Vaccine efficacies of elastase, exotoxin A, and outer-membrane protein F in preventing chronic pulmonary infection by *Pseudomonas aeruginosa* in a rat model

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Summary. The protective efficacies of eight vaccine preparations consisting of *Pseudomonas aeruginosa* outer-membrane protein F, elastase and exotoxin A toxoid, administered either individually or in various combinations, were determined in a rat model of chronic pulmonary infection. Rats were immunised intramuscularly at 2-week intervals (days 0, 14 and 28). On day 42, blood was collected and antisera were obtained from each vaccine group for use in an enzyme-linked immunosorbent assay which determined the titre of IgG antibodies elicited by each vaccine. Also on day 42, rats were challenged by intratracheal inoculation of a clinical isolate of *P. aeruginosa* encased within agar beads. On day 49, the animals were killed and the lungs were examined macroscopically for the presence of lesions and fixed for histological examination. When compared with control rats immunised with bovine serum albumin, rats immunised with protein F alone as a vaccine received significant protection against the development of severe pulmonary lesions. Elastase used alone as a vaccine provided some protection against severe lung lesions and reduced the incidence of microscopic peribronchial inflammation. However, the combination of protein F plus elastase as a vaccine did not afford protection from severe lesions, and there was an increased incidence of necrotising granulomas in the lungs from this vaccine group. Protection against lung lesions from the three-component vaccine consisting of protein F, elastase and exotoxin A toxoid was similar to that provided by the protein F vaccine. Neither macroscopic nor histological evidence showed any enhancement of protective efficacy for the three-component vaccine over that of the protein F vaccine. No combination of elastase or exotoxin A toxoid with protein F improved the protective efficacy of the protein F vaccine alone.

Introduction

Previous studies have shown that active immunisation with outer-membrane protein F of *Pseudomonas aeruginosa* prevents *P. aeruginosa* infections in various animal models, including a murine acute infection model,¹ a burned mouse model^{2,3} and a rat model of chronic pulmonary infection.^{4,5} Data from the chronic pulmonary infection model are of particular interest as one potential clinical use of a protein F vaccine might be for immunotherapy of children with cystic fibrosis (CF) to prevent the colonisation of their lungs by *P. aeruginosa*. Lung disease is responsible for > 95 % of the morbidity and mortality in CF patients,⁶ and *P. aeruginosa* is the predominant bacterial pathogen associated with chronic pulmonary infection in these patients.^{6,7} In the rat model, immunisation with purified protein F reduced the incidence of severe lung lesions from 74 % in control rats to 27 % in immunised rats. The incidence of pul-

monary colonisation was reduced from 77 % in control rats to 41 % in protein F-immunised rats⁴ challenged with heterologous immunotype strains of *P. aeruginosa*. These results suggested that continued development of a protein F vaccine for possible use in CF patients was warranted.

Opsonophagocytic killing antibodies are produced in response to immunisation with protein F.⁵ Such antibodies should promote clearance of *P. aeruginosa* from the lung. However, *P. aeruginosa* has several toxins and enzymes that promote inflammation, interfere with the host immune response and promote bacterial colonisation. Two of the most important pathogenic determinants in chronic lung infections by *P. aeruginosa* are exotoxin A⁸ and elastase.^{8,9} Elastase in particular is thought to play a major role in interfering with the host immune system¹⁰ by cleaving IgG and IgA, inhibiting the activity of various cytokines and interfering with the function of T cells and natural killer cells.

We investigated the possibility that a three-component vaccine consisting of outer-membrane protein

F, exotoxin A toxoid and elastase might enhance protection over that afforded by protein F alone. The rationale was that antibodies directed towards exotoxin A and elastase might reduce tissue injury and inflammation and reduce bacterial interference with the host immune response, so that antibodies directed towards protein F could promote clearance of the bacterial cells from the lung more effectively.

Materials and methods

Bacterial strains and growth conditions

The strains of *P. aeruginosa* used in the vaccine studies were the PAO1 strain, which typed as O-5 with Difco typing antisera, corresponding to a Fisher-Devlin immunotype 7, and a clinical isolate, which typed as Difco O-2, corresponding to a Fisher-Devlin immunotype 3, obtained from the Clinical Microbiology Laboratory, Louisiana State University Medical Center (LSUMC, Shreveport, Louisiana). Both strains were grown routinely in Nutrient Broth (Difco) at 32°C with shaking.

Screening for elastase and exotoxin A activities

Twenty clinical isolates of *P. aeruginosa* from patients with CF and 59 clinical isolates from non-CF patients were obtained from the LSUMC-S Clinical Microbiology Laboratory and screened for the production of elastase and exotoxin A. Each isolate was grown in a medium consisting of dialysed tryptic soy broth, glycerol 1% and 0.05 M monosodium glutamate¹¹ for 18 h at 32°C with shaking. The bacterial cells were pelleted by centrifugation at 12000 *g* for 10 min and the supernate was assayed for elastase and exotoxin A. Elastolytic activity was measured by determining the digestion of elastin congo red by the culture supernate relative to a standard curve generated with varying concentrations of purified elastase.¹² Strains that produced more than 50 µg of elastase/ml of supernate were considered positive for elastase production. The concentration of exotoxin A in the supernate was estimated in an enzyme-linked immunosorbent assay (ELISA) with mouse antisera to exotoxin A toxoid. A standard curve was generated with varying concentrations of native exotoxin A as the test antigen. The absorbance reading from the wells coated with culture supernate as test antigen was compared with the standard curve to estimate the exotoxin A concentration of the supernate. Strains producing ≥ 0.05 µg of exotoxin A/ml of supernate were considered positive. A positive and a negative control strain were included in all assays; *P. aeruginosa* strain M2 was positive for elastase and negative for exotoxin A; strain PA103 was negative for elastase and positive for exotoxin A.

Vaccine preparations

Protein F was purified from *P. aeruginosa* strain PAO1 by gel extraction as described previously.¹ Such

purified protein F preparations have been shown to consist predominantly of a homogeneous major protein F band, with several very minor bands of lower-mol.-wt proteins (which may represent degradative products of protein F) upon SDS-PAGE.¹ The lyophilised preparation of protein F contained *c.* 1.2% protein and *c.* 0.3% PAO1 lipopolysaccharide (LPS), with the remainder of the dried preparation consisting of SDS powder.⁵ Purified elastase was purchased from Nagase and Co., Ltd. (Tokyo, Japan). Purified exotoxin A was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA). Bovine serum albumin (BSA) was purchased from Sigma. All protein determinations were performed by the Lowry method as modified by Markwell *et al.*¹³ A toxoid of exotoxin A was prepared as follows: enough formalin was added to 1 mg of exotoxin A in a 1-ml volume containing 0.15 M NaCl and 0.01 M sodium phosphate, pH 7.0, to make a 4% solution of formalin. This formalin-exotoxin A solution was incubated at 37°C for 4 days. The solution was then diluted with phosphate buffered saline (PBS), pH 7.5, to a concentration of 400 µg of exotoxin A/ml. The diluted toxoid was dialysed against 2000 volumes of PBS, pH 7.5, for 18 h at 4°C in dialysis tubing with a 6000–8000 mol. wt cut-off. The toxoid was filtered through a 0.2-µm membrane filter. The protein concentration was determined, and adjusted to 200 µg of toxoid/ml PBS. The toxoid was stored in 1-ml volumes at –20°C until required.

Immunisation protocol

Young adult female, specific-pathogen-free Sprague-Dawley rats (175–200 g) (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were immunised on days 0, 14 and 28 by intramuscular injection into alternate hips. For the first two immunisations, all vaccine preparations were adsorbed to aluminium hydroxide (2.5 mg/ml) as adjuvant. The third immunising doses were all given without adjuvant. Protein F was administered at a concentration of 25 µg/immunising dose, elastase at 100 µg/dose, exotoxin A toxoid at 20 µg/dose, and BSA at 25 µg/dose. Each immunising dose consisted of 0.3 ml total volume of sterile PBS containing the appropriate vaccine preparation(s). Eight groups of rats were immunised with the following vaccine preparations: BSA alone, protein F alone, elastase alone, exotoxin A toxoid alone, protein F plus elastase, protein F plus exotoxin A toxoid, elastase plus exotoxin A toxoid, and protein F, elastase and exotoxin A toxoid combined.

Monitoring the immune response

On day 42 three rats/immunisation group were bled by cardiac puncture under sodium pentobarbital anaesthesia and the sera were pooled. Normal sera were obtained by bleeding five unimmunised rats. The titre of IgG (γ-chain-specific) antibodies for each of the

Fig. 1. Normal lung with alveoli, bronchi and blood vessels (H and E). Bar = 100 μ m.

Fig. 2. Bronchus with a surrounding subacute inflammatory infiltrate composed of lymphocytes and small numbers of neutrophils (H and E). Bar = 100 μ m.

serum pools was determined by ELISA performed as described previously.² The ELISA antigens used included elastase, exotoxin A, cell envelopes of strains PAO1 and the clinical isolate, and purified LPS of strains PAO1 and the clinical isolate. Four independent determinations were performed for each of the antisera and the median titre was calculated.

The ability of the antibodies elicited by immunisation with the exotoxin A toxoid to neutralise the lethal activity of native exotoxin A was demonstrated as follows. The minimum lethal dose of exotoxin A in mice was determined to be 0.25 μ g injected intraperitoneally in a 0.2-ml volume. One of the following was added to 2.5 μ g of exotoxin A in a 0.5-ml volume: 0.5 ml of normal serum, 0.5 ml of PBS or 0.5 ml of antiserum against the exotoxin A toxoid. The tubes were incubated for 18 h at 4°C. Groups of four mice were given 0.2 ml containing 0.5 μ g of the treated exotoxin A from each tube by intraperitoneal injection and observed for death within 72 h. The toxoided immunogen was judged to be satisfactory since all four mice died within 72 h after injection of exotoxin A treated with either PBS or normal serum whereas none of four mice given the exotoxin A treated with anti-toxoid serum died.

Chronic pulmonary infection model

The method of Klinger *et al.*¹⁴ was used with modifications as detailed previously.⁴ Two weeks after the third immunising dose (day 42), the rats were challenged with agar beads containing bacteria. The rats were first anaesthetised with an intraperitoneal injection of sodium pentobarbital and then inoculated *via* a tracheal incision with 60 μ l of bead slurry containing *c.* 5×10^4 cfu of the *P. aeruginosa* clinical isolate. A curved, beaded-tip 20-gauge needle was gently guided to favour 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 49), the rats were killed by an overdose of halothane (Ayerst Laboratories, Inc., New York, NY, USA). Before removal, the lungs were examined macroscopically for the presence of lesions and scored from 0 to 4+,⁴ depending on the presence and severity of the lesions. For histological examination, the tracheas were cut just below the larynx, and the heart-lung block was removed. Lungs were inflation-fixed with buffered formalin 10%. After fixation, the external surfaces of the lungs were again evaluated to determine whether there were gross visible lesions. A central longitudinal section of each lung was taken for microscopic examination. Right and left lungs were marked for identification with ink. The tissue was dehydrated in graded alcohols, cleared in HistoClear® and embedded in paraffin. Sections (4 μ m) were cut and stained with haematoxylin and eosin. Sections were then examined and the following parameters were evaluated: normal

Fig. 3. Blood vessels with a surrounding chronic inflammatory infiltrate composed of lymphocytes (H and E). Bar = 100 μ m.

Fig. 4. Rounded granuloma composed of large numbers of epithelioid cells and scattered lymphocytes. A small micro-abscess is seen in the centre of the granuloma (H and E). Bar = 100 μ m.

lung architecture (fig. 1), interstitial inflammation, peribronchial fibrosis and inflammation (fig. 2), perivascular fibrosis and inflammation (fig. 3), the presence of necrotising granulomas (fig. 4) and the presence of purulent bronchitis. Any other abnormalities were also recorded. Animals with disease in all lobes of the lungs were considered to be secondarily infected and were eliminated from the histological study.

All animals used in this research were handled in accordance with the Animal Care and Use Committee guidelines of LSUMC-Shreveport.

Statistics

All statistical analyses were performed with the IBM EpiStat Basic Statistics Program; *p* values were calculated by the one-tailed Fisher's exact test, values ≤ 0.05 were considered to be significant.

Results

Screening for elastase and exotoxin A production

Of 20 *P. aeruginosa* clinical isolates from the lungs of CF patients, six (30%) produced elastase, two (10%) produced exotoxin A and only one (5%) produced both. Forty-five (76%) of 59 *P. aeruginosa* clinical isolates from various sources in non-CF patients produced elastase, whereas 13 (25%) of 53 produced exotoxin A and 10 (17%) of the 59 produced both. Three of the isolates that produced both elastase and exotoxin A were tested in preliminary experiments to determine their ability to cause severe lung lesions in rats immunised with BSA. The isolate that caused the most severe lung pathology was chosen for use in the remainder of the vaccine experiments; it produced $> 1000 \mu$ g of elastase and *c.* 0.1 μ g of exotoxin A/ml of culture supernate.

Antibody response

Median IgG antibody titres to elastase, exotoxin A, cell envelopes of strain PAO1 and the clinical isolate, and LPS of strain PAO1 and the clinical isolate as determined by ELISA with antisera from each of the eight vaccine groups are given in table I. In each of the four vaccine groups receiving protein F there was a high titre (2560–5120) to cell envelopes of strain PAO1 but also a titre of 10240 to the homologous PAO1 LPS. However, protein F immunisation also produced titres ranging from 480–2560 to cell envelopes of the clinical isolate, even though the titre to the LPS of this heterologous immunotype strain was only 120–400. The antibodies reactive with the cell envelopes of the clinical isolate are thought to be predominantly against protein F. In a previous study in which immunisation with a protein F preparation purified from strain PAO1 afforded protection to rats against pulmonary lesions, the IgG antibody titres against cell envelopes of the heterologous immunotype strains in the pro-

Table I. Median IgG antibody titres in immunised rats on day 42

Vaccine group	Titre in ELISA against					
	elastase	exotoxin A	PAO1 CE*	clinical isolate CE	PAO1 LPS	clinical isolate LPS
BSA	0	0	0	0	0	0
Protein F	2.5	12.5	5120	2560	10240	160
Protein F plus elastase plus exotoxin A toxoid	6400	5760	2560	640	10240	340
Elastase	10240	10	10	15	0	0
Elastase plus protein F	7680	5	3840	480	10240	400
Elastase plus exotoxin A toxoid	10240	10240	0	0	0	0
Exotoxin A toxoid plus protein F	5	10240	5120	1280	10240	120
Exotoxin A toxoid	2.5	10240	10	2.5	0	0

*CE, purified cell envelopes.

Table II. Macroscopic evaluation of lung lesions in the eight vaccine groups of rats

Vaccine group	Number of rats with lung lesions scored*					Number of rats with severe lesions/ total challenged	p value
	0	1+	2+	3+	4+		
BSA	5	9	11	13	5	29/43	...
Protein F	15	9	7	3	2	12/36	0.002
Protein F plus elastase plus exotoxin A toxoid	5	10	7	2	0	9/24	0.017
Elastase	9	5	5	1	5	11/25	0.051
Elastase plus protein F	7	5	8	3	1	12/24	0.127
Elastase plus exotoxin A toxoid	1	9	4	4	2	10/20	0.147
Exotoxin A toxoid plus protein F	4	4	8	2	1	11/19	0.328
Exotoxin A toxoid	1	4	4	7	2	13/18	0.480

*Lung lesions were scored as follows: 0, normal 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 quarter lobe. Lesions scoring 2+ or greater were considered to be severe.

tected rats were 160–640.⁴ Thus, all four vaccine groups receiving protein F in the present study responded with a titre to cell envelopes of the clinical isolate that appeared to be capable of affording protection. However, the inclusion of elastase with protein F in any vaccine preparation resulted in reduced antibody titres against the cell envelopes of both strain PAO1 and the clinical isolate but an increased titre to LPS of the clinical isolate. For example, the two vaccines including both elastase and protein F induced titres of 640 and 480 to cell envelope and titres of 340 and 400 to the LPS of the clinical isolate. The two vaccines containing protein F without elastase had titres of 2560 and 1280 to cell envelope and titres of 160 and 120 to the LPS of the clinical isolate. The mechanism behind this apparent antagonistic effect of elastase on the antibody response to protein F is unknown. The use of elastase as an immunogen produced high titres (6400–10240) of antibodies reactive with elastase in the four vaccine

groups receiving elastase. Similarly, the four vaccine groups immunised with exotoxin A toxoid all responded with high titres (5760–10240) of antibodies reactive with exotoxin A. None of the vaccine components failed to elicit antibodies of the desired specificity, and none of the elicited antibodies were reactive at a significant level to the other vaccine components. Each of the eight vaccine combinations elicited antibodies as expected (table I).

Protection afforded by vaccines

When compared with the control rats immunised with BSA, the rats immunised with protein F alone were afforded significant protection from severe lung lesions upon subsequent challenge with the clinical isolate of *P. aeruginosa* encased within agar beads (table II); 67% of the rats immunised with BSA developed severe (2+ to 4+) lung lesions, whereas only 33% of the rats immunised with protein F

developed severe lesions ($p = 0.002$). The three-component vaccine consisting of protein F, elastase and exotoxin A toxoid also provided significant protection, as compared to the control rats immunised with BSA. Severe lesions were present in only 38% of the rats given the three-component vaccine ($p = 0.017$). However, the three-component vaccine did not provide any enhancement over the protection afforded by protein F alone as a vaccine (table II). Elastase alone as a vaccine provided protection of borderline statistical significance (severe lesions in 44% of immunised rats, $p = 0.051$). Exotoxin A toxoid alone provided no protection; severe lesions occurred in 72% of immunised rats ($p = 0.480$). The three vaccine combinations consisting of two preparations (elastase plus protein F, elastase plus exotoxin A toxoid or protein F plus exotoxin A toxoid) each appeared to exhibit an antagonistic effect which was not seen with the three-component vaccine. Surprisingly, although protein F alone provided statistically significant protection and elastase alone provided borderline statistically significant protection, the combination of the two preparations did not protect ($p = 0.127$). Furthermore, histological examination of the formalin-fixed lungs revealed that rats immunised with the combination of elastase plus protein F appeared to have a higher incidence of necrotising granulomas than the other groups. Necrotising granulomas were found in three of 15 lungs examined from rats immunised with protein F alone, whereas in rats immunised with elastase plus protein F, necrotising granulomas were found in nine of 14 lungs ($P = 0.019$). The incidence of necrotising granulomas (fig. 4) in all the remaining vaccine groups did not vary significantly from the incidence seen in the group immunised with protein F. Microscopic evaluation of the fixed lungs for the presence of peribronchial or perivascular inflammation and foci of interstitial chronic inflammation revealed no significant difference in the incidence of inflammation among rats immunised with the various vaccine preparations, with one exception. Rats immunised with elastase alone had a significantly lower incidence of peribronchial inflammation (fig. 2) than that found in rats immunised with any of the other vaccines. Only two of 11 rats immunised with elastase had peribronchial inflammation compared with 10 of 15 rats immunised with protein F ($p = 0.019$).

Discussion

There is considerable interest in the development of a vaccine for immunising CF children to prevent colonisation of their lungs by *P. aeruginosa*. The major antigens which have been investigated include LPS,¹⁵ alginate¹⁶ and outer-membrane protein F. Of these, only protein F is present throughout the entire colonisation process. LPS O-antigens are present initially but are lost during colonisation.¹⁷⁻¹⁹ Alginate is not produced initially but appears later.^{18,19} Fur-

thermore, since alginate is not tightly cell-associated but is released in copious amounts, it is difficult to imagine how antibodies to alginate could lead to clearance of the bacterial cells from the CF lung. Protein F appears to have many attributes that make it a prime vaccine candidate. It is an essential major outer-membrane protein²⁰ whose loss renders mutants avirulent.³ Protein F is surface-exposed in wild-type strains^{21,22} and is present and immunologically cross-reactive in all strains of *P. aeruginosa*.^{23,24} Antibodies to protein F do not cross-react with other genera of gram-negative rods²⁴ but do possess opsonophagocytic killing activity against *P. aeruginosa*.^{5,25} In two previous studies,^{4,5} active immunisation with either protein F purified from strain PAO1 or recombinant protein F afforded significant protection against both pulmonary lesions and pulmonary colonisation by *P. aeruginosa* in the rat model of chronic lung infection. All of these observations support the continued development of a protein F vaccine for possible use in CF patients to prevent chronic colonisation of their lungs with *P. aeruginosa*.

One possible approach for the development of a more effective vaccine to prevent *P. aeruginosa* pulmonary colonisation appeared to be the use of a two-pronged strategy of protection. Antibodies directed toward protein F should directly promote clearance of the bacterial cells from the lung. We reasoned that antibodies against certain toxins or enzymes might reduce the ability of the invading bacteria to cause inflammation and to retard the effectiveness of the host immune response. Thus, antibodies to these virulence determinants might aid the host in resisting initial colonisation and might enhance the effectiveness of the antibodies directed toward protein F in promoting bacterial clearance and reducing pulmonary lesions. Therefore, exotoxin A and elastase were selected as the virulence determinants to use as immunogens. Both exotoxin A^{8,26} and elastase^{8,9,26} had been shown to contribute to pathogenesis in chronic lung infections. Furthermore, Que and Woods²⁶ had proposed that CF patients might benefit from treatment with an anti-exoproduct regimen such as steroid therapy to inhibit the release of *P. aeruginosa* exoproducts and thus prevent lung injury due to their activity. More recently,^{7,10,27} an improved understanding of the pathological processes involved in chronic *P. aeruginosa* lung colonisation and tissue injury has further elucidated the important role played by elastase. Finally, the approach of trying to control specific inflammatory mechanisms at an early stage in order to halt tissue injury in the CF lung has now been widely endorsed,^{7,26,28,29} although the mechanism proposed involves the use of corticosteroid or non-steroidal anti-inflammatory agents and not the use of specific anti-elastase or anti-exotoxin A antibodies.

The rat model of chronic pulmonary infection established by the intratracheal instillation of *P. aeruginosa* encased in agar beads was used. Although no animal model truly represents the CF lung, this has

been widely recognised as the most appropriate animal model available for such studies. For the challenge strain of *P. aeruginosa*, a strain that produced both elastase and exotoxin A and that was a different LPS immunotype to the PAO1 strain from which protein F was purified was required. Clinical isolates of *P. aeruginosa* isolated from patients both with and without CF were screened to obtain this strain. Whereas it has been reported that *c.* 85% of all *P. aeruginosa* clinical strains produce elastase⁹ and 90% produce exotoxin A,³⁰ 76% of 59 clinical isolates from non-CF patients produced elastase and 25% of 53 isolates produced exotoxin A detectable by our assay methods. Only 17% of the 59 strains produced both elastase and exotoxin A. The clinical strains isolated from the lungs of CF patients exhibited a lower incidence of elastase production (30%), of exotoxin A production (10%), and of production of both elastase and exotoxin A (5%). This phenomenon of a reduced incidence of the production of various virulence determinants by CF strains is well documented.^{18, 31, 32} One strain was selected from those clinical strains producing both detectable elastase and exotoxin A for use in these vaccine studies; it typed with commercially available typing sera as Difco O-2 (Fisher-Devlin immunotype 3).

Rats were immunised with three doses of the eight different vaccine combinations administered intramuscularly into alternate hips at 14-day intervals. Two weeks after the third immunising dose (day 42), antisera were collected for determination of IgG antibody titres by ELISA. Each vaccine preparation performed as expected (table I), in that protein F, elastase and exotoxin A toxoid elicited high titres of antibodies that reacted specifically with the homologous immunogen without reacting significantly with the two heterologous immunogens. Combinations of two or three of the immunogens resulted in the appropriate mixture of antibodies as predicted (table I). Also on day 42, the rats were challenged with pulmonary inoculation of the clinical strain. Seven days later (day 49) the lungs were examined for the presence and severity of macroscopic lesions. Protein F provided significant protection from the development of severe (2+ to 4+) pulmonary lesions (table II), thus confirming its previously reported vaccine efficacy in this rat model.^{4, 5} Elastase alone provided protection of borderline statistical significance, but the exotoxin A toxoid given alone was ineffective in this model system against this challenge strain, even though it was shown to elicit antibodies capable of neutralising the lethal activity of native exotoxin A. The combining of any two of the three immunogens in a vaccine preparation resulted in a lack of protection from severe lesions (table II). This result was seen even with the combination of protein F plus elastase, although each of the two exhibited protective ability when used alone. The three-component vaccine did afford protection against the development of pulmonary lesions, but the level of protection was similar

to that provided by the protein F vaccine alone. Rats immunised with the three-component vaccine also had no better clearance of *P. aeruginosa* from their lungs than rats immunised with protein F only (data not shown). Thus, we concluded that the inclusion of elastase and exotoxin A toxoid into a protein F vaccine does not improve the protective efficacy over that of a protein F vaccine alone.

If the protection of the various vaccine groups against the most severe lung lesions (3+ and 4+) is considered, the following pattern is revealed. Eighteen (42%) of 43 control rats immunised with BSA had 3+ and 4+ lung lesions. Elastase alone did not protect from the most severe lung lesions, present in six (24%) of 25 rats immunised with elastase ($p = 0.110$), nor did exotoxin A toxoid—nine (50%) of 18 rats, $p = 0.38$. The combination of elastase and exotoxin A toxoid also failed to protect; six (30%) of 20 of these rats had 3+ and 4+ lung lesions ($p = 0.268$). However, all four vaccine combinations containing protein F afforded some degree of protection against 3+ and 4+ lung lesions. Protein F used alone reduced the number of rats with these most severe lung lesions to only five (14%) of 36, $p = 0.006$. In the three-component vaccine group, two (8%) of 24 of the rats had 3+ and 4+ lesions, $p = 0.003$. This is a comparable level of protection to that provided by the protein F vaccine alone. The addition of elastase to protein F retained protective efficacy—3+ and 4+ lesions in four (17%) of 24 rats, $p = 0.031$ —as did the addition of exotoxin A toxoid to protein F—three (16%) of 19 rats, $p = 0.040$. The conclusion remains the same when considering protection from 2+ to 4+ lesions, i.e., the vaccine efficacy of protein F is not enhanced significantly by the inclusion of either or both of these other virulence factors into the vaccine.

Histological examination of sections of fixed lungs was performed to ensure that protection at a microscopic level did not occur with some vaccine preparations but go undetected by our gross examination of lung lesions. Lungs were examined for deviations from normal lung architecture (fig. 1). The presence of peribronchial inflammation (fig. 2), perivascular inflammation (fig. 3) and the development of necrotising granulomas (fig. 4) were among those parameters noted. Rats immunised with either protein F alone or with the three-component vaccine displayed the same degree of inflammation and granuloma formation. No histological evidence was found to indicate that the three-component vaccine gave better protection than the protein F vaccine alone. The only vaccine preparation that reduced the degree of inflammation was elastase administered alone, which significantly decreased the incidence of peribronchial inflammation. This may indicate that elastase may possess some ability as a vaccine to protect lungs from inflammation and lesions related to inflammation. This observation may deserve further study. However, the combined vaccine of elastase with protein F resulted in a level of peribronchial inflammation similar to that of the

protein F and three-component vaccines and led to a significant increase in the incidence of necrotising granulomas.

In summary, although the combined vaccine approach, designed to elicit antibodies that reduce initial inflammation and thus make antibodies directed against protein F more effective appeared attractive,

we failed to find any evidence of enhanced efficacy of such a component vaccine in our studies and therefore conclude that this approach lacks promise for success.

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References

- Gilleland HE, Parker MG, Matthews JM, Berg RD. Use of a purified outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine in mice. *Infect Immun* 1984; **44**: 49–54.
- Matthews-Greer JM, Gilleland HE. Outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine against heterologous immunotype strains in a burned mouse model. *J Infect Dis* 1987; **155**: 1282–1291.
- Matthews-Greer JM, Robertson DE, Gilleland LB, Gilleland HE. *Pseudomonas aeruginosa* outer membrane protein F produced in *Escherichia coli* retains vaccine efficacy. *Curr Microbiol* 1990; **20**: 171–175.
- Gilleland HE, Gilleland LB, Matthews-Greer JM. Outer membrane protein F preparation of *Pseudomonas aeruginosa* as a vaccine against chronic pulmonary infection with heterologous immunotype strains in a rat model. *Infect Immun* 1988; **56**: 1017–1022.
- Gilleland HE, Gilleland LB, Hughes EE, Matthews-Greer JM. Recombinant outer membrane protein F of *Pseudomonas aeruginosa* elicits antibodies that mediate opsonophagocytic killing, but not complement-mediated bacteriolysis of various strains of *P. aeruginosa*. *Curr Microbiol* 1992; **24**: 1–7.
- Zach MS. Pathogenesis and management of lung disease in cystic fibrosis. *J Roy Soc Med* 1991; **84**(S18): 10–17.
- Høiby N, Koch C. *Pseudomonas aeruginosa* infection in cystic fibrosis and its management. *Thorax* 1990; **45**: 881–884.
- Woods DE, Cryz SJ, Friedman RL, Iglewski BH. Contribution of toxin A and elastase to virulence of *Pseudomonas aeruginosa* in chronic lung infections of rats. *Infect Immun* 1982; **36**: 1223–1228.
- Wretling B, Pavlovskis OR. *Pseudomonas aeruginosa* elastase and its role in *Pseudomonas* infections. *Rev Infect Dis* 1983; **5** Suppl 5: S998–S1004.
- Kharazmi A. Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. *Immunol Lett* 1991; **30**: 201–206.
- Iglewski BH, Sadoff JC. Toxin inhibitors of protein synthesis: production, purification, and assay of *Pseudomonas aeruginosa* toxin A. In: Moldave K, Grossman L (eds) *Methods in enzymology*. New York, Academic Press. 1979; **LX**: 780–793.
- Moriwaka K. Production of elastase and proteinase by *Pseudomonas aeruginosa*. *J Bacteriol* 1964; **88**: 745–757.
- Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978; **87**: 206–210.
- Klinger JD, Cash HA, Wood RE, Miler JJ. Protective immunization against chronic *Pseudomonas aeruginosa* pulmonary infection in rats. *Infect Immun* 1983; **39**: 1377–1384.
- Schaad UB, Lang AB, Wedgwood J *et al.* Safety and immunogenicity of *Pseudomonas aeruginosa* conjugate A vaccine in cystic fibrosis. *Lancet* 1991; **338**: 1236–1237.
- Pier GB, Small GJ, Warren HB. Protection against mucoid *Pseudomonas aeruginosa* in rodent models of endo-bronchial infections. *Science* 1990; **249**: 537–540.
- Hancock REW, Mutharia LM, Chan L, Darveau RP, Speert DP, Pier GB. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect Immun* 1983; **42**: 170–177.
- Luzar MA, Montie TC. Avirulence and altered physiological properties of cystic fibrosis strains of *Pseudomonas aeruginosa*. *Infect Immun* 1985; **50**: 572–576.
- Pier GB, DesJardins D, Aguilar T, Barnard M, Speert DP. Polysaccharide surface antigens expressed by nonmucoid isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients. *J Clin Microbiol* 1986; **24**: 189–196.
- Yoshimura F, Zalman LS, Nikaido H. Purification and properties of *Pseudomonas aeruginosa* porin. *J Biol Chem* 1983; **258**: 2308–2314.
- Lambert PA, Booth BR. Exposure of outer membrane proteins on the surface of *Pseudomonas aeruginosa* PAO1 revealed by labeling with [¹²⁵I]lactoperoxidase. *FEMS Microbiol Lett* 1982; **14**: 43–45.
- Mutharia LM, Hancock REW. Surface localization of *Pseudomonas aeruginosa* outer membrane porin protein F by using monoclonal antibodies. *Infect Immun* 1983; **42**: 1027–1033.
- Mutharia LM, Nicas TI, Hancock REW. Outer membrane proteins of *Pseudomonas aeruginosa* serotype strains. *J Infect Dis* 1982; **146**: 770–779.
- Counts GW, Schwartz RW, Ulness BK *et al.* Evaluation of an immunofluorescent-antibody test for rapid identification of *Pseudomonas aeruginosa* in blood cultures. *J Clin Microbiol* 1988; **26**: 1161–1165.
- Battershill JL, Speert DP, Hancock REW. Use of monoclonal antibodies to protein F of *Pseudomonas aeruginosa* as opsonins for phagocytosis by macrophages. *Infect Immun* 1987; **55**: 2531–2533.
- Que JU, Woods DE. Alteration of lung structure and function by *Pseudomonas aeruginosa*. *Pathol Immunopathol Res* 1987; **6**: 93–102.
- Williams JC, Lucas BJ, Knee C, Renzetti M, Donahue J. Acute lung injury induced by *Pseudomonas aeruginosa* elastase in hamsters. *Exp Lung Res* 1992; **18**: 155–171.
- Elborn JS, Shale DJ. Lung injury in cystic fibrosis. *Thorax* 1990; **45**: 970–973.
- Döring G. Microbial lung infections: new therapeutic strategies. *Pathol Biol* 1991; **39**: 587–591.
- Bjorn MJ, Vasil ML, Sadoff JC, Iglewski BH. Incidence of exotoxin production by *Pseudomonas* species. *Infect Immun* 1977; **16**: 362–366.
- Woods DE, Schaffer MS, Rabin HR, Campbell GD, Sokol PA. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. *J Clin Microbiol* 1986; **24**: 260–264.
- Burke V, Robinson JO, Richardson CJL, Bundell CS. Longitudinal studies of virulence factors of *Pseudomonas aeruginosa* in cystic fibrosis. *Pathology* 1991; **23**: 145–148.