Evaluation of a New Polyvalent Pseudomonas Vaccine in Respiratory Infections

JAMES E. PENNINGTON and JARMILA J. MILER

Infectious Diseases Division, Department of Medicine, Peter Bent Brigham Hospital, Boston, Massachusetts 02115, and the Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, England

Received for publication 27 June 1979

A new polyvalent, cell wall extract, Pseudomonas aeruginosa vaccine (PEV-01), was evaluated by using a guinea pig model of experimental Pseudomonas pneumonia. Guinea pigs routinely developed fourfold rises in serum hemagglutinating Pseudomonas antibodies after four vaccine injections given over 2 weeks. Vaccinated animals survived an intratracheal Pseudomonas challenge (1 × 10⁸ colony-forming units) significantly better (13 of 14 survived) than did a control group (5 of 14 survived) (P < 0.01). Clearance of viable Pseudomonas from lung tissue was significantly better in vaccines than controls at both 3 h (P < 0.02) and 6 h (P < 0.05) after infection. Both gross and histological examinations of lung tissue revealed less pulmonary tissue damage in vaccinated animals following Pseudomonas infection. Thus, PEV-01 Pseudomonas vaccine appears capable of eliciting a specific protective response in the guinea pig respiratory tract.

Certain patient populations are specifically at risk for infection with Pseudomonas aeruginosa (9, 25, 28, 31, 32, 34), and mortality is particularly high when infection involves the lung (19, 25, 31). The identification of these high-risk populations has lead to interest in immunoprophylaxis or immunotherapy for such patients against Pseudomonas sepsis (2, 14, 20, 23, 25). To date, the majority of experimental and clinical studies involving Pseudomonas vaccines have concentrated on protection for burn sepsis (2). Except for one clinical report (23) and one study in mink (29), there is no direct evidence that vaccination can elicit a specific protective immune response for Pseudomonas in the respiratory tract. A recently described model for experimental Pseudomonas pneumonia in guinea pigs (18) was employed in this study to assess the potential of a new polyvalent Pseudomonas extract vaccine (15), in inducing protection against fatal hemorrhagic Pseudomonas pneumonia.

MATERIALS AND METHODS

Animals. Hartley-strain guinea pigs (400 g), obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were housed in standard cages and fed guinea pig chow and water.

P. aeruginosa. The strain of P. aeruginosa used in this study was obtained from Mike Fisher and Carl Heifetz (Parke, Davis & Co., Detroit, Mich.) and is designated as strain P-4. This strain was isolated from a patient with sepsis and was maintained on brucella agar slants at 4°C. P-4 was found to be a Fisher immunotype 4 (6) and a Habs serotype 1 (7) (Typing was courtesy of M. Fisher and C. Heifetz and the Colindale Laboratory, Colindale, England.) This strain was serum resistant and produced relatively small quantities of exotoxin A (assay courtesy of M. Pollack, Naval Medical Research Institute, Bethesda, Md.). To determine whether strain P-4 produced protease, culture filtrates of an overnight growth of P-4 in Trypticase soy broth were injected intradermally into guinea pigs, as previously described (24). An early (ca. 10 min) hemorrhagic skin reaction occurred with three separate filtrate batches, indicating production of protease by our challenge strain (24). The preparation and quantitation of Pseudomonas for experimental lung infection has been described (18).

Vaccine protocol. The polyvalent Pseudomonas extract vaccine used in this study was designated PEV-01, prepared by Miler and Spilsbury at the Wellcome Research Laboratories, Beckenham, Kent, England (15). This vaccine contains immunogens derived from 16 Pseudomonas serotypes (including Habs serotype 1 through 12, Veron 13, Meitert 10, and Homma types 11 and 13) (10). Thus, strain P-4 corresponded antigenically to one (Habs type 1) of the antigens included in PEV-01 vaccine.

PEV-01 was supplied in glass vials as a freeze-dried material. Vaccine was reconstituted with 2.5 ml of sterile isotonic saline, warmed to 37°C. Final PEV-01 suspensions contained 210 μg of antigen per ml. Guinea pigs were vaccinated by using either three, four, or six subcutaneous injections (0.25 ml/injection) during a 2-week period. Control groups received injections of isotonic saline in identical volumes.

Since past studies have indicated that protective immune responses in mice vaccinated with PEV-01 correlate with hemagglutinating (HA) Pseudomonas antibody titers (10), the passive HA assay was used to detect immunological reactivity in vaccinated guinea pigs. Serum specimens were obtained before vaccina-
tion and during the week after the final vaccine injection in vaccinees. Control values were obtained from saline-injected animals. Blood was obtained by cardiac puncture and aspiration, as previously described (18). In addition to serum HA titers, selected vaccinated and control animals were sacrificed (intraperitoneal pentobarbital) 1 week after finishing the vaccine regimen and underwent tracheobronchial lavage (17). Bronchial lavage fluid specimens were concentrated (4°C) from initial volumes of approximately 80 ml, to final volumes of 5 ml, using a positive-pressure ultrafiltration device (Amicon Corp., Lexington, Mass.) with a UM-10 filter (Amicon). Optical densities at 280 nm (Gilford Spectrophotometer) of 1:10 saline dilutions of the fluid concentrates revealed similar values among vaccinees (0.28 ± standard error 0.02) and controls (0.34 ± standard error 0.03). Serum and bronchial fluid specimens were frozen to −20°C until the HA antibody assays were done.

The HA assays were done by using modifications of a previously described technique (10). Specimens were first heated to 56°C for 30 min and then were reacted in twofold dilutions with sheep erythrocytes, coated with PEV-01 polyvalent Pseudomonas vaccine antigens. In addition, HA assays were repeated on chosen samples after reduction of disulfide bonds with dithiothreitol (22). Results are expressed as reciprocals of the highest dilutions showing hemagglutination.

**Pseudomonas** respiratory infection and protection studies. Experimentally induced hemorrhagic Pseudomonas pneumonia can be produced in guinea pigs by directly instilling a Pseudomonas-saline suspension into the tracheobronchial tree (18). The rationale for instillation rather than aerosolization has been given (18). For this study, guinea pigs were anaesthetized with intraperitoneal pentobarbital, and the trachea was surgically exposed by using an aseptic technique. A 0.5-ml portion of Pseudomonas P-4 suspended in isotonic saline was instilled into the trachea by using a syringe with a no. 25 needle. The proximal trachea was briefly occluded during instillation. Necks were then sutured, and the animals were positioned upright until awake. Strain P-4 produced fatal pneumonia in six consecutive untreated normal guinea pigs when challenge inocula of 1 × 10⁸ colony-forming units (CFU) were used. Of 10 animals, 7 survived challenge with 5 × 10⁸ CFU of P-4. Heat-killed (60°C for 90 min) P-4 inocula of 1 × 10⁷ CFU did not produce a fatal outcome in four animals challenged in a similar manner. To compare the relative virulence of our *Pseudomonas* challenge strain with other *Pseudomonas* strains, we obtained two *Pseudomonas* strains isolated from clinical cases of septicaemia. These strains (DC220 and DC86) were kindly provided by M. Pollack (Naval Medical Research Institute, Bethesda, Md.), who characterized each strain as potent producers of protease and exotoxin A by using previously described techniques (24). Intratracheal challenge of guinea pigs with various inoculum sizes of each strain was carried out, and survival rates were determined. Strains DC220 and DC86 produced 100% mortality at 5 × 10⁷ and 3 × 10⁷ CFU, respectively. Animals routinely survived challenges of 1 × 10⁷ CFU with either strain. Thus, these strains were only slightly more virulent than P-4, despite their increased potential for exotoxin A production.

To evaluate the potential of PEV-01 to protect guinea pigs from fatal pneumonia, a series of vaccinated and control animals underwent lung challenge with 1 × 10⁶ CFU of P-4 *Pseudomonas* (as described above) 5 to 10 days after final vaccine injections. Animals were placed in cages, allowed to awaken (about 3 h after infection), and observed for survival without further treatment. Autopsies were carried out on all fatalities.

The effect of PEV-01 vaccination on pulmonary clearance of viable *Pseudomonas* was assessed by using serial quantitative lung cultures after *Pseudomonas* challenges. For these studies, a sublethal challenge inoculum was used (5 × 10⁶ CFU in 0.5 ml of saline), and groups of vaccinated and control animals were sacrificed at 0, 3, 6, and 24 h after intratracheal challenge. After the neck and thorax were exposed surgically, the trachea and lungs were removed intact, and the right lung was resected. Lungs were individually placed into a commercial-grade Waring blender (Waring Products Division, Dynamics Corp. of America, New Hartford, Conn.) with 25 ml of sterile isotonic saline and homogenized at low speed for 1 min. Quantitative cultures were then made by using serial dilutions of homogenate, as previously described (18).

Gross and histopathological observations were also made on the lung tissues from vaccinees and controls after these sublethal lung challenges. In addition to gross tissue inspection at 3, 6, and 24 h after infection, histological preparations of lungs were made on all lungs removed 24 h after lung challenge. After the lungs were resected, the right mainstem bronchus was tied off, the right lung was resected distal to the ligature, and quantitative cultures were performed as described above. The trachea was then cannulated with a no. 18 Ranfac Special needle (Randall-Faichney Corp., Boston, Mass.), and the left lung was expanded by using a syringe with 10% Formalin (about 5 ml). The trachea was then tied off, and the lungs were fixed for 48 h in 10% Formalin, then sectioned longitudinally and embedded in paraffin. Tissue sections (5 μm) were prepared and stained with hematoxylin-eosin stain.

**Non-specific protection studies.** To evaluate whether PEV-01 vaccination provided *Pseudomonas*-specific lung protection or whether protective effects might be related to "non-specific" immune enhancement, additional lung challenges were carried out by using a nonpseudomonad, gram-negative bacillus, *Escherichia coli*. A clinical isolate (septicaemia) of *Escherichia coli* was obtained from the clinical microbiology laboratory at the Peter Bent Brigham Hospital (strain no. 30355) and was handled in an identical fashion to *Pseudomonas* P-4. After normal animals were challenged with various inocula sizes, it was determined that 3.0 × 10⁶ CFU of *E. coli* no. 30355 would reliably produce mortality by hemorrhagic pneumonia, as verified by autopsy and lung cultures. All animals survived 1 × 10⁶-CFU challenges. Groups of control and PEV-01 vaccinated guinea pigs were then challenged with 3.0 × 10⁶ CFU of *E. coli*, and survival was determined.

**RESULTS**

Guinea pigs tolerated PEV-01 vaccination well. There were no discernible differences in behavior, appearance, or appetite between vac-
cinated and control animals. All vaccinated guinea pigs exhibited increased serum anti-
Pseudomonas HA antibody titers during the week after completing vaccination with PEV-01.
Among 30 animals assayed, 29 had a fourfold or greater rise in HA titer above the control value
of 4. No significant difference in HA antibody development was noted among groups receiving
three, four, or six injections over a 2-week period (Table 1). A regimen of four injections was
arbitrarily selected for the survival and tissue protection studies carried out below. The majority
of serum HA activity appeared to be of the immunoglobulin M (IgM) class, since inactivation
of macroglobulins with dithiothreitol abolished much of the HA antibody activity (Table 1). In contrast to serum, an HA response was not detected in bronchial fluid concentrates.

Survival from Pseudomonas pneumonia was significantly better in the vaccinated animals ($P$
< 0.01 by the chi-square test with Yates correction) (Table 2). Deaths often (6 of 10) occurred
within 24 h of infection. Each surviving vaccinated animal had at least a fourfold elevation of
serum HA titers. Thus, increased serum HA titers appeared to correlate with an improved outcome
from pneumonia (Table 2). The single vaccine fatality also had an elevated HA titer. Autopsies from all fatalities showed extensive bilateral hemorrhagic pneumonia. Cultures of lung tissues grew heavy P. aeruginosa in each case.

In contrast to these studies with Pseudomonas, lung challenge with E. coli produced 100% fatality in both control ($n = 6$) and PEV-01-vaccinated ($n = 6$) groups. Thus, we were unable to detect nonspecific immune protection in lung tissues after PEV-01 vaccination by using an in vivo infectious challenge.

Clearance of viable Pseudomonas from lung tissues was more efficient in PEV-01-vaccinated animals than in controls (Fig. 1), with a prompt decrease in counts to 2.5% of base line occurring as early as 3 h after infection in the vaccinees. Lung Pseudomonas counts in vaccinees were significantly lower than controls at both 3 h ($P$
< 0.02 by Student's t test) and 6 h ($P < 0.05$) after infection. By 24 h, there were no significant differences between the two groups. Thus, improved early Pseudomonas lung clearance was a prominent effect of PEV-01 vaccination.

Gross inspection of lungs resected at several time intervals after a sublethal Pseudomonas challenge revealed differences in the extent of hemorrhagic pneumonia between controls and vaccinees. As early as 3 h after infection, bilateral pleural surface petechiae were present in both groups. However, in at least half of the controls, lobar hemorrhage was also noted. By

![FIG. 1. Pulmonary Pseudomonas clearance in vaccinated (●) and control (○) guinea pigs. Each value represents the mean ± standard error for six animals.](http://iai.asm.org/)

<table>
<thead>
<tr>
<th>TABLE 1. Serum HA antibody titers for PEV-01 antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of vaccine injections</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>Controls</td>
</tr>
</tbody>
</table>

* Each HA antibody titer is expressed as the reciprocal of the geometric mean ± standard error of mean.

<table>
<thead>
<tr>
<th>TABLE 2. Survival from Pseudomonas pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>Vaccinees</td>
</tr>
<tr>
<td>Controls</td>
</tr>
</tbody>
</table>

a Numbers in parentheses indicate percentage of group surviving infection.
b Reciprocal of geometric mean HA antibody titer.
c $P < 0.01$ by chi-square test with Yates correction.
FIG. 2. Lung sections (×250) from guinea pigs sacrificed 24 h after intratracheal instillation of $5 \times 10^6$ CFU of Pseudomonas (hematoxylin-eosin stain). (A) Control animal. Extensive PMN inflammatory reaction. Moderate alveolar wall edema with capillary congestion and some intraalveolar hemorrhage. (B) Vaccinated animal. Minimal polymorphonuclear leukocyte reaction with relatively little alveolar wall edema or parenchymal hemorrhage.
DISCUSSION

Despite the availability of newer antibiotics with potent in vitro activity for P. aeruginosa, the mortality for Pseudomonas sepsis remains high (25). Host defense for Pseudomonas depends upon the presence of adequate numbers of functional phagocyte cells plus serum opsonic activity (36). Optimal phagocytosis of Pseudomonas occurs in the presence of type-specific, heat-stable Pseudomonas antibody (4, 33, 35). Thus, it is theoretically advantageous for patient groups at particular risk of Pseudomonas infection to possess preformed, specific anti-Pseudomonas antibodies. A number of Pseudomonas-antigenic components have been considered for vaccine preparations, including cell wall lipopolysaccharides (5) and polysaccharides (21), a protein moiety of endotoxin (1), and a toxic biological substance, exotoxin A, secreted by certain strains of Pseudomonas (13, 24, 30). Until recently, production and clinical availability of Pseudomonas vaccine has been limited to a single heat-stable lipopolysaccharide preparation (8). Although this vaccine has proven of some value in both burn (2) and cancer patients (37), a considerable incidence of endotoxin-like effects has been reported with its use (14, 20, 37). Recently, a new polyvalent Pseudomonas vaccine (PEV-01) has become available for investigational purposes (15). Antigens for this vaccine were obtained from organisms grown under special conditions, and were extracted from live organisms by using an ethylendiaminetetraacetic acid–glycine mixture (15). This resulted in obtaining type-specific and cross-protective immunogens with a limitation of compounds associated with toxic side effects (15). PEV-01 has proven effective both in actively and passively transferred protection of mice from intraperitoneal challenge with Pseudomonas (10, 15) and has been well tolerated and immunogenic in human volunteer studies (11). Since pulmonary host defense mechanisms often operate independently from systemic immune mechanisms (12), it is important that the potential value of any new vaccine for pulmonary protection be specifically evaluated by direct pulmonary challenges. No information currently exists regarding the ability of PEV-01 to produce a protective immune response in respiratory tissues; thus, a study was carried out with this vaccine in an animal model of Pseudomonas pneumonia.

PEV-01 routinely produced a systemic immune response in guinea pigs after a 2-week immunization (Table 1) and was found to be effective in protecting animals from fatal Pseudomonas pneumonia (Table 2). Vaccinees also demonstrated decreased lung tissue damage (Fig. 2A and B). Although the vaccinated group did not show significant increases in HA Pseudomonas antibodies in bronchial secretions, this assay is most sensitive to IgM antibody, an Ig class frequently absent from bronchial fluids (26). Studies demonstrating superior in vivo protection with IgG Pseudomonas antibodies (3), as well as prolonged periods of protection related to IgG rather than IgM antibodies (16), further illustrate the value of IgG-specific Pseudomonas antibody analysis.

The mechanisms for PEV-01-induced pulmonary protection were not defined in this study but may have been related to the increased early lung clearance of viable organisms (Fig. 1). Others have shown that Pseudomonas lipopolysaccharide antigens can elicit Pseudomonas-specific local opsonins in the respiratory tract (26). Non-specific activation of alveolar macrophages by Pseudomonas antigens was not shown, however (27). It is possible that PEV-01 vaccination led to increased serum and local bronchial secretion opsonic activity, which in turn enhanced alveolar macrophage and polymorphonuclear leukocyte (PMN) phagocytosis and killing of Pseudomonas. It was significant that fewer PMNs were observed in the lungs of vaccinees 24 h after infection, and it may be that in the presence of increased Pseudomonas-specific antibodies fewer PMNs were needed for effective Pseudomonas clearance. Reduced tissue damage may have been partially related to decreased PMN-derived lysosomal enzymes.

The present study demonstrates that PEV-01, a new and potentially less toxic polyvalent Pseudomonas vaccine, has the capability of inducing specific respiratory tract protection. Whether these findings will prove of benefit for patients at risk of the acute Pseudomonas pneumonia associated with cancer or respiratory care units, or from the chronic Pseudomonas bronchitis associated with cystic fibrosis, will await appropriate clinical trials.

ACKNOWLEDGMENTS

I thank Diana Kuchmy for her technical assistance and William F. Hickey and Betty Flammio, Department of Pathology, Peter Bent Brigham Hospital, for help with the histological preparations. I also thank J. Mellor, Wellcome Research Laboratories, for his advice and assistance with this project.

This work was supported in part by research grants from the Cystic Fibrosis Foundation and the National Foundation—March of Dimes and by Public Health Service grant AI15411 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


