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Protective Immunization Against Chronic *Pseudomonas aeruginosa* Pulmonary Infection in Rats

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Rats were immunized systemically with various doses of the polyvalent *Pseudomonas aeruginosa* vaccine PEV-01. After a series of two or three doses (25 to 50 μg each) at 8- to 11-day intervals, animals were challenged intratracheally by the agarose bead technique with a serotype 5 *P. aeruginosa* strain at periods of 9 to 42 days. Immunized animals developed circulating antibodies (primarily immunoglobulin M) against vaccine components at levels significantly higher than challenged, nonimmunized controls ($P < 0.005$). Eight to ten days postinfection, histological sections of lungs from immunized animals showed only minimal inflammation associated with infectious foci (agarose beads) as compared with the extensive pathological changes of airways and parenchyma seen in infected nonimmunized control animals. However, no significant reduction in bacterial numbers was observed. Such protection lasted at least 6 weeks after the final immunization. It is speculated that the vaccine may contain components of cell surface proteins and virulence exoproducts.

Pseudomonas aeruginosa continues to be the principal cause of severe (ultimately life-threatening) chronic pulmonary infections in patients with cystic fibrosis (CF) (9, 40). Conventional combination aminoglycoside-semisynthetic penicillin chemotherapy is of clinical value but is virtually never adequate for elimination of the infecting organisms. Previously, the efficacy of new chemotherapeutic or immunological approaches to *Pseudomonas* pulmonary infection has been difficult to evaluate. However, as an animal model for chronic *P. aeruginosa* lung infection recently has become available (4), it was important to determine the effectiveness of a new vaccine currently under commercial development. Active immunization (1, 6, 30) and passive immunotherapy (25) with lipopolysaccharide (LPS) vaccines have been attempted in a number of patient groups (e.g., burn, leukemia, and intensive-care patients) with variable outcome. A small number of older CF patients with existing *Pseudomonas* pulmonary infections were immunized with a polyvalent *P. aeruginosa* LPS vaccine (Warner Lambert/Parke, Davis & Co.) with no apparent benefit (7, 30). This vaccine was described as a mixture of chemically extracted LPS from the five most common immunotypes of *P. aeruginosa* (7). Local and systemic adverse reactions in response to this preparation have been reported by most investigators. Pennington and Miler (27) have shown that a less toxic cell surface vaccine (PEV-01) protects guinea pigs against acute overwhelming pulmonary challenge with *P. aeruginosa*. Additionally, the heptavalent LPS vaccine has been shown effective in protecting against chronic *Pseudomonas* pulmonary infection in guinea pigs (27). Homma et al. have shown some protection of mink against naturally occurring or experimental (12) acute *P. aeruginosa* hemorrhagic pneumonia by using broadly reactive original endotoxin protein preparations. Recently, original endotoxin protein and toxoids of *Pseudomonas* exotoxin, combinations of protease, and elastase have been used in limited trials among select adult patients having a variety of chronic pulmonary diseases complicated by various degrees of colonization and infection by *P. aeruginosa* (A. Tachibana, K. Suzuki, K. Nakata, H. Okano, and H. Tanimoto, Proc. Jpn. *Pseudomonas aeruginosa* Soc. **14**:14, 1980).

Because of problems of toxicity, native LPS vaccines will probably have very limited use in active immunization of susceptible patient populations. In this study, we used the recently developed vaccine PEV-01 (24). The safety of this multivalent *P. aeruginosa* vaccine was demonstrated in human volunteers (16), and its lack of toxicity and efficacy were shown in trials in infected burn patients in India and Great Britain (14, 15). We report here successful protection

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against chronic pulmonary challenge with *P. aeruginosa* in rats immunized with the less toxic multivalent cell surface extract vaccine, PEV-01.

(A preliminary portion of this work was presented at the 1981 Annual Meeting of the American Society for Microbiology [J. D. Klinger and H. A. Cash, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B14, p. 17].)

MATERIALS AND METHODS

Infection model. The method of Cash et al. (4) was used with slight modifications to establish experimental pulmonary infections in young adult (150 to 250 g) barrier-sustained male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Random lots of these animals were screened by the supplier for antibody against *Mycoplasma pulmonis*; only groups showing no evidence of mycoplasma infection were used in these studies. These animals were isolated from other rats and housed in wire-bottom cages after infection. Slurries of agarose spherules 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 0.05 ml of an 18-h tryptic soy broth culture and 50 ml of 2% agarose in phosphate-buffered saline (PBS) (pH 7.2) (FTA buffer; BBL Microbiology Systems, Cockeysville, Md.) was forcefully pipetted into a 600-ml beaker containing 150 ml of light paraffin oil at 50°C; the oil was stirred rapidly with a 50-mm Teflon-coated stirring bar. This mixture was stirred at 22°C for approximately 6 min to form an emulsion and then slowly cooled by the addition of crushed ice to the outside of the beaker over the next 7 to 10 min. After this short cooling period, the oil-agarose mixture was poured into 500-ml separatory funnels containing 150 ml of 0.5% sodium desoxycholate in PBS. Bacteria-containing agarose beads (mean diameter, 100 to 150 μm) settled out rapidly, and the oil layer was removed by aspiration. The washing process was repeated in 0.25% sodium desoxycholate and finally through three 200-ml changes of PBS. A 1-ml amount of the washed slurry was homogenized in 49 ml of PBS with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, N.Y.) and then serially diluted and spread on tryptic soy agar plates to confirm actual bacterial counts.

Rats were lightly anesthetized with ether and inoculated via tracheal incision with 0.05 to 0.06 ml of bead slurry containing 10^4 to 10^5 CFU with beaded-tip 20-gauge needles. The needle was gently guided to favor inoculation of the left lung. The challenge strain of *P. aeruginosa* (strain JR-1) used in this study was an International serotype 5 (typing sera from Difco Laboratories, Detroit, Mich.) originally isolated from the sputum of a 5-year-old CF patient in San Antonio, Tex. On original isolation, JR-1 was characterized as having mucoid colony morphology; however, as used in this study, it had classical smooth colony morphology (39). Strain JR-1 was chosen from several CF isolates and in an earlier study was shown to produce extracellular protease (primarily elastase); exotoxin A synthesis was demonstrated by reaction of concentrated culture supernatants against reference antitoxin in a microimmunodiffusion assay (J. D. Klinger, D. C. Straus, and J. A. Bass, Am. Rev. Respir. Dis. **119**:253, 1979).

Animals were sacrificed by exsanguination via cardiac puncture after sodium pentobarbital anesthesia. Both lungs were removed with sterile technique and homogenized in 50 ml of PBS for quantitative bacteriology. Identification of organisms recovered from lung homogenates 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% Formalin in PBS. After fixation, samples were sectioned and stained by standard hematoxylin-eosin procedures. Coded sections were assessed for relative extent of inflammation (alveolar and interstitial), alteration of bronchial epithelia, focal hemorrhage, necrosis, fibrosis, and abscess formation. A minimum of 10 to 20 fields per section were viewed to obtain a representative analysis of uniform tissue alteration. Protection was assumed only when a majority of histological sections from infected (i.e., bead-containing) lobes showed absence of such pulmonary pathology. Serum from both groups of animals were assayed for anti-*Pseudomonas* antibody.

Immunization and antibody assay. Multivalent cell extract vaccine (lot PEV-01; The Wellcome Research Laboratories, Beckenham, Kent, England) was provided in vials containing 525 μg of lyophilized bacterial extract. This material represents approximately equal portions of EDTA-glycine extracts from each of 16 *P. aeruginosa* serotypes (including Habs serotypes 1 to 12, Veron type 13, Meitert type 10, and Homma types 11 and 13) (24). The contents of each vial were dissolved in 2.5 ml of sterile saline and used for intramuscular immunization of rats. Rats were immunized by intramuscular injection of 0.1 to 0.25 ml of vaccine containing 21 to 52.5 μg of PEV-01. Immunization schedules ranged from two to four injections at 7- to 10-day intervals followed by periods of 10 to 42 days before intratracheal challenge, as shown in Table 1.

Anti-*Pseudomonas* antibody was measured in serum heated at 56°C for 30 min by passive hemagglutination (HA) of sheep erythrocytes (SRBC) sensitized with either PEV-01 vaccine (13, 29) or trichloroacetic acid-extracted serotype-specific *P. aeruginosa* LPS (38). Strains representing all International serotypes were kindly provided by P. V. Liu, University of Louisville Health Science Center. HA assays with vaccine-sensitized SRBC were also repeated on dithiothreitol-treated sera to differentiate immunoglobulin G (IgG) and (IgM) levels (31).

RESULTS

Antibody responses. Table 1 shows results for four immunized and matched control groups of rats and for one nonimmunized nonchallenged group. PEV-01 vaccine appeared to have no adverse effects in these animals. There were no changes in activity, eating habits, or coat condition; there were no local reactions. Pyrogenicity in rats was not determined. Animals in group I received initial vaccine doses of 52.5 μg fol-

TABLE 1. Intratracheal challenge of PEV-01 vaccine-immunized rats

Group	Dosage[a]		Challenge[b]	Immunization-challenge interval (days)	Challenge-sacrifice interval (days)	Bacterial persistence[c]	Titer at sacrifice[d]		Pulmonary histology[e]
	μg/dose	Period between doses (days)					IgM	IgG	
I	52.5, 25	7 (10)	8.0×10^4	9	8	3.9×10^6	27.9 ± 1.45	11.3 ± 1.22	−
I-control	52.5, 25	7 (8)	8.0×10^4	9	8	5.7×10^5	4.0 ± 1.44	2.0 ± 1.33	+
II	52.5, 25, 25	9 (18)	1.1×10^5	12	9	4.8×10^5	50.9 ± 1.25	9.0 ± 1.16	−
II-control	52.5, 25, 25	9 (11)	1.1×10^5	12	9	4.9×10^5	1.9 ± 1.21	2.1 ± 1.34	+
III	42.5, 25, 25	10 (9)	3.8×10^5	28	10	4.7×10^5	50.9 ± 1.16	14.8 ± 1.22	−
III-control	42.5, 25, 25	10 (8)	3.8×10^5	28	10	4.1×10^5	17.4 ± 1.39	5.7 ± 1.31	+
IV	42.5, 25, 25	10 (7)	2.0×10^4	42	10	8.1×10^5	52.4 ± 1.12	13.1 ± 1.20	−
IV-control	42.5, 25, 25	10 (8)	2.0×10^4	42	10	1.4×10^6	10.4 ± 1.64	4.8 ± 1.43	+
Trial control	0 (10)		None	NA	NA	NA	0	0	NA

[a] Initial and booster doses; numbers in parentheses are numbers of rats (n) per group. Note: control groups received identical doses of bovine serum albumin on the same schedule.
[b] CFU per 0.05 ml of inoculum slurry (strain JR-1).
[c] Mean CFU of *P. aeruginosa* in lung homogenates.
[d] Geometric mean HA titer (± standard error) with PEV-01-sensitized SRBC.
[e] Presence (+) or absence (−) of indicators of pulmonary pathology (see text). NA, Not applicable.

lowed in 7 days by a 25-μg booster. Groups II to IV were given 43 to 52.5-μg doses and two additional 25-μg doses at 9- or 10-day intervals. Sera from rats bled before any immunization (trial control) had no anti-PEV-01 antibody detectable in this HA assay. Reciprocal titers in sera from immunized animals obtained at sacrifice ranged from 8 to 256. Corresponding controls ranged from 0 to 64, reflecting responses to pulmonary challenge alone. Geometric means for all groups are shown in Table 1. In all instances, immunized groups had significantly greater responses than nonimmunized, challenged animals ($P < 0.005$ by Student's t test). Titers in sera from immunized animals were 2.5- to 5.6-fold higher before dithiothreitol reduction of disulfide bonds, indicating relatively greater levels of IgM. A similar pattern was found in sera from control animals. Somewhat higher IgG levels were found in groups in which the interval between the final immunization and challenge was lengthened (groups III and IV).

Response to infection. Animals tolerated intratracheal instillation of agarose beads very well. Within a few minutes after the procedure, rats were mobile and alert. There was no significant morbidity over the experimental period. During this entire time (8 to 10 days postchallenge), only one death (within the first 24 h) occurred among the 79 infected animals. Within 3 days, postchallenge surgical wounds were nearly completely healed. Wound infections were extremely rare, presumably due to housing in wire-bottom cages.

Bacteriology. Mean bacterial levels in the lungs remained within $\pm 10^2$ times the original inoculum (Table 1). Somewhat surprisingly, the numbers of organisms recovered were not significantly different between immunized and control animals in any of the groups. Organisms grown from lung homogenates retained the smooth colonial morphology and serotype (type 5) of the original infecting strain. Mucoid colonies did not appear. In some instances, growth of organisms from homogenates appeared to be slower than that of strain JR-1. In such cases, at 24 h of incubation, colonies were smaller than expected; by 48 h, such differences were no longer observed. Superinfecting non-*P. aeruginosa* organisms were not found.

Pathology. Extensive macroscopic changes were evident in lungs from infected, nonimmunized animals. Hemorrhagic regions were prominent, involving from as little as part of one lobe to an entire lung. In many instances, lungs were dark red-brown in color, had hard mottled surfaces, and were very difficult to inflate with Formalin. On sectioning, microabscesses were occasionally found. Larger airways were occasionally visibly plugged with grayish-white exudate. Lungs from immunized animals lacked these gross alterations and were readily distinguishable from those of nonimmunized controls. Microscopic examination of coded sections by coinvestigators unaware of code definitions showed equally pronounced differences between immune and nonimmune animals from all experimental groups (I to IV).

In marked contrast to controls, sections of lungs from immunized animals revealed only minimal inflammatory responses associated with alveolar walls and relatively localized around beads. Figure 1B shows a bead lodged in a small airway. Most alveoli remained normal in thick-

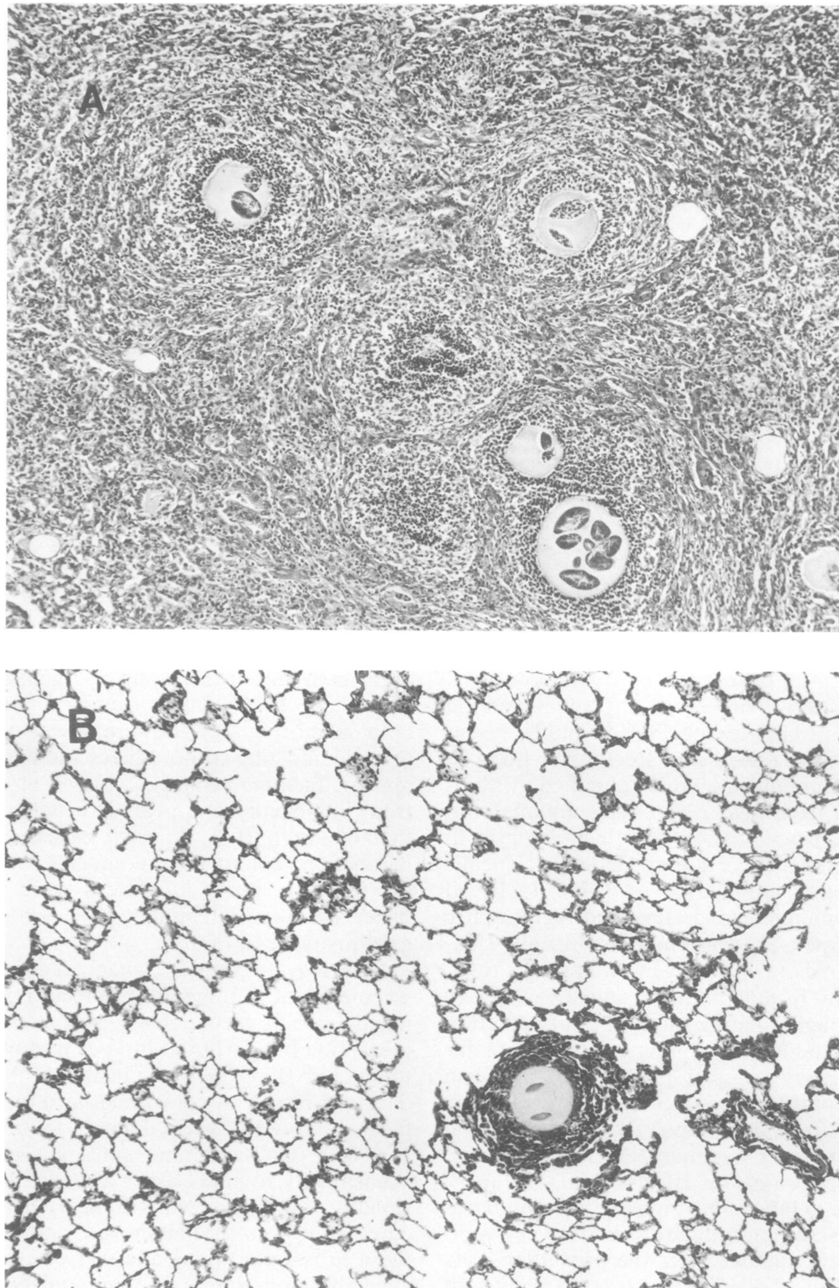

FIG. 1. Hematoxylin–eosin-stained lung sections from animals immunized with three doses of PEV-01 vaccine (B) and nonimmunized controls (A). Magnification, 80×. Note bacterial microcolonies in agarose beads in both fields. Intense inflammatory events have nearly obliterated normal airway and alveolar structures in the control lung. An identical inoculum evoked only mild cellular influx generally restricted to the area around infected beads. Sections are from animals in group II (see Table 1).

ness. Two elliptical bacterial microcolonies are evident in the agarose bead. In Fig. 1A (nonimmune lung), inflammatory cells can be seen associated with these microcolonies. The differences between changes seen in sections from immunized versus nonimmunized animals was so great that no quantitative assessment was required.

Sections of lung from nonimmune rats showed nearly complete obliteration of normal structure

(Fig. 1A). Acute and chronic inflammatory cells were found in all areas of the parenchyma and often occluded airways. Alveolar septae were thickened and often showed infiltration of polymorphonuclear leukocytes and erythrocytes. Both polymorphonuclear leukocytes and alveolar macrophages were concentrated on the surfaces of bacteria-containing beads. Damage to small airways was evident as disruption of ciliated respiratory epithelium (often sloughing as a sheet) with subsequent squamous metaplasia and inflammatory infiltrate in submucosal and peribronchial regions. Eosinophilic-staining edema fluid was often found in these airways. These processes evolved rather quickly, and by 10 days after infection, some evidence of fibrosis was present. Cumulatively, these changes often involved 50 to 100% of the left lung. Tolerance of such extensive pathology was made possible at least in part by success in confining the inoculum primarily to one lung. Significant vasculitis, as seen in *Pseudomonas* pneumonia of hematogenous origin, was not observed.

Although the pathological involvement was less extensive in group IV (challenge = 2.0×10^4), pulmonary protection was evident when these animals were infected 6 weeks after the final immunization (Fig. 2). The number of organisms used in this group reflects variation in the challenge culture and agarose bead preparations rather than experimental design calling for a smaller challenge dose.

DISCUSSION

The introduction of the multivalent cell extract *P. aeruginosa* vaccine PEV-01 prompted our interest in determining its usefulness in prevention or alteration of chronic *Pseudomonas* pulmonary infections. In the human trials reported thus far (14–16), untoward local and systemic reactions have been minimal, presumably owing to a relative lack of LPS. The vaccine does contain serotype-specific carbohydrates which have been shown to act as principal immunizing surface antigens (2, 24). Additionally, a significant portion (11.6%) of the dry weight of the vaccine is protein, presumably extracted from outer membrane-associated or structural proteins or LPS-associated proteins. The novel culture and mild extraction procedures used in the preparation of PEV-01 (24) allow recovery of cell surface, outer membrane, and periplasmic membrane antigens from living organisms with minimal physical or chemical alteration. As an example, LPS fragments in PEV-01 differ in molecular weight and ultrastructure from *P. aeruginosa* LPS obtained by Westphal (phenol-water) or EDTA extraction techniques (19; J. J. Miler and J. F. Spilsbury, First Internatl. Symp. Infect. Immunocompromised Host, Veldhoven, Netherlands, 1980).

In an earlier series of experiments, rats were immunized with relatively low doses of PEV-01, i.e., up to 5 μg. Even when repeated up to four times at weekly intervals, no pulmonary protection was engendered. However, when initial vaccine doses were increased to approximately 50 μg followed by one or two additional doses, substantial protection against infection-related lung pathology was obtained. Occasional low levels of cross-type protection were previously described with vaccines from individual serotypes (24). The extent of such an effect in the present study is unknown, so the net protective dose for a single serotype might be as low as 3 μg (i.e., $1/16 \times 50$ μg; extracts of 16 serotypes in vaccine). In the present study, protection was demonstrated against a single serotype 5 *P. aeruginosa* strain. Although it is noteworthy that strain JR-1 was originally an isolate from CF sputum, it will be important to demonstrate that PEV-01 immunization protects against pulmonary challenge by other serotypes. We chose postchallenge sera from 16 immunized animals (groups I to IV) and determined HA titers against SRBC sensitized with LPS preparations from the seven serotypes (3, 6–11) most commonly isolated from CF patients in our hospital. Reciprocals of the means of these titers ranged from 5 (serotype 9) to 24 (serotype 3). The reciprocal of the geometric mean titer of these sera against JR-1 LPS-sensitized SRBC was 56. Whether a similar increase would appear in response to infection by strains of other serotypes and whether such increase would be protective remain to be shown.

Pathological changes in the lungs of nonimmunized animals were highly reminiscent of those seen in lungs of infected CF patients. Pulmonary protection has been judged by very marked reduction in these changes. Such alterations include peribronchial and parenchymal inflammation, epithelial metaplasia, focal hemorrhage, bronchiectasis, and occasional microabscess formation. An as yet unresolved technical problem with this model concerns the location and relative numbers of challenge organisms residing in beads versus those in the lung parenchyma following infection. Lam et al. (18) employed scanning electron microscopy to show bacterial cells in the alveolar spaces of lungs of rats infected by the agarose bead technique. Additionally, both organisms and soluble exoproducts (e.g., proteases) have been demonstrated in pulmonary sites outside of beads by fluorescent-antibody techniques; however, the precise proportion of bead-associated and free organisms remains unknown (H. A. Cash, Ph.D. Dissertation, University of Texas Health Science Cen-

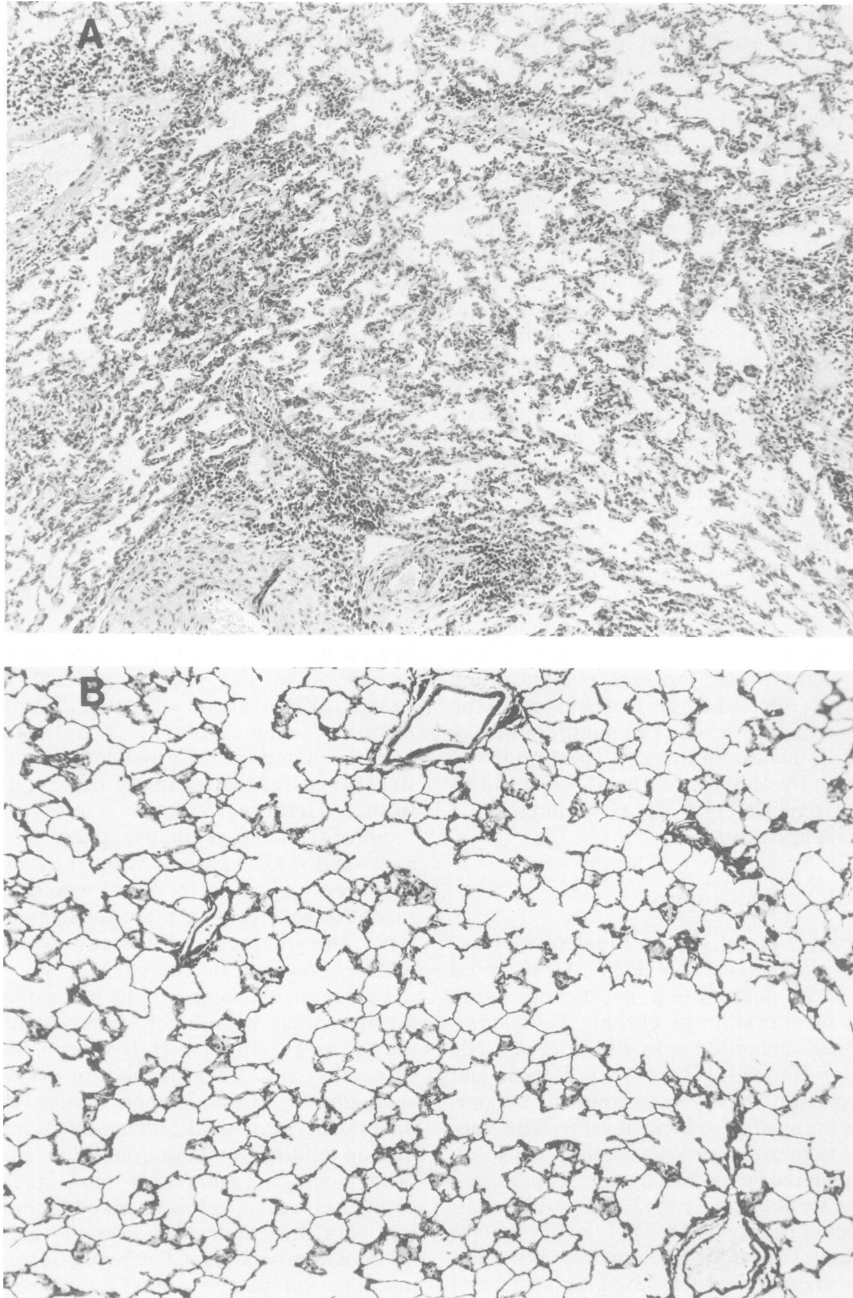

FIG. 2. Hematoxylin–eosin-stained lung sections from animals immunized with three doses of PEV-01 (B) and nonimmunized controls (A). Magnification, 80×. The immunization-challenge interval was 6 weeks. Note extensive alveolar thickening and cellular infiltrate in (A) compared with very minimal cellular influx and negligible alveolar changes in the immunized lung. Sections are from animals in group IV (see Table 1).

ter, San Antonio, 1979 [H. A. Cash, D. C. Strauss, and J. A. Bass, Can. J. Microbiol., in press]). This situation is probably analogous to that of mucus plugs in lungs of CF patients, in which organisms are relatively sequestered, but from which toxic products may diffuse.

Pennington and Duchmy (26, 28) have related pulmonary protection in acute experimental challenges in guinea pigs to IgM-facilitated clearance by pulmonary macrophages. We were therefore intrigued by the finding of reduced pathology in PEV-01-immunized animals in the

presence of virtually unaltered numbers of challenge organisms. A possible explanation relates to the potential for immunization against bacterial proteins included as part of the vaccine extraction process.

Miler and Spilsbury (Miler and Spilsbury, First Internatl. Symp. Infect. Immunocompromised Host) further characterized a portion of the protein content of PEV-01 vaccine as outer membrane components. Fernandes and Cundy (5) have demonstrated that *P. aeruginosa* exotoxin A can exist as a cell envelope-bound protein as well as a soluble exoproduct. The toxic potential of exotoxin A for a variety of cells (including macrophages) has been demonstrated by several authors (23, 32). Infected CF patients or animals with experimental *Pseudomonas* infections develop circulating antibodies against exotoxin A (17, 33, 34, 37), and immunization with toxoids of exotoxin A affords protection in certain animal models of local (e.g., burned rodent) or systemic challenge (20, 37). Such materials have not been used alone to specifically protect against pulmonary challenges. Klinger and Shuster (K. W. Klinger and C. W. Shuster, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B93, p. 32) have recently described a nontoxic macromolecular structure purified from *P. aeruginosa* culture supernatants which cross-reacts immunologically with soluble exotoxin A. When sera from goats or rats immunized against these nontoxic cross-reactive structures were included as blind controls in HA assays with PEV-01-sensitized SRBC, we found high titers (1:128 to 1:512) (data not shown). Thus, it would appear possible that at least part of the pulmonary protection seen in PEV-01-immunized animals in the absence of bacterial clearance may be due to immunization against protein exoproducts (or related fragments) serendipitously included during vaccine preparation. Although PEV-01 has been characterized as a cell surface vaccine, perhaps the term "cell extract" is more apt. A possible clinical corollary to these speculations comes from the work of Pollack and Young (34) showing more favorable outcome in *P. aeruginosa* sepsis in patients having antibody to both cell surface components (LPS) and exotoxin A.

Although we have no data suggesting that protease-related materials are also included in PEV-01 vaccine, a potentially protective role for antiprotease activity can be envisioned based on probable proteolytic destruction of complement components (36) and the recent demonstration by Heidbrink et al. (8) of the critical importance of complement-dependent phagocytic killing of *P. aeruginosa*. Clearly, much additional effort should be directed at evaluating the protective potential of immunization with toxoids of purified exoproducts in alteration of *P. aeruginosa* pulmonary infection.

CF patients apparently have no inherent defects in local or systemic immune functions, but the potential consequences of any augmentation of immune responses must be a major consideration. Matthews et al. have observed that in an age-matched population, CF patients with decreased gamma globulin levels had less severe lung disease than those with normal or elevated levels, suggesting a possible role for immune-modulated pulmonary damage (21). A few studies have shown the presence of circulating immune complexes or pulmonary deposition of IgG-complement complexes in CF patients (3, 10, 11, 22). Høiby and Schiotz and Schiotz et al. (10, 35) have found (i) immune complexes in serum and sputum from infected CF patients, (ii) complement activation in sputum evidenced by split product C3c, and (iii) potential for liberation of vasoactive amines in in vitro basophil histamine release assays with CF serum. Berdischewsky et al. (3) studied circulating immune complexes in hospitalized CF patients and found LPS as the major *Pseudomonas* antigen; exotoxin A was infrequently involved in complexes, despite high antitoxin titers. Immune complexes are found more frequently in patients with severe pulmonary disease, but their precise significance remains uncertain.

In a most important experimental study in this area, Pennington et al. (27) have shown that immunization with LPS vaccine provided substantial protection against lung damage in a guinea pig model for chronic pulmonary infection only if animals were immunized before challenge. Immunization after infections were established was not beneficial, although there appeared to be no increase in circulating immune complexes. It is clear that before designing immunization trials (with any vaccine) in CF patients, long-term animal studies addressing the potential of inadvertent or increased immune-mediated damage must be pursued. We have recently adapted the agarose bead challenge technique for use in larger laboratory animals (rabbits, cats, etc.), allowing serial immunization, challenge, and hyperimmunization in the same animals. This will facilitate the extended animal experiments which could form the basis for human trials. The positive findings reported in the present study with PEV-01 vaccine suggest that immunization of CF patients before natural infection could prevent or substantially ameliorate the life-threatening component of their disease.

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