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Outer Membrane Protein F Preparation of *Pseudomonas aeruginosa* as a Vaccine against Chronic Pulmonary Infection with Heterologous Immunotype Strains in a Rat Model

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Outer membrane protein F (porin) was purified by extraction from polyacrylamide gels of cell envelope proteins of the *Pseudomonas aeruginosa* PAO1 strain. Rats were immunized intramuscularly with 25 μg of protein F on days 1 and 14 and then challenged on day 28 via intratracheal inoculation of bacterium-containing agar beads. On day 35 the lungs were either fixed for histological examination or submitted for quantitation of the bacteria present. Protein F immunization afforded significant protection against challenge with six of six heterologous lipopolysaccharide immunotype strains of *P. aeruginosa*. By an enzyme-linked immunosorbent assay, the protein F-immunized rats had both immunoglobulin G and M antibody responses to cell envelopes of all six of the heterologous immunotype strains. Protein F immunization greatly enhanced the ability of the rats to clear the inoculated *P. aeruginosa* from the lungs and significantly reduced the incidence and severity of pulmonary lesions as compared with those in bovine serum albumin-immunized control rats. These data show the efficacy of outer membrane protein F as a protective vaccine in a rat model of chronic pulmonary infection.

Pseudomonas aeruginosa is the principal cause of life-threatening chronic pulmonary infection in patients with cystic fibrosis (CF) (22) and has been called the "harbinger of death" in children with CF (19). Therapy of *P. aeruginosa* chronic pulmonary infection in CF patients is difficult, with conventional antibiotic chemotherapy virtually never succeeding in eliminating the organism from the infected lungs (10). Achievement of effective immunotherapy of *P. aeruginosa* pulmonary infection in CF patients would represent a major breakthrough in the therapy of these patients. However, two currently available commercial *P. aeruginosa* vaccines, both of which are based on the cell wall lipopolysaccharide (LPS) antigens (8, 10), have been reported (12, 18) to have no apparent benefit when administered to CF patients. Successful immunotherapy would appear to await the development of alternative vaccines for *P. aeruginosa*.

For the past several years we have been conducting studies into the vaccine potential of outer membrane protein F of *P. aeruginosa*. Protein F is the major porin protein of the outer membrane, is exposed on the cell surface (11, 16), and is conserved and antigenically related in all serotype strains (17) of *P. aeruginosa*. We previously reported that a purified protein F (porin) preparation used as a vaccine to actively immunize mice afforded significant protection in both an acute infection model (5) and a burn model (15) from subsequent challenge with strains of *P. aeruginosa* of heterologous LPS immunotypes. The results reported in this paper demonstrate the ability of a purified protein F (porin) preparation to protect rats against challenge with each of the six heterologous LPS Fisher-Devlin immunotypes in a rat model of chronic pulmonary infection established upon intratracheal challenge with bacteria encased in agar beads. This rat model for chronic *P. aeruginosa* lung infection is the best available animal model to represent chronic pulmonary *P. aeruginosa* infection in CF patients.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *P. aeruginosa* used included the following: the PAO1 strain (Difco O-5; Difco Laboratories, Detroit, Mich.) that corresponds to a Fisher-Devlin immunotype 7, ATCC 27584 (Difco O-6, Fisher-Devlin immunotype 1), ATCC 27313 (Difco O-11, Fisher-Devlin immunotype 2), ATCC 27314 (Difco O-2, Fisher-Devlin immunotype 3), ATCC 27315 (Difco O-1, Fisher-Devlin immunotype 4), ATCC 27585 (Difco O-10, Fisher-Devlin immunotype 5), ATCC 27579 (Difco O-7,8, Fisher-Devlin immunotype 6), ATCC 27318 (which upon receipt was typed as a Difco O-untypable strain), and H296 (Difco O-5, Fisher-Devlin immunotype 7). All ATCC strains were obtained from the American Type Culture Collection, Rockville, Md. The H296 strain was a gift from R. E. W. Hancock (Vancouver, British Columbia). All strains were grown at 30°C with stirring in nutrient broth (Difco).

Purification of protein F. Protein F was purified only from the PAO1 strain by the gel-extraction method of Kabir (9), modified as previously described (5). Cell envelopes were harvested, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (5). Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell envelope proteins, undenatured protein F migrates at 36 kilodaltons, whereas denatured protein F migrates at 39 kilodaltons and thus is referred to as F′ (6). Our purified, gel-extracted protein F usually migrated in the F′ position. Protein determinations were performed by the Lowry method as modified by Markwell et al. (14). The 2-keto-3-deoxyoctulosonic acid content was assayed by using the method of Dröge et al. (3). Various protein F preparations used for immunization contained 1.5 to 4.0% protein. An immunizing dose of protein F preparation containing 25 μg of protein contained 1.7 μg of contaminating LPS.

LPS extraction. LPS was extracted from all nine strains

used in the enzyme-linked immunosorbent assays (ELISAs) by using the phenol-water extraction method of Westphal and Jann (21).

ELISA. The ELISA was performed as previously described (15). The immune response after outer membrane protein F immunization was monitored by ELISA in the following manner. Ten rats were actively immunized with protein F (see below) on days 1 and 14 and then bled by cardiac puncture on day 28, and the sera were pooled. These antisera from protein F-immunized rats were titered against the purified protein F preparation and against the cell envelopes and purified LPS from the six heterologous Fisher-Devlin immunotypes, the untypable rough LPS mutant, the homologous Fisher-Devlin immunotype 7 strain H296, and the PAO1 strain to determine the immunoglobulin M (IgM) (μ-chain specific) and the IgG (γ-chain specific) antibody responses. Normal sera were obtained by cardiac bleeding of 10 unimmunized rats. The antibody titers were confirmed in three separate ELISA determinations.

Active immunization of rats. Young adult (200- to 225-g) female, specific-pathogen-free Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were routinely immunized on days 1 and 14 by intramuscular injection into alternate hips. Rats designated as F immunized received 0.2 ml of sterile saline containing a protein F preparation dose corresponding to 25 μg of sterile, purified protein F. Rats designated as control received 0.2 ml of sterile saline containing 25 μg of bovine serum albumin. In one experiment, rats were immunized with 0.2 ml of sterile saline containing 7 μg of purified PAO1 LPS on days 1 and 14.

Chronic pulmonary infection model. The method of Klinger et al. (10) was used with modification. Slurries of Ionagar beads containing approximately 10^6 CFU of the challenge organism per ml were prepared as follows. A 25-ml portion of a 50°C mixture containing approximately 1 ml of an 18-h nutrient broth culture and 30 ml of 2% Ionagar in phosphate-buffered saline (PBS), pH 7.2, was forcefully pipetted into a 600-ml beaker containing 150 ml of heavy mineral oil at 50°C. This mixture was stirred rapidly at 50°C with a Teflon-coated stir bar for approximately 2 min to form an emulsion. The oil mixture was transferred to another magnetic stirrer; while the mixture continued to be rapidly stirred, crushed ice was added to the outside of the beaker, and the emulsion was allowed to cool for 5 min. After this cooling period, the oil-Ionagar mixture was poured into a 500-ml separatory funnel containing 150 ml of 0.5% sodium desoxycholate in PBS. Large agar beads settled out rapidly and were collected and discarded. The smaller beads were allowed to settle over 20 to 30 min and then were collected. The oil-detergent mixture was poured out of the funnel, and the funnel was rinsed two times with PBS to remove any residual oil. The collected agar beads were returned to the funnel and then washed with 150 ml of 0.25% sodium desoxycholate, followed by two 200-ml washes with PBS. With each wash, the first layer of beads to settle was discarded as representing the larger beads in that bead sample. The final slurry of bacteria-containing Ionagar beads consisted of beads of a fairly uniform size (mean diameter, 100 to 150 μm). A 0.1 ml amount of the washed slurry was homogenized in 4.9 ml of PBS with a Ten Broeck tissue grinder and then serially diluted and spread on nutrient agar plates to confirm actual bacterial counts.

Two weeks (day 28) after the second immunizing dose of protein F or bovine serum albumin, the rats were challenged with the bacteria-containing agar beads. The rats were first anesthetized with an intraperitoneal injection of 0.175 ml of sodium pentobarbital (25 μg/g of body weight) and then inoculated via a tracheal incision with 0.05 ml of bead slurry containing 10^4 to 10^5 CFU with a curved, beaded-tip 20-gauge needle. The needle was gently guided to favor inoculation of the left lung. The incision was closed with sterile wound clips. Afterwards the rats were housed in individual wire-bottom cages to reduce the likelihood of wound infection.

Seven days after challenge (day 35), the rats were sacrificed by an overdose of halothane (Ayerst Laboratories, Inc., New York, N.Y.). In one group of rats (both control and protein F immunized), both lungs were aseptically removed and homogenized in a 1:10 (wt/vol) dilution of sterile PBS with a Ten Broeck tissue grinder. Dilutions of these lung tissues were plated onto nutrient agar for quantitative determination of the numbers of *Pseudomonas* organisms present. Identification of organisms recovered from the lung homogenates as being the challenge strain of *P. aeruginosa* was confirmed by Gram stain, oxidase reaction, and serotyping. In matched animals, the tracheas were cut just below the larynx, and the heart-lung block was removed for histological examination. Lungs were inflation fixed with 10% buffered Formalin. Before removal of lungs from both groups of rats (for quantitation of organisms or for histological examination), the lungs were observed macroscopically for the presence of lesions and scored 0 to 4+, depending on the presence and severity of the lesion(s). After Formalin fixation, the lungs were sectioned from the area showing lesions, or, in lungs showing no obvious pathology, sections were made from the bottom of the left lung (the site of inoculation). After paraffin infiltration, tissue blocks were cut into sections of 5-μm thickness. Several sections from different depths into the tissue were picked up onto three or four separate slides. These slides were stained by the standard hematoxylin-eosin procedure. All sections on each slide were examined microscopically (a minimum of 20 to 30 fields).

Statistics. Fisher's exact test was used to calculate two-tailed *P* values for significant levels of protection. Values of ≤ 0.05 were considered significant.

RESULTS

Rat immune response after protein F immunization. The IgG and IgM immune responses in protein F-immunized rats were determined for day 28, the day of challenge in the active immunization protection experiments. A summary of the individual IgG and IgM antibody titers in antisera from protein F-immunized rats against the protein F preparation and against cell envelopes and purified LPS from the six heterologous Fisher-Devlin immunotypes, the untypable rough *P. aeruginosa* strain, the homologous Fisher-Devlin 7 strain, and the PAO1 strain is given in Table 1. On day 28 these antisera had an IgG antibody titer of 80 but no detectable titer of IgM antibody, to the protein F preparation. Protein F-immunized rats had a detectable IgG response to four of the six heterologous immunotype purified LPSs and a minimal IgM response to four of the six heterologous immunotype LPSs. The use of purified LPS as the test antigen allowed us to determine whether antibodies to LPS were present in the antisera of protein F-immunized rats. However, this reactivity to heterologous immunotype LPS is most likely due to epitopes exposed in the core region of the purified LPS, and its ability to play any meaningful role in protection against challenge with smooth, whole cells of heterologous immunotype is doubtful. More indicative of

TABLE 1. Cross-reactivity of antisera from day 28 protein F-immunized rats

Antigen	IgG titer	IgM titer
Protein F	80	0
27584 CE[a] (FD 1)[b]	160	20
27313 CE (FD 2)	320	20
27314 CE (FD 3)	640	40
27315 CE (FD 4)	320	40
27585 CE (FD 5)	640	20
27579 CE (FD 6)	640	10
27318 CE (untypable)	5,120	160
H296 CE (FD 7)	5,120	160
PAO1 CE (FD 7)	5,120	320
27584 LPS (FD 1)	0	0
27313 LPS (FD 2)	0	5
27314 LPS (FD 3)	320	0
27315 LPS (FD 4)	10	5
27585 LPS (FD 5)	40	5
27579 LPS (FD 6)	20	5
27318 LPS (untypable)	5,120	80
H296 LPS (FD 7)	2,560	160
PAO1 LPS (FD 7)	10,240	320

[a] CE, Purified cell envelopes.
[b] FD, Fisher-Devlin immunotype.

protective antibodies is the reaction obtained against cell envelopes of the heterologous immunotype strains. Protein F-immunized rats had both an IgG and an IgM response to all six of the heterologous immunotype cell envelopes. These data suggest that protein F-immunized rats produce a significant immune response which is capable of reacting with cells of the heterologous immunotype strains. That this response is due to the protein in the protein F preparation is indicated by the fact that a reactive antibody titer was obtained to several of the heterologous immunotype cell envelopes in the absence of any antibody titer to those immunotype LPSs.

Macroscopic evaluation of lung lesions in control versus protein F-immunized rats. With the rat chronic pulmonary infection model we saw that statistically significant protection against severe lung lesions was afforded to the protein F-immunized rats challenged with six of six heterologous LPS immunotype strains of *P. aeruginosa* as compared with control rats (Table 2). The scoring of lung lesions from 0 to 4+ could be accomplished readily (Fig. 1). Klinger et al. (10) previously reported that macroscopic changes evident in infected lungs made them readily distinguishable from uninfected lungs in their study as well. In rats immunized with 7-μg doses of purified PAO1 LPS, protection against severe lung lesions (2+ to 4+) was afforded when rats were challenged with the homologous immunotype 7 H296 strain (3 of 10 LPS-immunized rats with severe lung lesions versus 9 of 10 control rats; $P = 0.02$) but not when rats were challenged with the heterologous immunotype 2 strain 27313 (9 of 10 LPS-immunized rats with severe lung lesions versus 8 of 10 control rats; $P = 0.5$).

Quantitation of bacteria in lungs on day 35. In 35 of 59 (59%) of the lung homogenates from the protein F-immunized rats submitted to bacterial quantitation, no growth of organisms resulted. In contrast, only 14 of 60 (23%) of the lung homogenates from control rats yielded no bacterial growth. Thus, protein F-immunized rats were significantly ($P = 0.00008$) more efficient in clearing the inoculated *P. aeruginosa* from their lungs compared with the control rats. Of the lung homogenates yielding bacterial growth, the mean bacterial counts per gram of lung tissue were 4.9×10^5 for the control rats and 8.6×10^4 for the protein F-immunized rats, which represents less than a sixfold difference in the mean bacterial count between infected lungs in the two groups of rats. There was no significant difference in bacterial counts between lungs with differing severities of lesions; i.e., lungs with 1+ lesions contained the numbers of bacteria equivalent to those in lungs with 4+ lesions. This was true for both control and F-immunized rats. Thus, whereas protein F immunization resulted in a significant increase in the number of rats which had completely cleared their lungs of inoculated organisms, in those protein F-immunized rats whose lungs remained colonized there was no significant

TABLE 2. Macroscopic evaluation of lung lesions in control versus protein F-immunized rats

Rat group	Challenge strain	No. with lung lesions[a]					No. 2+ to 4+/total	P^b
		0	1+	2+	3+	4+		
Control	27584 (FD 1)[c]	2	4	6	5	2	13/19	
F immunized	27584 (FD 1)	14	2	1	1	0	2/18	0.0006
Control	27313 (FD 2)	5	5	11	8	1	20/30	
F immunized	27313 (FD 2)	22	3	1	1	2	4/29	0.00005
Control	27314 (FD 3)	2	4	2	6	5	13/19	
F immunized	27314 (FD 3)	9	4	5	1	0	6/19	0.050
Control	27315 (FD 4)	1	1	6	4	1	11/13	
F immunized	27315 (FD 4)	6	3	1	2	1	4/13	0.015
Control	27585 (FD 5)	3	4	14	0	0	14/21	
F immunized	27585 (FD 5)	11	9	6	0	0	6/26	0.0036
Control	27579 (FD 6)	1	0	2	3	14	19/20	
F immunized	27579 (FD 6)	4	5	7	5	0	12/21	0.009

[a] Lung lesions were scored as follows: 0, normal-appearing lung with no visible lesion; 1+, one or two small lesions not exceeding 1 mm in diameter; 2+, three to eight small lesions or medium lesion of 2 to 5 mm; 3+, two or more medium lesions or large lesion exceeding 5 mm; 4+, lesions covering at least one-fourth of lobe.
[b] P values ≤.05 were considered statistically significant. P values were determined by Fisher's exact test.
[c] FD, Fisher-Devlin immunotype.

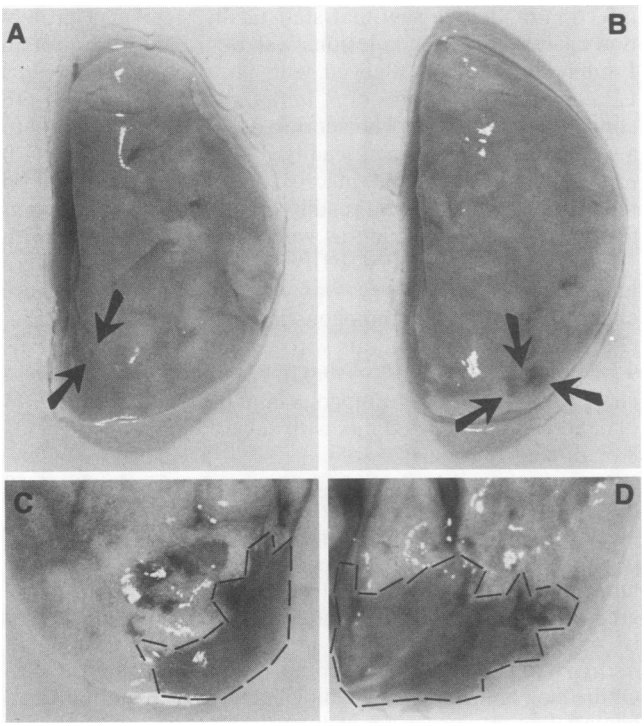

FIG. 1. Macroscopic evaluation of lung lesions. (A) Lung from protein F-immunized rat showing a 1+ lesion (arrows); (B) lung from control rat showing a 2+ lesion (arrows); (C) lung from control rat showing a 3+ lesion (dashed lines); (D) lung from control rat showing a 4+ lesion (dashed lines).

reduction in the number of organisms present as compared with those in the colonized lungs of control rats.

Histological evaluation of lung sections. The histological findings upon examination of sections of fixed lungs agreed exceedingly well with our macroscopic evaluation of the lungs (Table 2). In all cases in which macroscopic evaluation of the lungs resulted in a score of 2+ or greater, the sections of these lungs revealed extensive inflammation resulting in nearly complete obliteration of the normal airway and alveolar structures in the lung (Fig. 2). In contrast, the vast majority of lungs scored as 0 had no pathology visible in the sections but had the appearance of normal lung tissue (Fig. 3A). An occasional lung scored as 0 by macroscopic evaluation was found to have very minimal cellular influx and negligible alveolar changes. Lungs scored as 1+ were predominantly found to have inflammatory responses localized around the bacteria-containing beads, with some thickening of alveolar walls (Fig. 3B). The differences in appearance between sections of severely inflamed lungs (Fig. 2) and sections from lungs scored as 0 to 1+ (Fig. 3) were so striking that differentiation between the two categories was straightforward. Our findings from the histological examination of lung sections agreed completely with the findings of Klinger et al. (10) regarding the changes seen in sections of severely inflamed lungs.

DISCUSSION

The major finding of this study is that active immunization of rats with a protein F preparation affords protection against subsequent challenge with strains of a heterologous immunotype in a rat model of chronic pulmonary infection with

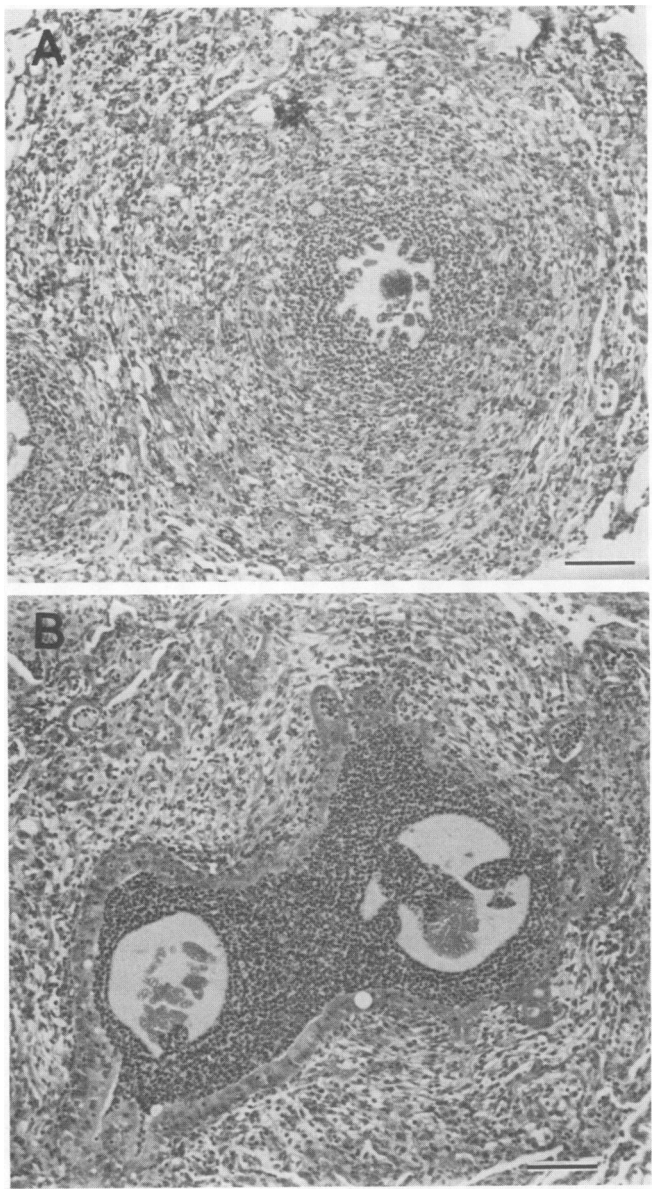

FIG. 2. Hematoxylin-eosin-stained sections of lungs from control rats. (A) Section from lung scored as 2+; (B) section from lung scored as 3+. Note in both sections an intense inflammatory response has resulted in nearly complete obliteration of the normal airway and alveolar structures of the lung. Polymorphonuclear leukocytes and alveolar macrophages are concentrated around the two agar beads in each section. Bars, 100 μm.

agar-encased *P. aeruginosa* that is representative of pulmonary infection by *P. aeruginosa* in patients with CF. Protein F-immunized rats had a greatly enhanced ability to clear the inoculated bacteria-containing agar beads from their lungs compared with the control rats, and the incidence and severity of lung lesions was significantly reduced in the protein F-immunized rats (Table 2) after challenge with each of the six strains with heterologous Fisher-Devlin immunotypes.

Protein F-immunized rats had both IgG and IgM antibodies, which reacted with the cell envelopes of all six heterologous immunotype strains (Table 1) on the day of challenge in the protection experiments. There are several lines of

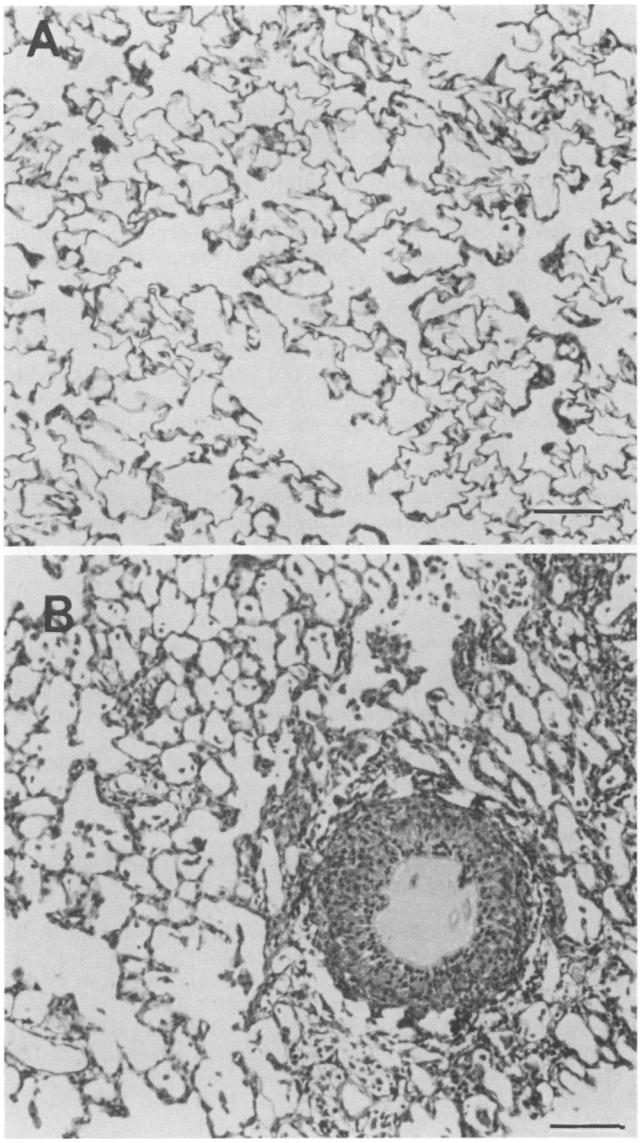

FIG. 3. Hematoxylin-eosin-stained sections of lungs from rats immunized with protein F. (A) Section from lung scored as 0 showing normal lung architecture with very minimal cellular influx and negligible alveolar changes; (B) section from lung scored as 1+ showing mild cellular influx concentrated around the agar bead with some thickening of alveolar walls evident. Bars, 100 μm.

evidence to indicate that these reactive antibodies are directed toward the protein in the protein F preparation and not toward any contaminating LPS. First, antisera from protein F-immunized rats were capable of reacting with cell envelopes of several heterologous immunotype strains in the absence of any detectable reactivity with purified LPS from these strains. (Antibodies that did react with the other heterologous purified LPSs would be expected to be directed to core epitopes on the LPS which would not be expected to be exposed on the surface of cell envelopes or the whole cell. Such antibodies should be able to bind to purified LPS but not to the cell surface of smooth cells.) Second, active immunization of rats with 7-μg doses of PAO1 LPS (four times the LPS concentration contained in an immunizing dose of the protein F preparation) afforded immunotype-specific protection but failed to protect against challenge with a heterologous immunotype strain. Third, Cryz et al. (2) failed to demonstrate cross-immunotype protection against multiple heterologous immunotype strains of *P. aeruginosa* after active immunization with both smooth and rough LPS from *P. aeruginosa* and concluded that protection elicited by active immunization with *P. aeruginosa* LPS "is predominantly serotype specific." We know of no published study that has shown that active immunization with *P. aeruginosa* LPS of a given immunotype can elicit antibodies that protect animals against challenge with smooth strains of multiple heterologous immunotypes. Thus, we conclude that protein F has the ability to elicit an immune response which is capable of providing protection against challenge with each of the six heterologous Fisher-Devlin immunotype strains in this rat model of chronic pulmonary infection.

On a theoretical basis, protein F would appear to have much more potential as a candidate vaccine antigen than do LPS antigens for use in patients with CF. The prevailing view (13, 20) is that colonization of the lungs of CF patients is initiated upon contact from environmental contamination with nonmucoid, LPS smooth strains expressing the immunotype-specific O antigen. However, upon interaction with the CF lung and host defenses, the colonizing strain loses its O antigen to become an LPS rough strain (7, 13, 20) and later begins to produce alginate to become a mucoid strain. An LPS vaccine could offer protection against the colonizing strain upon initial contact but theoretically would lose its effectiveness when the strain lost its O antigen to become a rough strain. A protein F vaccine, on the other hand, not only would protect against the colonizing strain upon initial contact but also would continue to protect when the strain converted to a rough strain. Another candidate vaccine antigen is alginate, the major component of the mucoid material from mucoid strains. However, a study of its use as a vaccine in the rat model of chronic pulmonary infection with *P. aeruginosa* indicated that alginate is most likely not a good candidate as a protective immunogen (24). Once the CF patient has become colonized by *P. aeruginosa*, administration of any *P. aeruginosa* vaccine is apt to be of little benefit and may actually be deleterious due to the formation of immune complexes (4, 19, 24). Administration of a *P. aeruginosa* vaccine to CF patients before colonization of their lungs with *P. aeruginosa* to prevent subsequent colonization by *P. aeruginosa* appears to be the best strategy for effective immunotherapy. We believe that protein F has the most potential of all of the available antigens of *P. aeruginosa* to be capable of providing protective immunity in this clinical situation.

This study provides evidence that a protein F vaccine is capable of protecting against pulmonary infection by *P. aeruginosa* in an animal model. The continued development of protein F as a possible human vaccine for use in CF patients is clearly appropriate. Human trials of a protein F vaccine will necessitate purifying large quantities of protein F free from impurities. The recent cloning of the gene for protein F (23) should assist in this purification. There is also the need for caution in extrapolating successful protection in the rat model to guarantees of success with human patients. The PEV-O1 vaccine protected in this rat model against lung pathology (10) but was subsequently reported to be of little benefit when used in CF patients (12). However, it is worth noting that, unlike our protein F vaccine, the PEV-O1 vaccine did not result in bacterial clearance from the lungs of the immunized rats (10). It was postulated (10) that at least part of the pulmonary protection in PEV-O1-immunized rats was due to the presence of protein toxins serendipitously

included in the vaccine preparation. The inclusion of toxoids of exotoxin A and elastase might be a means to substantially enhance the effectiveness of a protein F vaccine. Both exotoxin A (1) and elastase (26) are produced by the majority of strains of *P. aeruginosa*, and both have been shown (25, 26) to contribute to pathogenesis in chronic lung infections. A component vaccine containing protein F, elastase toxoid, and exotoxin A toxoid might provide twofold protection against *P. aeruginosa* pulmonary infection by eliciting antibodies capable of attacking the bacterial cell surface and capable of inactivating two of the virulence factors thought to mediate tissue damage in the lung. Certainly, such a component protein F vaccine represents an attractive candidate for further study as a *P. aeruginosa* vaccine for possible future use in therapy of CF patients. The favorable results with a protein F vaccine obtained in the present study indicate that further development of a protein F vaccine for use in CF patients to prevent colonization of their lungs with *P. aeruginosa* is warranted.

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