Immunochemical and Biochemical Analysis of the Polyvalent Pseudomonas aeruginosa Vaccine PEV

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The Pseudomonas aeruginosa polyvalent vaccine PEV and its 16 constituent monovalent extracts from International Antigenic Typing System serotypes 1 through 13 and 15 through 17 (J. J. Miller, J. F. Spilsbury, R. J. Jones, E. A. Roe, and E. J. L. Lowbury, J. Med. Microbiol. 10:19-27, 1977) were subjected to biochemical analysis and to detailed immunochemical analysis with rabbit anti-PEV immunoglobulins. The results of chemical analysis, of analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed in conjunction with silver staining, and of analysis by crossed immunoelectrophoresis, sodium dodecyl sulfate-polyacrylamide gel-crossed immunoelectrophoresis, and Western blotting showed clearly that lipopolysaccharide was a major constituent of each monovalent extract and that it was probably the dominant antigen present in at least 15 of the 16 monovalent extracts. A 16.2-kilodalton protein, which was promiscuous and nonsedimentable at 105,000 × g and which appeared to be biochemically and antigenically unrelated to pili, was a common although minor antigen for all extracts. Several other proteins, some of outer membrane origin, were also detected in unformalized extracts, but these were also minor antigenic constituents of the vaccine. Neither pili nor flagellin appeared to be major antigenic constituents of tested monovalent extracts, although anti-flagella antibodies could be demonstrated in rabbit anti-PEV by Western blotting. Preliminary analysis by crossed immunoelectrophoresis of serum raised in volunteers to PEV also indicated the presence therein of antibodies to lipopolysaccharide antigens.

Pseudomonas aeruginosa is an opportunistic pathogen which is frequently associated with nosocomially acquired infections. Certain groups of patients, for example, those suffering from cystic fibrosis or those who are immunocompromised as a result of severe burn wounds, malignancy, or immunosuppressive therapy, appear to be particularly susceptible to infection by this organism (3). Moreover, even with the availability of improved antibiotic therapy, the mortality rate of patients succumbing to infection by P. aeruginosa is still relatively high (see reference 9 for review). Immunotherapy is one promising method of treatment of patients at high risk to infection by P. aeruginosa. Therefore, in the past decade, much attention has been focused on the protective properties of the surface antigens of this bacterium and on the development of a safe and effective vaccine.

Lipopolysaccharide (LPS) has probably received the most attention as a protective surface antigen of P. aeruginosa. It has been clearly established that antibody directed against this outer membrane component protects mice against infection by homologous strains of P. aeruginosa (10, 11). Anti-LPS antibody appears to be important also in the protection of humans against infection by this organism. High anti-LPS titers have been correlated with increased rates of survival among patients with pseudomonic bacteremia (50), and in addition, during a 5-year clinical trial, the rate of Pseudomonas-related mortality among severely burned patients was found to be greatly reduced after immunization with a heptavalent LPS-based vaccine called Pseudogen (Parke, Davis & Co., Detroit, Mich. [1, 25]). Severe adverse reactions to this vaccine, however, were common (1, 45). The major outer membrane pore-forming protein, protein F, is another surface-expressed antigen of P. aeruginosa for which the ability to elicit a protective antibody response in mice has been demonstrated (18, 38). In contrast to LPS, protein F is antigenically related in all 17 serotypes of P. aeruginosa (39). In addition, at least three other surface components of P. aeruginosa have been reported to function as protective antigens. These include flagella (27, 37), a toxic glycolipoprotein (2, 14, 54), and a high-molecular-weight polysaccharide (47, 49, 51) which shares immunological identity with the O-side chains of homologous LPS (46, 48).

PEV-01 (Wellcome Biotechnology Ltd., Beckenham, United Kingdom) is a polyvalent vaccine which is composed of pooled surface antigen extracted, under mild conditions, from viable cells of 16 different serotypes of P. aeruginosa (36). This vaccine has been tested in volunteers (29) and has undergone preliminary clinical trials, notably at Safdarjung Hospital, New Delhi, India (28, 52). The results indicate that immunization of burn patients with either the polyvalent vaccine or human anti-PEV immunoglobulins provides protection, with few adverse side effects, against fatal infection by P. aeruginosa (52). Despite these encouraging results, the serologically active component of the PEV vaccine remains unidentified. However, in view of the fact that the Pseudomonas cells retain viability after extraction (with EDTA-glycine buffer [36]), and in view of the well-recognized sensitivity of the outer membrane of this organism to chelating agents (25), one might suspect that surface components would be important constituents of PEV.

Bearing in mind the well-documented role of antibody in protection against Pseudomonas infection (9), we have undertaken, as a preliminary step towards identifying the protective antigen(s) in PEV-01, a high-resolution immunochemical analysis of the vaccine and its component monovalent extracts. We report here on the results of this

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investigation and show that LPS is a major constituent of the vaccine.

(Preliminary accounts of this work have been presented at the 100th and 103rd Ordinary Meetings of the Society for General Microbiology, Warwick, United Kingdom, 1984, P30 and 1985, P33, respectively, and at the Lutunren Lectures on Molecular Genetics, The Netherlands, 1984, p. 30.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The 16 strains of *P. aeruginosa* that were used to prepare the original vaccine PEV-01 (36) were obtained from Wellcome Biotechnology Ltd. They correspond to virulent strains of the International Antigenic Typing System (IATS) serotypes 1 through 13 and 15 through 17. For preparation of monovalent extracts, *P. aeruginosa* was grown as described (36) in 4-liter fermentors containing minimal medium supplemented where appropriate with [35S]methionine (1.4 to 1.7 μCi/ml). For the preparation of some cellular components, bacteria were grown at 37°C in 500 ml of minimal medium (36) in 2-liter Erlenmeyer flasks, with agitation at 200 rpm. Cells were harvested at early stationary phase (A660, approximately 2.5) and were either (i) washed once in distilled water, lyophilized, and stored at −70°C until required for isolation of LPS or (ii) washed once in 30 mM Tris hydrochloride (pH 8.0) and used immediately for preparation of membrane fractions. A multipilated strain (DB2) of *P. aeruginosa PAO* (IATS serotype 2) was used for the preparation of pili and was grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) as described (44).

Monovalent extracts and polyvalent vaccine PEV. The polyvalent vaccine PEV and its component monovalent extracts were prepared at Wellcome Biotechnology Ltd. as previously described (36). Briefly, bacteria from 16 serotype strains were extracted at 3 × 10^10 to 5 × 10^10 viable organisms per ml in EDTA-glycine buffer at 37°C, and the cell-free supernatant fractions were sterilized by filtration and by the addition of Formalin to 0.3% (vol/vol). The polyvalent vaccine PEV was derived by pooling equal volumes of all 16 monovalent extracts (36). The number ascribed to each monovalent extract denotes the IATS serotype of the strain from which the extract was prepared, with the exception that extract 14 is derived from IATS serotype 17. (IATS serotype 14 is rarely isolated and is not used in the production of PEV.) For chemical analyses and analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, PEV and the monovalent extracts were dialyzed against 10 mM Tris hydrochloride (pH 7.5) for 36 h at 4°C. Undialyzed extracts were used for analyses by crossed immuno-electrophoresis (CIE). The routine treatment of monovalent extracts with formaldehyde was omitted in experiments designed to assess the effect of this reagent on the resolution of constituent antigens.

Preparation of LPS. LPS was extracted from whole lyophilized cells by the hot aqueous phenol procedure of Westphal and Jann (57). After extensive dialysis against water, the aqueous phase was lyophilized and then resuspended in 0.5 M NaCl to a final concentration of 0.75% (wt/vol). Nucleic acid was removed by fractional precipitation with cetyl-trimethylammonium bromide (57), and the detergent was eliminated by twice precipitating LPS from 0.5 M NaCl with 10 volumes of ethanol at 4°C for 2 h. The final precipitate was suspended in a small volume of distilled water and dialyzed extensively against distilled water at 4°C. Yields of LPS isolated in this manner from *P. aeruginosa* serotypes were generally in the range of 3.2 to 8.9% of the cell dry weight and contained less than 1% protein or nucleic acid.

Isolation of cell fractions. Outer and inner membranes were isolated from *P. aeruginosa* IATS serotype 6 by French press lysis, followed by centrifugation of the lysate on two consecutive sucrose density gradients, as described by Hancock and Nikaido (22). The procedure yielded an inner membrane fraction (density, 1.17 g/ml) together with two outer membrane fractions at densities of 1.23 g/ml (OM2) and 1.248 g/ml (OM1). Both OM1 and OM2 displayed the same protein profiles on analysis by SDS-polyacrylamide gel electrophoresis.

Flagella were sheared from cells of the same strain by blending in a Silverson V5099 mixer-emulsifier at high speed with 10 1-min bursts. After whole cells were removed by centrifugation at 12,000 × g for 20 min at 4°C, flagella were harvested from the supernatant fraction by centrifugation at 105,000 × g for 3 h at 4°C. The pelleted material gave one major protein band with a molecular weight of 53,000 when analyzed by SDS-polyacrylamide gel electrophoresis, and the material revealed characteristic flagellar structures when viewed by negative staining in the electron microscope.

Pili were isolated from *P. aeruginosa* PAO/DB2 essentially as described by Paranchych et al. (44), but with the omission of the final centrifugation steps on CsCl gradients. Pili isolated in this manner gave a major band at 17 kilodaltons, which reacted strongly in immunoblotting experiments with anti-PAO pili serum. When analyzed by SDS-polyacrylamide gel electrophoresis, the pilin preparation showed a contaminant at 53 kilodaltons attributable to flagelin.

Anti-PEV immunoglobulins. Six New Zealand White rabbits were initially injected intradermally with the polyvalent vaccine (54 μg of protein, 4.2 μg of 2-keto-3-deoxyoctonate acid [KDO]) emulsified in Freund complete adjuvant. Booster injections with Freund incomplete adjuvant were given both subcutaneously and intradermally on days 14 and 28 and then at monthly intervals. Serum was collected and pooled at fortnightly intervals over a period of 5 months. Human anti-PEV serum raised in volunteers was kindly supplied by Wellcome Biotechnology Ltd. Immunoglobulins G and M (IgG and IgM) were isolated and concentrated 10-fold with respect to the original serum concentration as previously described (42a, 55).

Adsorption of rabbit anti-PEV serum with LPS derived from serotype 6 (LPS-6) was achieved by mixing 250 μl of purified and concentrated immunoglobulins with 50 μl of LPS-6 (2 mg/ml) and 150 μl of phosphate-buffered saline (pH 7.4). Incubation was continued for 1 h at 37°C, and the turbid suspension was then held at 20°C for 18 h. Precipitated LPS was removed by centrifugation at 10,000 × g for 5 min. The supernatant fraction was removed and redorsed with an additional 100 μg of LPS-6 as above. Negligible precipitation was observed, and residual LPS was finally removed by centrifugation at 41,000 × g and 4°C for 1 h.

Electrophoretic techniques. CIE was performed at 20°C in 1% agarose (Seakem LE; relative mobility [−m], 0.01 to 0.15; Miles Laboratories, Stoke Poges, United Kingdom) dissolved in CIE running buffer (barbital hydrochloride [pH 8.6] containing 1% [vol/vol] Triton X-100), as previously described (42a, 55). Samples, suspended in 1% Triton X-100-50 mM Tris hydrochloride-5 mM EDTA (pH 8.6), were routinely subjected to electrophoresis at 5.5 V/cm for 75 min in the first dimension and at 2 V/cm overnight (18 to 22 h) in the second dimension. Unless otherwise indicated,
the second-dimension gel contained 4.72 μl of rabbit anti-PEV immunoglobulin per ml (equivalent to 0.78 mg of protein per ml). Gels were washed, pressed, and dried, and the immunoprecipitates were stained with Coomassie brilliant blue as described previously (42a, 55). The method of precipitate excision and analysis used to determine the nature of protein antigens contained within radiolabeled CIE immunoprecipitates has been detailed elsewhere (42, 42a).

SDS-polyacrylamide gel CIE was performed essentially as described by Chua and Blomberg (8) with the following modifications. In the first dimension, duplicate samples containing 75 ng of KDO were subjected to electrophoresis until the dye front reached 9.0 cm into the separating gel. Half of the polyacrylamide gel was then fixed and stained for carbohydrate (16). The other half was washed three times in 200 ml of distilled water (3 times for 15 min each) and once in 200 ml of CIE running buffer (15 min) before excision of the individual lanes (width, 6 mm) for immunoelectrophoresis into antibody. In the second dimension, immunoelectrophoresis was performed on glass plates (106 by 80 by 2 mm) by using the Triton X-100 barbital buffer system routinely used for CIE (see above). Polyethylene glycol was omitted from the antibody gel, which contained 11.32 μl of rabbit anti-PEV immunoglobulins per ml (1.9 mg of protein per ml). After electrophoresis at 4 V/cm for 16 to 18 h, gels were soaked for several hours in distilled water before pressing, washing, drying, and staining with Coomassie brilliant blue (42).

SDS-polyacrylamide gel electrophoresis with 12.5% (wt/vol) polyacrylamide separating gels (32) and 15 protein molecular weight standards (43) and procedures for autoradiography (4) and for silver staining for protein (41) and carbohydrates (16) were performed as described previously. Western blotting was done by the procedure of Burnette (5) with BA85 nitrocellulose membrane filters (pore size, 0.45 μm; Schleicher & Schuell, Dassel, Federal Republic of Germany) and either standard-sized or mini gels (Marysol Ind. Co. Ltd., Tokyo, Japan). Peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories) was used as second antibody, and reacting antigens were visualized by incubating blots in freshly prepared solutions containing 18 ml of 50 mM Tris hydrochloride (pH 7.5), 2 ml of 0.6% (wt/vol) 4-chloro-1-naphthol in methanol, and 10 μl of 30% (100 volumes) H₂O₂.

Electron microscopy. Samples were negatively stained with 1% (wt/vol) potassium phoshgenate state (pH 7.0) and examined in a Hitachi HU-12A electron microscope.

Analytical procedures. Protein content was determined by a modified Lowry procedure (35) with bovine serum albumin as the standard. KDO was assayed by the thiobarbituric acid method after hydrolysis of samples in 0.1 N sulfuric acid for 30 min (30). Carbohydrate was estimated by the phenolsulphuric acid procedure with a glucose standard (15). Nucleic acid was estimated from A₂₆₀ by using an extinction coefficient (E₂₆₀) for a 1-mg/ml solution of 50.8/cm (13).

RESULTS

Biochemical analysis of monovalent extracts. Chemical analyses revealed the presence of various amounts of total carbohydrate, KDO, and protein in all 16 extracts (Table 1). Calculations based on KDO content indicate that about 20% of the cellular LPS was removed from IATS serotype 6 by the extraction procedure. The presence of high levels of LPS was further substantiated by analysis of the extracts by SDS-polyacrylamide gel electrophoresis. When gels were stained for carbohydrate, banding patterns characteristic of heterogeneously sized LPSs (19) were observed with 15 of the 16 extracts (Fig. 1A). The remaining extract (explant 15) contained only two resolvable species. The species close to the track dye was presumed to correspond to core-lipid A. Whether the other high-molecular-weight component corresponds to uniformly sized LPS, free O-antigen chains, or a polymer unrelated to LPS is unclear at present. The banding patterns observed for most of the extracts were unique, and several extracts (e.g., extracts 2, 5, 10, and 13) evidently contained groups of LPS species which fell into two or more discrete size ranges. These highly characteristic profiles appeared to be true reflections of the sizes of the O-antigen repeats (19) and not artifacts caused by LPS oligomers or by the presence of nucleic acid, since they were unaffected by pretreatment of the extracts with 40 mM EDTA or with nuclease or by increasing the concentration of SDS in the polyacrylamide gels from 0.1 to 0.5% (wt/vol). Furthermore, for those serotypes examined (IATS serotypes 1, 2, 5, 6, 8 through 10, 13, 15, and 17), the characteristic banding profile observed for each monovalent extract correlated almost precisely with that obtained for LPS purified from lyophilized cells of the corresponding serotype (not shown). For most extracts, the dominant species of LPS had between 10 and 35 O-antigen repeating units. The similarities in the banding profiles for extracts 7 and 8 may in part reflect the close chemical and antigenic structure of the LPS of these two serotypes (7, 34; see the legend to Fig. 5).

The protein profiles of all 16 monovalent extracts were also analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1B). A major protein with a molecular weight of 16,200 was resolved for 15 of the 16 extracts. It could also be detected in the remaining extract (extract 7) at increased loadings of sample. At least half of the extracts also contained a second protein with a molecular weight of approximately 21,400, and most extracts contained several other minor proteins. From a consideration of protein- and carbohydrate-stained gels (Fig. 1A and B), neither the 16.2- nor the 21.4-kilodalton protein appeared to be glycosylated. A band located close to the dye front was also consistently stained by the protein silver staining procedure (Fig. 1B).

<table>
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<tr>
<th>Extract</th>
<th>Protein (µg/ml)</th>
<th>Total carbohydrate (µg/ml)</th>
<th>KDO (µg/ml)</th>
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<td>2</td>
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<td>50.9</td>
<td>21.6</td>
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<tr>
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<tr>
<td>PEV</td>
<td>72.2</td>
<td>24.2</td>
<td>5.6</td>
</tr>
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</table>

* This value does not include amino sugars, which are not detected by the procedure used to quantitate total carbohydrate (15).
FIG. 1. Carbohydrate and protein profiles of the monovalent extracts. Dialyzed monovalent extracts 1 through 16 were subjected to SDS-polyacrylamide gel electrophoresis, and the fixed gels were stained with silver for carbohydrate (A) and protein (B). For carbohydrate analysis, the applied samples contained 0.7 μg of carbohydrate (0.09 to 0.29 μg of KDO); for protein analysis, samples contained 2.0 μg of protein. Extracts 1 through 16 are analyzed in numerical order as indicated. The banding profile characteristic of smooth LPS becomes more evident for extract 16 (panel A, lane 16) at higher loadings of carbohydrate. The apparent sizes of salient polypeptides are indicated in kilodaltons at the side of the figure.

However, as this band was stained much more intensely for carbohydrate (Fig. 1A) and was not stained by Coomassie brilliant blue, it was assumed more likely to be carbohydrate or lipid in nature than protein.

In addition to the above well-resolved components, the monovalent extracts also contained various amounts of a high-molecular-weight component which remained unresolved towards the top of the separating gel. This material was stained for both protein (Coomassie blue and silver) and carbohydrate (Fig. 1A and B) and, unlike the 16.2-kilodalton protein, could be degraded in part by pronase (data not shown). To ascertain whether this unresolved material was related to treatment of vaccine extracts with Formalin and to assess the likely identity of proteins, unformalinized extracts were prepared from serotype 6 organisms, and their polypeptide profiles were compared with those of formalinized preparations and with those of fractionated membranes, flagella, and pili (Fig. 2). Freshly prepared extract 6 displayed a polypeptide profile considerably more complex than that observed for similar extracts that were stored in Formalin, and the unresolved material observed for the latter preparation can be attributed to Formalin-induced polymerization of most of these proteins (with the apparent exception of the 16.2- and 21.4-kilodalton proteins; Fig. 2, lanes 3 and 4). Similar results were observed for other unformalinized extracts examined (data not shown). The dominant 16.2-kilodalton polypeptide of monovalent extract

purified outer membranes (20 μg of protein); lane 10, purified outer membranes (20 μg of protein) plus unformalinized extract 6 (20 μg of protein); lane 11, purified inner membranes (20 μg of protein). Sizes of the protein standards are indicated in kilodaltons at the side of the figure.

FIG. 2. Comparison by SDS-polyacrylamide gel electrophoresis of formalinized and unformalinized extract 6 and of various subcellular fractions derived from P. aeruginosa IATS serotype 6. Lane 1, Molecular weight standards; lane 2, supernatant fraction obtained after Silverson blending of cells and centrifugation at 105,000 × g for 3 h (35 μg of protein); lanes 3 and 8, unformalinized extract 6 (20 μg of protein); lane 4, formalinized extract 6 (19 μg of protein); lane 5, formalinized extract 6 (19 μg of protein) plus crude pili preparation (10 μg of protein); lane 6, crude pili preparation (10 μg of protein); lane 7, flagella (2.5 μg of protein); lane 9, protein; lane 10, purified outer membranes (20 μg of protein); lane 11, purified inner membranes (20 μg of protein). Sizes of the protein standards are indicated in kilodaltons at the side of the figure.
FIG. 3. Analysis by CIE of the polyvalent *P. aeruginosa* vaccine PEV. A sample of PEV, 15 μL containing 0.07 μg of KDO and 0.89 μg of protein, was subjected to CIE as described in Materials and Methods. Immunoprecipitates are numbered (in multiples of 10) in order of decreasing electrophoretic mobility. Visualization of immunoprecipitates formed by minor antigens 20, 40, and 41 is difficult at these loadings of antigen and antibody. The immunoprecipitates formed by antigens designated 130/140 were heterogeneous and difficult to resolve by CIE. Therefore, for the purpose of this study, they were considered as one (no. 130/140). The anode is to the left and top of the gel.

6 was not sedimentable by centrifugation at 105,000 × g for 3 h (Fig. 2, lane 2) and did not correspond to pilin, as judged by coelectrophoresis experiments (Fig. 2, lanes 4 through 6) and by Western blotting experiments conducted with anti-PAO or anti-PAK pili antibody (data not shown). In addition, flagella did not appear to be a major constituent of the extracts (Fig. 2, lanes 3, 4, and 7), although anti-flagella antibody could be demonstrated in anti-PEV serum by Western blotting (see Fig. 11). Comparison of the polypeptide profiles of unformalinized extracts and isolated outer membrane fractions (Fig. 2, lanes 8 through 10) and studies of heat modifiability (not shown) suggested that some vaccine proteins, notably those with apparent molecular weights of 74,000, 46,000, and 24,500, were probably outer membrane in origin and that the 24,500-molecular-weight protein probably corresponded to protein G* described by Hancock and Carey (20).

Analyses of PEV and monovalent extracts by CIE. The antigenic compositions of PEV and all 16 monovalent extracts were analyzed by CIE. The standard pattern routinely obtained with the polyvalent vaccine, together with the number assigned to each resolved immunoprecipitate, is shown in Fig. 3. Thus, 10 major antigens (no. 50 through 140) and 5 minor antigens (no. 10 through 41) were identified in PEV. Few additional antigens were detected by manipulation of the antibody concentration over an eightfold range, by using agarose of lower endoosmotic flow (−m, < 0.1) or by screening for cathodally migrating antigens (not shown). In contrast to PEV, the monovalent extracts had extremely simple CIE profiles, displaying, under standard conditions, only one or two immunoprecipitates (Fig. 4). The immunoprecipitates formed by the major antigen in at least 10 of the extracts (extracts 1 through 3, 5 through 8, 10, 13, and 16) had similar overall morphologies in their possession of an anodal shoulder or double peak. After application of four to

FIG. 4. CIE profiles of monovalent extracts 1 through 16 (panels 1 through 16, respectively). Volumes of antigen applied were as follows: 15 μL, extracts 4, 9, and 11; 12 μL, extract 14; 10 μL, extracts 12 and 16; 2 μL, all other extracts. Loadings corresponded to 6.4 to 88 μg of KDO and 0.08 to 1.1 mg of protein. Each extract number corresponds to the IATS serotype from which the extract is derived, with the exception that extract 14 is obtained from IATS serotype 17. The anode is to the left and top of all immunoplates.
eight times more antigen, several additional immunoprecipitates, notably one designated no. 41, could be detected with all 16 extracts (see Fig. 9B). The profiles of CIE immunoprecipitates resolved for unformalized extracts resembled those of the corresponding formalinized vaccine extract and were dominated by the same analogous major immunoprecipitin band (see Fig. 9A).

The origins of major antigens in PEV were determined by coimmunoelectrophoresis of PEV with each individual extract. A few representative examples are shown in Fig. 5, and the results for all 16 extracts are summarized in the legend to that figure. Thus, it was demonstrated that six of the extracts (extracts 1, 3, 5, 6, 10, and 13) each contributed to PEV an immunologically distinct antigen. In contrast, the major antigenic components derived from extracts 2 and 16 cross-reacted, as did those from extracts 7 and 8. Interestingly, the major antigen of extracts 2 and 16 not only cross-reacted with antigen no. 50 of PEV but also affected the height of the immunoprecipitate (no. 90) formed by extract 5 (Fig. 5). This may indicate the existence of common determinants between these components. Antigen no. 130/140 was apparently derived from six different extracts, i.e., extracts 4, 9, 11, 12, 14, and 15. This immunoprecipitate, however, was heterogeneous in nature and difficult to resolve with clarity. Further analysis of pairs of these extracts by coimmunoelectrophoresis and immunodiffusion indicated, at best, reactions of only partial identity between their major antigens.

To ascertain whether a similar antibody response is elicited against PEV in humans, the polyvalent vaccine was analyzed by CIE with an intermediate gel containing human anti-PEV immunoglobulins. All of the major antigens precipitated by rabbit anti-PEV immunoglobulins also reacted to some extent with human immunoglobulins (Fig. 6).

**Identification and properties of antigens resolved by CIE.**

The effect of protease, detergent, and heat treatment on the CIE profile of PEV is shown in Fig. 7. Analysis indicated that antigens 50 through 120 were largely resistant to *Streptomyces griseus* protease (Fig. 7B). The anodic shoulders of the immunoprecipitates did disappear after protease treatment, but this was paralleled by an increase in the peak heights of the main immunoprecipitin arcs and may reflect an association between the major antigens and some minor protease-sensitive vaccine component. In contrast, the antigen complex designated no. 130/140 was apparently affected by protease (Fig. 7B). When Triton X-100 was omitted from the buffer system, individual immunoprecipitates were not resolved (Fig. 7C), indicating that these antigens are likely to be membrane derived. In addition, antigens 50 through 120 were shown to be remarkably resistant to heat. After exten-

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**FIG. 5.** Origin of antigens 50 through 140 established by coelectrophoresis. Monovalent extracts 1, 2, 10, 11, and 16 (2 to 10 μl) were analyzed by CIE either alone (Fig. 4) or mixed with 13 μl of PEV (B through F, respectively). The resulting immunoprecipitin profiles were then compared to the control CIE profile of the polyvalent vaccine alone (A). In each case, after coimmunoelectrophoresis, the peak height of a single (arrowed) immunoprecipitate of PEV increased, thus identifying the origin of the corresponding antigen. After similar experiments (data not shown) were conducted with the remaining monovalent extracts, the origins of all major resolved antigens were established as follows: antigen no. 50, extracts 2 and 16; antigen no. 60, extract 10; antigen no. 70, extract 13; antigen no. 80, extract 6; antigen no. 90, extract 5; antigen no. 100, extracts 7 and 8; antigen no. 110, extract 1; antigen no. 120, extract 3; antigen no. 130/140, extracts 4, 9, 11, 12, 14, and 15.

**FIG. 6.** Analysis of human anti-PEV immunoglobulins by CIE with intermediate gel. PEV (0.07 μg of KDO, 0.89 μg of protein) was analyzed by CIE with a main reference gel containing rabbit anti-PEV immunoglobulins (4.72 μl/ml) and either no antibody (A) or 125 μl of human anti-PEV immunoglobulins per ml (B). All immunoprecipitates resolved in panel A are depressed to some extent in panel B, indicating their reaction with the human immunoglobulins. The anode is to the left and top of both gels.
sive boiling, they still retained their ability to bind antibody, although they evidently underwent some physical or chemical alteration which affected their electrophoretic mobilities in agarose (Fig. 7D). Furthermore, this ability to bind antibody was unaffected by extended incubation (18 h at 20°C) of boiled samples with any one of three different proteases, viz., S. griseus protease, staphylococcal V8 protease, and trypsin (not shown).

As the properties of antigens 50 through 120 (viz., their serotype specificity [Fig. 5] and heat and pronase resistance [Fig. 7]) are all reminiscent of LPS, the relationship between resolved antigens and LPS purified from selected serotypes was examined by CIE. LPS preparations from serotypes 1, 2, 5, 6, 8, 10, and 13 were each shown to contain a major antigenic component which shared immunological identity with antigens no. 110, 50, 90, 80, 100, 60, and 70, respectively (Fig. 8). The anodic shoulders of the immunoprecipitates, however, were consistently less prominent for the LPS preparations than for the vaccine extracts. Outer membranes isolated from P. aeruginosa serotype 6 were also analyzed by CIE with rabbit anti-PEV immunoglobulins. One major antigen was precipitated, and this was also identified as LPS (not shown).

The identity of the minor antigen (no. 41) detected for all monovalent extracts at elevated loadings (Fig. 9A and B) was established by the method of precipitate excision and analysis (42, 42a). Thus, CIE analysis of unformalized [35S]methionine-labeled extract 6, followed by excision of the relevant immunoprecipitate (Fig. 9B and C) and analysis by SDS-polyacrylamide gel electrophoresis and fluorography (42, 42a), identified a polypeptide with a molecular weight of 16,200 as the sole protein constituent of the antigen (Fig. 9D, lane 2). Identical results were obtained for the corresponding antigen present in unformalized [35S]methionine-labeled extract 12 (not shown). By the same procedures, two other minor antigens (x and y) present in extract 6 (Fig. 9B) were also identified. Antigen x contained a 42-kilodalton polypeptide (Fig. 9D, lane 3), the presence of the 16.2-kilodalton protein in this preparation being attributed to antigen entrapment (42, 42a). Antigen y, which formed an incomplete immunoprecipitate, contained a single protein (probably protein G*) with a molecular weight of 24,500 (Fig. 9D, lanes 5 and 6).

Analysis of monovalent extracts by SDS-polyacrylamide gel CIE. Antigens which retain or regain their antigenicity after SDS-polyacrylamide gel electrophoresis may be detected by electrophoresis in a second dimension into an antibody-containing gel. By using this technique it was demonstrated that the rabbit anti-PEV serum contained high levels of antibody directed against the major carbohydrate component of each monovalent extract tested (viz., extracts 2, 4, 6,
were present, anti-PEV serum was exhaustively adsorbed with LPS-6. The adsorbed serum was then used in Western blotting experiments to detect reactive antigens in several cellular fractions derived for IATS serotype 6 (Fig. 11). Strong reactions were observed for flagella (Fig. 11, lane 6), the protein with a molecular weight of 16,200 (lane 8), and for three outer membrane components with molecular weights of approximately 38,000, 19,000, and 10,000 (lane 4). The two outer membrane proteins of highest molecular weight we assume to be proteins F and H1/H2 (20). The identity of the 10-kilodalton component is unclear, but it may correspond to protein 1 or to glycolipid. Several other reacting proteins in the 53- to 42-kilodalton range were detected for the Silverston supernatant fraction (Fig. 11, lane 8). Some of these may be related to flagella and to degradation products thereof. It is notable that reactions attributable to pili (Fig. 11, lane 7) or to inner membrane components (lane 6) could not be discerned. Furthermore, analysis of a lysis supernatant containing soluble components of the cell revealed that, of over 55 proteins resolved in this fraction (data not shown), only about 9 reacted with antibody (Fig. 11, lane 9). Of these, only the three with molecular weights in excess of 53,000 could not be attributed to either an outer membrane (lane 4) or a surface origin (lane 8).

It is notable that, of the reacting species documented above, only proteins with molecular weights of 42,000, 16,200, and 10,000 featured prominently in reactions for extract 6 (Fig. 11, lanes 2 and 3). Significantly, both the 42- and 16.2-kilodalton proteins fractionated into surface extracts and not with outer membrane (Fig. 2 and 11). The diffuse staining reactions observed for formalized but not unformalinized extract 6 probably represent interaction of antibody with polymerized components of the extract.

### DISCUSSION

For the following reasons, there seems little doubt that LPS is the major antigenic constituent of most if not all of the 16 monovalent extracts which together constitute the *Pseudomonas* vaccine PEV. First, as judged by their KDO content (Table 1) and carbohydrate profile (Fig. 1A), all extracts contained roughly comparable concentrations of LPS. Second, the major antigen observed for each of the monovalent extracts 1, 2, 5, 6, 8, 10, 12, and 13 shared immunological identity with LPS purified from the corresponding IATS serotype (Fig. 8). Third, the results of SDS-polyacrylamide gel CIE indicated that LPS was a major antigenic constituent for extracts 2, 4, 6, 7, 10, 11, 13, and 14 (Fig. 10). Western blotting confirmed these observations and extended them to include all extracts with the possible exception of extract 15. In addition, the major antigens resolved for PEV by CIE displayed properties of heat and protease resistance anticipated for LPS (Fig. 7). Finally, the major antigens resolvable by CIE for the individual extracts showed immunological interrelationships comparable to those established for the corresponding IATS O-serotypes (7, 34).

Although LPS was clearly the dominant antigen for most of the monovalent extracts, other antigens were present. Notable among these was a 16.2-kilodalton protein which was common to all extracts and which generated CIE immunoprecipitate no. 41. The identity of this protein is unclear at the present time. However, from the following considerations, a relationship with pili (17, 44) appears unlikely. First, cells were grown under conditions (36) which usually suppress the production of pili. Moreover, neither formalized nor unformalinized extracts showed evidence

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**FIG. 9.** Minor antigens of extract 6 and their identification by precipitate excision and polypeptide analysis. Unformalinized [35S]methionine-labeled extract 6 (1-μl [A] and 16-μl [B] samples) was analyzed by CIE against rabbit anti-PEV immunoglobulins (18.9 and 4.72 μU/ml, respectively). Immunoplates in panels A and B were stained with Coomassie brilliant blue. Panel C shows an autoradiogram of the immunoplate in panel B. Antigen no. 80 (LPS) was clearly the dominant antigen for the unformalinized extract (panel A). Minor antigens (no. 41, x, and y), detected at elevated loadings of extract (panel B), were located by autoradiography on unstained dried gels and marked for excision (panel C). Panel D shows an analysis of excised immunoprecipitates by SDS-polyacrylamide gel electrophoresis and fluorography. Lanes 1 and 6, [35S]methionine-labeled extract 6 (0.3 μl); lane 2, immunoprecipitate no. 41; lane 3, immunoprecipitate x; lane 4, immunoprecipitate no. 80; lane 5, immunoprecipitate y. Note that labeled polypeptides cannot be detected for immunoprecipitate 80 (LPS). The sizes of salient proteins are indicated in kilodals at the side of the fluorogram.

7, 10, 11, and 13 through 15). The profiles obtained for eight of these extracts are shown in Fig. 10. In each case, the position and height of the immunoprecipitate corresponded precisely to that predicted from the position and intensity in the polyacrylamide gel of the bands of smooth LPS (or other polysaccharide in the case of extract 15). Little or no immunoprecipitate corresponding to rough LPS or to any of the protein components could be detected. It is worth emphasizing that these results demonstrate that at least four (viz., extracts 4, 11, 14, and 15) of the six monovalent extracts which contributed to the ill-resolved CIE immunoprecipitate no. 130/140 (Fig. 3) possessed LPSs or polysaccharides which readily formed immunoprecipitates in agarose.

**Analysis by Western blotting.** Analysis of all 16 monovalent extracts by Western blotting with rabbit anti-PEV serum generated a profile almost identical to that obtained after silver staining of the extracts for carbohydrate (Fig. 1A). For each serotype, by far the most dominant reactions could be attributed to heterogeneously sized LPS. Few other bands could be discerned among the intensely labeled LPS species (not shown). Since it was important to clearly establish whether antibodies to other *Pseudomonas* antigens

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FIG. 10. Analysis of monovalent extracts by SDS-polyacrylamide gel CIE. Monovalent extracts were analyzed as described in Materials and Methods. Control lanes from the first-dimension polyacrylamide gel were stained for carbohydrate and have been aligned in the correct position with respect to the second-dimension CIE gel. The anode is to the left and the top of all gels. In each case, numbers refer to the extract under investigation.

of pilus structures when viewed in the electron microscope. Second, the 16.2-kilodalton protein had a similar size in all 16 extracts (Fig. 1B) but was clearly distinguishable from PAO pilin by SDS-polyacrylamide gel electrophoresis (Fig. 2). Third, as judged by immunoblotting, anti-PEV serum recognized the 16.2-kilodalton proteins but not PAO pilin (Fig. 11). Conversely, antiserum raised to PAO pili reacted with PAO pilin but not with the 16.2-kilodalton proteins. In addition, the 16.2-kilodalton proteins were not recognized by antiserum raised to PAK pili.

The properties of this 16.2-kilodalton protein and also the 21.4-kilodalton polypeptide, viz., their presence in all or many of the serotypes, their ready removal from the cell surface by mild extraction or by homogenization, their apparent absence from purified outer membranes, their retention in supernatant fractions after centrifugation at 105,000 × g, and the apparent absence of sugar residues, may suggest a relationship with (the proteinaceous moiety of) the slime glycolipid proteins which have been described for this organism (2, 14, 40, 54). An alternative candidate is the so-called original endotoxin protein described by Tanamoto et al. (56). This protein has a molecular weight of about 22,000 and is reported to be a common surface antigen for most serotypes of P. aeruginosa (9, 56).

The antigenic and molecular composition of all 16 monovalent extracts appeared to remarkably simple, a phenomenon which can be attributed, at least in part, to the selective removal of a limited number of cell-surface antigens during extraction with EDTA-glycine buffer. This was not unexpected. Hedstrom et al. (25) found release of LPS and outer membrane proteins D, E, G, and H1 but not F, H2, or I on exposure of sucrose-stabilized P. aeruginosa to Tris-EDTA, and Hancock et al. (21) have documented the release of LPS and protein E under similar conditions. The proclivity of the organism to shed parts of its outer membrane is also underscored in a recent report documenting the spontaneous release of LPS during growth in defined medium (6). However, the simplicity of the polypeptide profiles observed for this vaccine belies to some extent the complexity of the original monovalent extracts. Analyses of freshly prepared unformalized extracts by SDS-polyacrylamide gel electrophoresis, CIE, and Western blotting clearly suggest the presence of outer membrane proteins and flagella within the original extracts. Although these do not seem to be major constituents of the vaccine, some, e.g., flagella and proteins F and H1/H2, appear capable of eliciting significant antibody responses (18, 24, 27). As a consequence, the possible contribution of these components to the protection afforded by PEV should not be ignored.

Formalization appeared to impair analysis of the extracts by causing aggregation of some of the protein components. Aggregation is slow, but it becomes a significant problem after a few months of storage at 4°C (unpublished observations). The effect can probably be attributed to
reaction of the hydrated methylene glycol form of formaldehyde [CH2(OH)2] with free primary amino groups in the proteins. The resultant aminomethylol derivatives can then undergo further reaction with an amide, hydroxy, sulphydryl, guanidinium, or imidazolyl group to function as proteins cross-linked by methylene bridges (53). An important practical consequence of these observations and the fact that polymerization is accelerated by heating at 100°C is the necessity of removing excess Formalin by dialysis before analysis on SDS-polyacrylamide gels; otherwise, additional cross-linkage is effected.

Another factor which may contribute to the time-dependent reduction in complexity of stored vaccine extracts is proteolytic degradation caused by trace amounts of extracellular proteases. However, it should be stressed that the protective capacity of the (formalinized) vaccine is virtually unaffected by prolonged storage (2 to 3 years) at 4 or 37°C (unpublished observations).

Why the major antigens present in extracts 4, 9, 11, 12, 14, and 15 resolved as an ill-defined immunoprecipitate (no. 130/140) during CIE of the polyvalent vaccine is not clear. The results of SDS-polyacrylamide gel CIE clearly showed that, for four of these extracts (extracts 4, 11, 14, and 15), LPS-polysaccharide was a major antigen. Furthermore, other CIE studies of Pseudomonas preparations confirm that LPS can form immunoprecipitates of a morphology very similar to that observed here for antigen 130/140 (26, 33). In the present instance, we suspect that antigen 130/140 represents an unresolved complex of either LPS species or of LPS and protein molecules derived from the six extracts in question. Formaldehyde-induced cross-linking of LPS and protein molecules may play a role here, as it might in the formation of the protease-sensitive anodal leg of some of the LPS antigens when resolved by CIE.

As severe adverse reactions (including malaise, fever, and localized induration and pain) were observed after administration of another LPS-based Pseudomonas vaccine, it may appear remarkable that PEV-01 contains LPS as the major antigen but yet elicits few toxic side effects (52). The reason for this apparent discrepancy most likely related to the CIE studies of LPS preparations. Assuming, as we have found, that KDO represents 2.0 to 4.8% of the dry weight of P. aeruginosa LPS (see also references 12 and 31), then one human dose of PEV contains at most about 30 μg of LPS. In contrast, approximately 1.75 mg of LPS was administered to 70-kg patients with each dose of the heptavalent LPS vaccine Pseudogen (1). In additions, as the high-molecular-weight polysaccharide described by Pier and co-workers (46, 49) shares immunological identity with the O-antigenic side chains of LPS but contains no lipid A, it is capable of eliciting an anti-LPS antibody response with no toxic side effects. Although this surface polysaccharide is approximately 1,000-fold less immunogenic than intact LPS (11), the possibility that it may have a role in PEV-induced immunity cannot be excluded and is now under investigation. Certainly, a major antigenic epitope elicited in human volunteers immunized with PEV seemed to be directed against the LPS components of this vaccine (Fig. 6). However, it remains to be established whether LPS can account for some or all of the protection afforded by PEV and what role, if any, is played by other constituents of the vaccine. Also, in the light of results presented herein, a careful comparison of the relative efficacies and toxicities of two vaccine products, PEV and Pseudogen, would appear to be warranted.


