and LPS-B. Otherwise, it contained a spectrum and relative concentration of smooth LPS species (with 9 to ≈50 O-antigen repeats) which resembled much more closely that of LPS-A than that of LPS-B.

Protective properties of extract-6 and purified LPS. The

TABLE 1. Comparison of the protective properties of extract-6, LPS-A, and LPS-B

Sample	No. of mice surviving per group with the indicated immunizing dose (pmol of KDO)					PD_{50}[a] (pmol of KDO)
	670	67	6.7	0.67	0.067	
Extract-6	ND[b]	9/10	8/10	4/10	0/8	2.09 (0.48–6.91)
LPS-A	10/10	9/10	8/10	5/10	ND	0.96 (0.13–3.21)
LPS-A + PL[c]	8/8	8/9	9/10	8/10	ND	<0.67
LPS-B	8/10	6/10	3/10	0/10	ND	56.2 (14.5–266.7)
LPS-B + PL[c]	9/10	8/10	6/10	0/10	ND	13.7 (3.2–46.6)

[a] The corresponding 95% confidence limits are given in parenthesis below each PD_{50}.

[b] ND, Not determined.

[c] PL, Phospholipid; the molar ratio of lipid phosphate to KDO was 50:1. When phospholipid was assayed alone at identical 10-fold dilutions (but in the absence of LPS), the protection pattern was 1/10, 0/10, 0/10, and 0/10.

degree of protective immunity afforded by extract-6, LPS-A, and LPS-B was compared by using a mouse challenge model, and the results of a typical experiment are shown in Table 1. On a KDO basis, extract-6 gave a level of protection which lay between that afforded by LPS-A and LPS-B, being very much closer to that induced by the former. In turn, LPS purified by hot phenol extraction (LPS-A) was approximately 50 times more protective than LPS purified by the method of Darveau and Hancock (LPS-B; 10). The results of enzyme-linked immunosorbent assay titrations (data not shown) performed on serum samples collected the day before challenge correlated with the protection results in that anti-LPS and anti-extract-6 immunoglobulin G titers similar to those elicited by either LPS-A or extract-6 were only detected in mice immunized with LPS-B when 10-fold-higher concentrations of the latter vaccine were used. These differences in the protective properties of the two LPS preparations could not be fully accounted for by differences in the physical state of the immunogens. Thus, although the PD_{50}s for both LPS-A and LPS-B were decreased somewhat by presentation in the form of liposomes (Table 1), the level of protection afforded by LPS-B in liposomes was still poorer than that afforded by LPS-A alone or by extract-6.

These results, together with those presented in Fig. 1, indicate that the concentration of LPS in the univalent vaccine is sufficient to account in full for the observed level of protection.

Protective properties of the 16,200-dalton protein. We have previously shown that a 16.2-kilodalton polypeptide is a major protein component of each monovalent extract, including extract-6, and that antiserum raised to PEV contains significant titers of immunoglobulins directed against it (28). Thus, it was of interest to determine the role, if any, of this antigen (termed antigen no. 41; reference 28) in PEV-induced protection against pseudomonas infection. A key

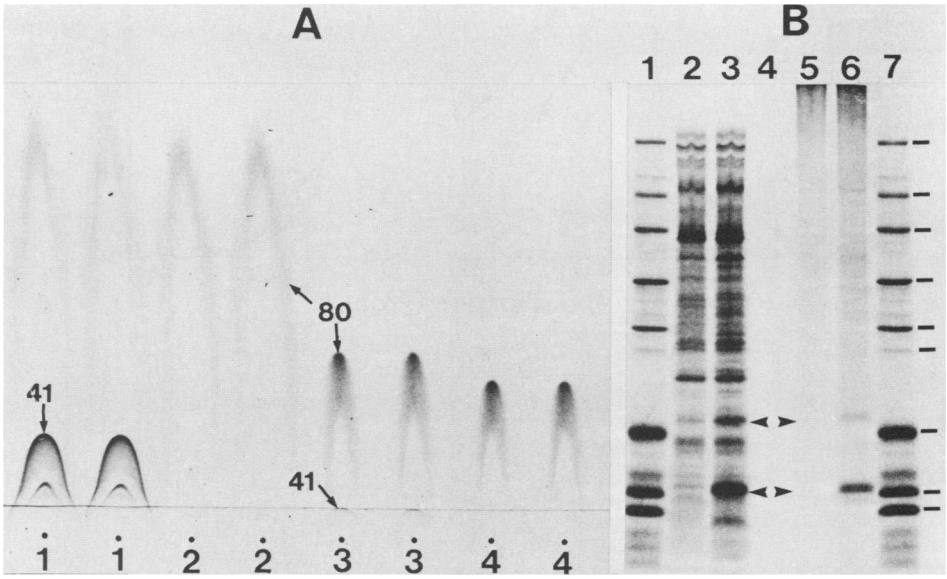

FIG. 2. Effect of filtration through Sep-Pak C_{18} cartridges on the antigen and polypeptide profiles of extract-6. Dialyzed extract-6 was filtered through Sep-Pak C_{18} cartridges as detailed in the text. The resultant filtrate was compared on an equal volume basis with untreated extract-6 by RIE (A) and by SDS-polyacrylamide gel electrophoresis (B). (A) Wells 1 and 3 contain 15 and 0.7 μl, respectively, of unfiltered (formalinized) extract-6. Wells 2 and 4 contain corresponding volumes, respectively, of filtered (formalinized) extract-6. The identities of immunoprecipitates corresponding to LPS (antigen no. 80) and to the 16.2-kilodalton protein (antigen no. 41) are indicated. Note the complete absence of antigen no. 41 and of a second minor antigen from filtered extracts (wells 2 and 4) and the relatively small decrease in peak height of the immunoprecipitate no. 80 after filtration (wells 3 and 4). The system used to number immunoprecipitates follows that detailed by MacIntyre et al. (28). The center of each well is indicated by a dot, and the anode is to the top of the immunoplate. (B) Lanes 1 and 7, molecular weight standards; lanes 2 and 3, unformalinized extract-6 after and before filtration, respectively (equivalent to 225 μl of original extract); lane 4, blank; lanes 5 and 6, formalinized extract-6 after and before filtration, respectively (equivalent to 225 μl of original extract). The identities of the 16.2- and 21.4-kilodalton polypeptides are indicated by arrowheads on the gel which has been stained with Coomassie brilliant blue. Also marked are the positions of the following molecular weight protein standards: phosphorylase *a* (94,000), catalase (61,000), fumarase (48,5000), ompF protein (37,200), carbonic anhydrase (30,000), chymotrypsinogen A (25,100), trypsin inhibitor (20,100), myoglobin (17,200), and lysozyme (14,300).

TABLE 2. Effect of filtration through Sep-Pak C_{18} cartridges on the protective properties of extract-6

Sample[a]	Vol of extract-6 (nl)	
	PD_{50}[b]	95% confidence limits
Before filtration	64	21–230
After filtration	66	15–320

[a] Before filtration the concentration of KDO in the extract was 16.6 nmol/ml.

[b] Data determined from mouse protection experiments with five serial, 10-fold dilutions of extract.

finding in this respect was the observation that antigen no. 41, together with another minor 21.4-kilodalton polypeptide, could be completely removed from extract-6 by filtration through Sep-Pak C_{18} hydrophobic cartidges. Neither protein could be detected in the resultant filtrate by either RIE (Fig. 2A) or SDS-polyacrylamide gel eleçtrophoresis (Fig. 2B, lanes 5 and 6). LPS, on the other hand, was largely unaffected by filtration; quantitative estimates of antigen content indicated that about 85% of the LPS could be recovered in the filtrate (Fig. 2A). SDS-polyacrylamide gel electrophoresis performed in conjunction with silver staining confirmed these results and in addition revealed identical spectra of LPS subspecies for the filtered and control preparations (data not shown).

Binding of the two proteins to Sep-Pak C_{18} cartridges appeared to be strong and quite specific. Neither polypeptide could be recovered by elution with buffer at pH 9 or with any of several different nondenaturing agents, including 10 to 95% (vol/vol) ethylene glycol, 1% (vol/vol) Triton X-100, and 1% (wt/vol) octyl glucoside. Furthermore, filtration through Sep-Pak C_{18} cartridges of unformalinized extract-6, for which over 40 polypeptides could be resolved, again resulted in the selective removal of the 21.4- and 16.2-kilodalton proteins, together with two other minor polypeptides (Fig. 2B, lanes 2 and 3).

To ascertain the contribution of the 16.2- and 21.4-

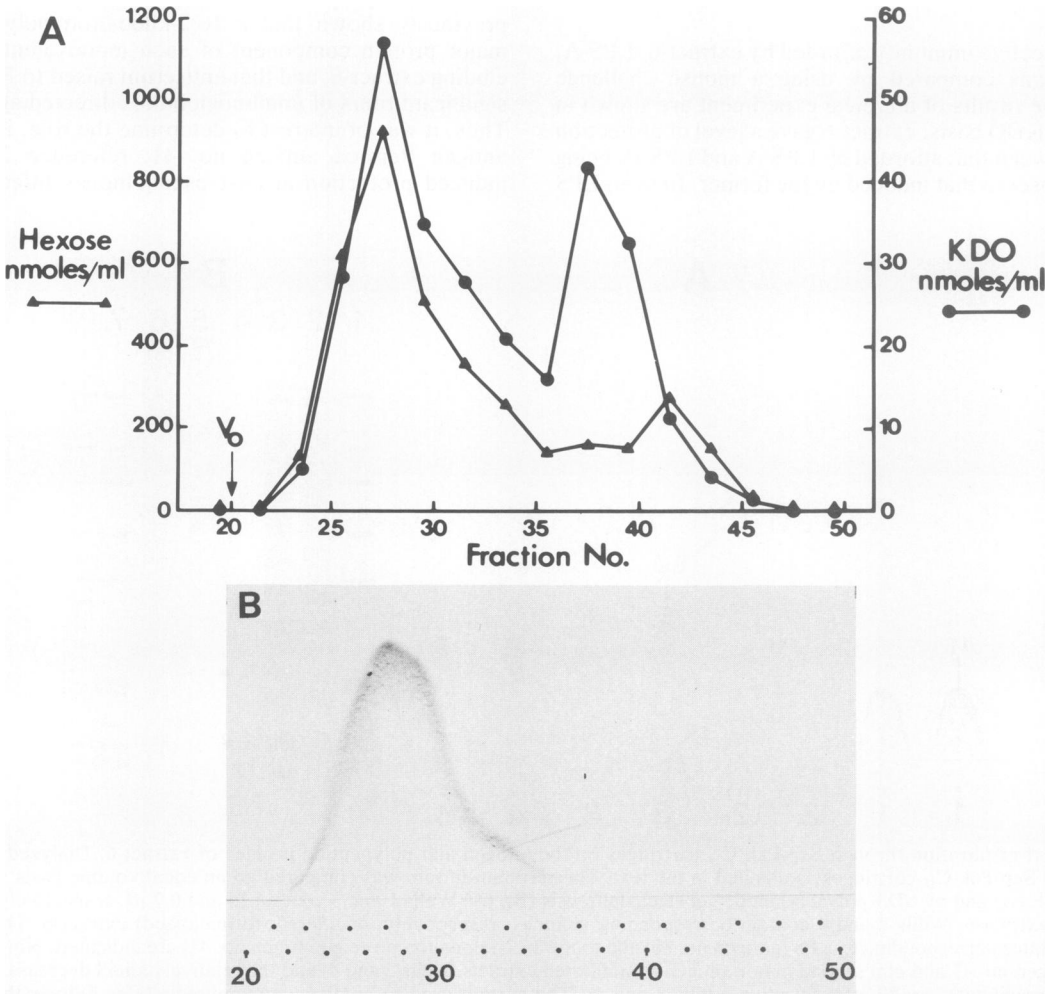

FIG. 3. Subfractionation of LPS-A by column chromatography. LPS-A (22 mg; 1.13 μmol of KDO) was applied to a column of Sephacryl S-300 and eluted with 0.25% buffered deoxycholate as described in the text. Eluted fractions were analyzed for KDO and total hexose (A), by fused RIE (B) and by SDS-polyacrylamide gel electrophoresis in conjunction with silver staining for carbohydrate (Fig. 4). Panels A and B have been arranged to allow precise alignment of fractions. (A) Symbols: ●, KDO; ▲, total hexose; V_0, void volume. (B) Equal volumes (0.5 μl) of column fractions 20 to 50 were each diluted with 4.5 μl of 1% (vol/vol) Triton X-100 in 50 mM Tris hydrochloride buffer containing 5 mM EDTA (pH 8.6) and analyzed by fused RIE against anti-PEV serum. The center of each well has been indicated by a dot, and the anode is to the top of the gel.

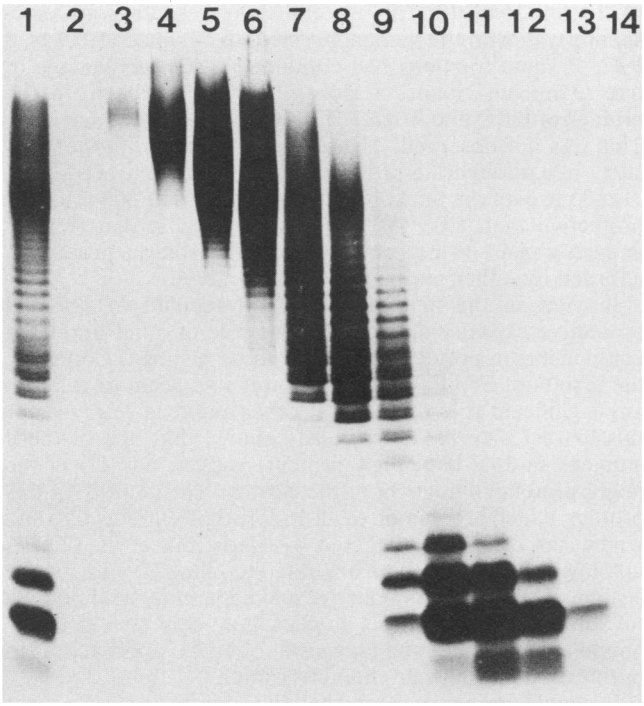

FIG. 4. Analysis of subfractionated LPS-A by SDS-polyacrylamide gel electrophoresis. LPS-A was fractionated by gel filtration as described in the legend to Fig. 3. Eluted fractions were analyzed and were resolved by silver staining for carbohydrate. Lane 1, LPS-A (511 pmol of KDO); lanes 2 to 14 contain (in order) 20 μl of fractions 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, and 45 (possessing 0, 86, 498, 972, 596, 474, 357, 274, 717, 556, 196, 67, and 39 pmol of KDO, respectively).

kilodalton proteins to PEV-induced immunity, filtered and unfiltered (formalinized) extract-6 were compared in the mouse challenge assay. The degree of protection afforded by both samples was indistinguishable (Table 2), thus eliminating the involvement of either protein in protection afforded by the vaccine extract in this model system.

Subfractionation of LPS. On balance, the evidence presented above strongly suggests that LPS is the major protective antigen in extract-6 of PEV. The molecule itself, however, is clearly heterogeneous with respect to size in both the extract and the purified form (Fig. 1). To pinpoint the major species involved in protection, LPS-A was subfractionated on a Sephacryl S-300 column equilibrated in a deoxycholate buffer (4, 46). The presence of LPS in column fractions was monitored by determination of KDO and total hexose, by fused RIE, and by SDS-polyacrylamide gel electrophoresis (Fig. 3A, 3B and 4, respectively).

Two major peaks of KDO were observed. The first, centered at fraction 27 (Fig. 3A), corresponded in profile with the single LPS immunoprecipitate obtained in fused RIE (Fig. 3B) and with the main peak of hexose (Fig. 3A). The second peak of KDO was centered at fraction no. 37 and corresponded to a minor shoulder in the hexose profile (Fig. 3A). These fractions generated no corresponding immunoprecipitate in fused RIE (Fig. 3B). It is notable that the apparent ratio of hexose/KDO decreased regularly from an initial value of about 25 (fraction 23) to about 4 (Fraction 37), a feature consistent with the fractionation of LPS on the basis of a decreasing number of repeating units in the O-antigen side chain. The significance of a minor peak of hexose eluting after the second peak of KDO is unclear at the present (Fig. 3A). As judged by the almost quantitative recovery of KDO (91%) and by the correspondence in carbohydrate profiles between the applied sample and the sum of the eluted fractions (Fig. 4), all LPS species were recovered in fractions 23 through 46. Certainly, the void volume fraction contained no LPS (or related material) detectable by RIE, by SDS-polyacrylamide gel electrophoresis, or by determination of KDO or total hexose (Fig. 3 and 4), indicating the absence of molecular species or aggregates of very high molecular weight.

Analysis of column fractions by SDS-polyacrylamide gel electrophoresis, followed by silver staining, clearly revealed a subfractionation of LPS species based on progressively decreasing numbers of repeating units in the O-antigen side chains (Fig. 4). The first peak of KDO corresponded largely to LPS species containing more than 15 O-antigen repeating units. These high-molecular-weight molecules were not well resolved by the gel system used here (Fig. 4). However, they did not appear to be artifacts caused by incomplete dissociation of aggregated lower-molecular-weight species (32, 37) since the banding patterns were unaffected by inclusion of 40 mM EDTA in the sample buffer or by increasing the concentration of SDS in the separating gel to 0.5% (wt/vol). In part at least, the poor resolution may be related to relative loadings; the characteristic LPS banding profiles can be discerned for such samples at the leading (i.e., low-molecular-weight) front (see Fig. 4, lanes 4 through 6). The second peak of KDO (fractions 37 to 41) was shown to largely represent LPS molecules with zero to two O-antigen repeating units. The absence of extended side chains and possibly of significant anti-core antibody in the anti-PEV serum, presumably accounts for the inability of these molecules to form electrophoretically stable immunoprecipitates during RIE (a similar phenomenon has been observed after SDS-polyacrylamide gel–CIE; reference 28). The population of LPS species of intermediate size (containing between 7 and 15 repeat units) was eluted from the column between fractions 31 and 35 (Fig. 4).

The demonstrable ability to fractionate pseudomonas LPS on the basis of size, i.e., the number of O-antigen repeating units, allowed the protective potencies of the subspecies to be compared in the mouse challenge assay (Table 3). It is clear from Table 3 that LPS molecules with extended (>18) O-antigen side chains induced a level of protection 50- to 100-fold superior to that afforded by species with predominantly zero to two or zero to three repeats. Given the above

TABLE 3. Effect of O-antigen chain length on the protective capacity of LPS

Sample[a]	Range of O-antigen repeats[b]	PD_{50}[c] (pmol of KDO)	95% confidence limits
LPS-A	0–≈50	1.98	0.67–6.01
Fraction 25	28–≈50	0.95	0.24–3.74
Fraction 27	18–≈50	0.69	0.19–2.88
Fraction 31	8–45	4.38	1.64–12.0
Fraction 37	0–10 (1–3)	56.2	9.93–532
Fraction 41	0–2 (1)	67.9	21.1–717

[a] The unfractionated (LPS-A) and fractionated samples (no. 25, 27, 31, 37, and 41) correspond to those described in Fig. 3.

[b] Where practicable, the major species of LPS present in the fractions are given in parentheses.

[c] Data determined from six serial, 10-fold dilutions of samples at an initial concentration of 815 pmol of KDO per ml.

data and the fact that about 45% of the total KDO partitioned in fractions 21 through 30, it is apparent that the bulk of the protection afforded by LPS-A can be accounted for by LPS-species possessing about 10 or more O-antigen repeating units. Indeed, making certain reasonable assumptions (based on profiles of LPS heterogeneity and known PD_{50}s) as to the likely protective capacity of fractions not tested, it can be estimated that over 85% of the protection afforded by LPS-A can be accounted for by LPS molecules with 10 or more O-antigen repeating units. In our experimental challenge system, as few as 2×10^{11} of these molecules of LPS are sufficient to confer 50% protection against one 95% lethal dose of virulent *P. aeruginosa* serotype 6.

DISCUSSION

Several recent studies have established that immunization with purified LPS protects mice against infection by *P. aeruginosa* (6-9). For the following reasons, there can be little doubt that LPS is also responsible in large part for PEV-induced protection of mice against infection by *P. aeruginosa* serotype 6. First, LPS is the major antigenic component of extract-6 (28). Second, extract-6 contains a spectrum of smooth LPS species with 9 to ≈50 O-antigen repeating units, which is qualitatively and quantitatively similar to that of purified LPS-A. In addition, protection studies performed on LPS-A subfractionated by column chromatography clearly demonstrate a relationship between protection and the number of O-antigen repeating units and indicate that LPS species possessing over about 10 O-antigen repeating units account for the bulk of the protection afforded by the preparation. Moreover, on a KDO basis, LPS-A can account in full for the protective properties of the vaccine extract. Finally, the protective capacity of extract-6 is unaffected by removal of two of its major protein antigens (16,200 and 21,400 daltons).

It is possible that the mild aqueous EDTA-glycine extraction technique used to obtain the vaccine extract may selectively extract from the bacterial cells a spectrum of LPS molecules with enhanced protective capacity (i.e., increased average length of O-antigen side chain). Certainly, it results in an LPS preparation with a much higher ratio of smooth/rough LPS than that isolated by the method of Darveau and Hancock (10). Irrespective of this, it is clear from the different heterogeneity profiles and PD_{50}s for LPS-A, LPS-B, and extract-6 that different extraction procedures can generate LPS preparations with quite different chemical and biological properties and that caution must be exercised in the interpretation of protection data involving LPS preparations of undefined heterogeneity.

Several lines of evidence suggest that the high-molecular-weight PS described by Pier and his coworkers (38-41) is not present in significant amounts in our preparations. Certainly, there is no trace in either purified LPS-A or LPS-B of the two hexoses, arabinose and galactose, reported to be characteristic constituents of this O-antigen-like polymer. (It should be stressed, however, that it has not been clearly established whether these additional sugars are covalently bound to O-antigen [and dependent for expression on the strain or growth conditions] or whether they derive from a contaminating PS [39, 40]). Moreover, KDO is present in each hexose-containing fraction eluted after Sephacryl chromatography of LPS-A (Fig. 3A), the observed decrease in molar ratio of hexose/KDO being consistent with the size-separation demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 4). It is also significant that the KDO profile of the first peak (of LPS-A) eluted by gel filtration corresponds well with the immunoprecipitate obtained after fused RIE. If some fractions had contained a high percentage of free O-antigen chains, a lack of correlation between the profiles of KDO and fused RIE might have been anticipated. This was not observed. These data, together with the similarity in carbohydrate profiles of LPS-A and extract-6 (Fig. 1) and the experimental observation that PS is 1,000-fold-less protective than LPS (8), on balance suggest that free O-antigen chains do not play a significant role in protection afforded by either purified LPS or extract-6.

In view of the fact that LPS can account for the full protective capacity of extract-6, the role of other unrelated components in protection appears to be minimal. Certainly, the results of Sep-Pak C_{18} filtration would seem to rule out any significant role for the two other antigens resolved for this extract. We have previously shown that one of these antigens (a 16.2-kilodalton protein, antigen no. 41) is the major protein component of the extract. The identity of this protein, which is common to all 16 serotype extracts (28) and which has also been detected by Hedstrom et al. (20), is unknown. It appears to be unrelated to pilin (28) and may be a slime component. Its selective and apparently avid binding to Sep-Pak C_{18} cartridges implies that it is an extremely nonpolar molecule and suggests obvious approaches to purification and further characterization.

It should be appreciated that the ability to assess what minor role, if any, other constituents of extract-6 play in protection is complicated somewhat by the fact that most of the proteins present in the formalinized vaccine extracts (with the notable exceptions of the 16.2- and 21.4-kilodalton proteins) are poorly resolved by SDS-polyacrylamide gel electrophoresis. The covalent polymerization of proteins which occurs on storage of the Formalin-treated vaccine extract is the major cause of this phenomenon (28). Some proteolysis may also occur. However, proteins which are susceptible to proteolysis are unlikely to play a role in protection since the protective capacity of the freshly prepared vaccine is undiminished by storage at 4 or 37°C for 2 years or by treatment with pronase (data not shown). Antibodies directed against flagella and outer membrane proteins (F and H1/H2) have also been detected in anti-PEV serum, but the corresponding antigens cannot be detected (even by Western blotting) in either formalinized or unformalinized extract-6 (28). Furthermore, neither flagella (21) nor these outer membrane proteins (19, 34) exhibit the mainly serotype-specific protection afforded by the constituent monovalent extracts of PEV (31). Clearly, we cannot rule out a role for these constituents in protection afforded by other serotype extracts. However, LPS is the major antigen for at least 14 of the other 15 extracts (28). Thus, in view of the results presented here and elsewhere (see reference 6 for review) on the protective potential of pseudomonas LPSs in a number of different animal systems, it seems likely that LPS also plays a major role in protection afforded by at least some of the other extracts comprising PEV.

The relevance of these results to protection of humans against infection by *P. aeruginosa* is indicated by the presence of significant titers of anti-LPS immunoglobulin G in hyperimmune serum of volunteers immunized with PEV (28; unpublished observations) and a body of largely circumstantial evidence (e.g., references 5, 6, 8, and 45) which suggests that intravenous transfer of anti-LPS immunoglobulins is a useful, although not necessarily sufficient strategy in controlling pseudomonas infection in the critically ill or immunocompromised patient.

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