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Effect of Immunization with Freund's Adjuvant and Pneumolysin on Histologic Features of Pneumococcal Infection in the Rat Lung In Vivo

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Immunization with Freund's adjuvant and pneumolysin and stimulation with Freund's adjuvant alone both reduced the severity of the pneumonia caused by injections of bacteria into the apical lobe bronchi of rats. Neither protocol influenced the incidence of pneumococcal bacteremia. Illness sufficiently severe to require sacrifice was delayed from 2.8 days in nonimmunized animals to 5.7 days in those immunized with Freund's adjuvant and pneumolysin ($P < 0.05$) and 4.5 days in those stimulated with Freund's adjuvant alone (P, not significant).

Diseases caused by *Streptococcus pneumoniae* are still very important in both the developing world and the Western world (1, 2). There is evidence that certain pneumococcal protein products contribute to the disease process (5). Pneumolysin is a thiol-activated toxin that is found in the cytoplasm of the pneumococcus and is released upon autolysis (3, 11). Pneumolysin affects the activities of human polymorphonuclear leukocytes, lymphocytes, and platelets (9, 12, 14, 15) and activates the classic pathway of complement independent of specific pneumococcal antibody (13, 17). The toxin also disturbs the structure and function of ciliated respiratory epithelium in vitro (7, 19). When pneumolysin is instilled into the partially ligated apical lobe bronchus of the rat lung in vivo, it causes a lobar pneumonia which is indistinguishable from that produced by live bacteria (8). We have investigated the effect of pneumolysin immunization on the severity of the histologic features of lobar pneumonia in our established rat model of lobar pneumonia.

The bacterial strain used was triple-washed *S. pneumoniae* (GB05, a serotype 3 strain) in phosphate-buffered saline (PBS) (10^5 CFU/ml). Recombinant pneumolysin was prepared from *Escherichia coli* JM109 harboring the pneumolysin gene in the expression vector PKK233-2, as previously described (16). The hemolytic titer of the purified toxin in PBS was approximately 1.5×10^6 hemolytic units/mg of protein (19).

Specific-pathogen-free Wistar rats (Charles River, Margate, United Kingdom) were used, and at the time of the operation they each weighed about 300 g. Rats were immunized intraperitoneally as described by Paton et al. (18). Immunization consisted of 0.5 ml of pneumolysin (70 μg/ml) in complete Freund's adjuvant (Sigma, Poole, Dorset, United Kingdom) injected intraperitoneally. Then, 10 days later, 0.5 ml of pneumolysin (70 μg/ml) in incomplete Freund's adjuvant was injected intraperitoneally. The second injection was repeated after a further 10-day interval. Eight days later, the operative procedure was carried out.

Animals were operated on in groups of 6 to 12 as previously described (8). Briefly, the thoracic cage was opened through the fourth intercostal space to reveal the apical lobe of the lung. A 7/0 prolene suture (Ethicon, Edinburgh, United Kingdom) was tied around the apical lobe bronchus as close as possible to its origin from the right main bronchus. The suture was tightened to narrow but not occlude the apical lobe bronchial lumen. Twenty microliters (2×10^3 bacteria or 800 ng of pneumolysin) of test solution was injected through the bronchial wall into the lumen of the apical lobe bronchus toward the lung periphery distal to the suture. The apical lobe was reinflated by gently inflating the lungs manually via the endotracheal tube, and the wound was repaired.

Seven groups of animals were studied, as shown in Tables 1 and 2. Animals used for histologic study were sacrificed 24 h postoperatively. Six of the animals in each group had blood samples taken by direct cardiac puncture for culture and measurement of pneumolysin antibody levels (13). The lungs were fixed in formalin before being processed routinely and embedded in paraffin wax for histology. A single longitudinal section exposing the two main axial bronchi was prepared and stained with hematoxylin and eosin. Slides were independently coded before histologic assessment. The overall appearance and tissue response were assessed by examination of 22 parameters, each scored on a semiquantitative scale of 0 to 3 as previously described (8). The scores for alveolar edema, alveolar infiltration, and the presence of interstitial and alveolar neutrophils (not macrophages) and a value reflecting the extent of the spread of the lesion throughout the lobe were summed to give a "pneumonitis" score. Further animals were observed for 1 week to assess the overall effects of immunization. Animals were sacrificed as described above when the independent observer, who was unaware of the treatment protocol, considered them sufficiently sick for death to be inevitable. Histological scores were analyzed by using a Kruskall one-way analysis of variance of ranks, with adjustments for multiple comparisons. Results were considered significant at the 5% level. Survival results were analyzed by the Mann-Whitney U test.

Five animals died from the operative procedure and were excluded from subsequent analysis. Pneumococcal bactere-

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TABLE 1. Results of experimental procedures leading to illness requiring sacrifice of rats

Group (treatment)[a]	No. of rats sacrificed on the following postoperative day:						No. of rats not sacrificed by day 7	Mean postoperative period by which sacrifice was required (days)
	1	2	3	4	5	6		
1 (immunization with FR and PL; no operation)	0	0	0	0	0	0	6	
2 (immunization with FR and PL; PBS injection)	0	0	0	0	0	0	6	
3 (no immunization; PL injection)	0	0	0	0	0	0	6	
4 (immunization with FR and PL; PL injection)	0	0	0	0	0	0	6	
5 (no immunization; pneumo injection)	1	3	0	1	0	1	0	2.8
6 (immunization with FR; pneumo injection)	0	0	3	0	0	3	0	4.5
7 (immunization with FR and PL; pneumo injection)	0	0	0	0	2	4	0	5.7[b]

[a] FR, Freund's adjuvant; PL, pneumolysin; pneumo, *S. pneumoniae* GB05. Six rats from each group were used.
[b] $P < 0.05$ versus group 5 by the Mann-Whitney U test.

mia was found at 24 h in the following number of animals from which blood samples had been taken in the three groups injected with live bacteria: five of the six animals in group 5 (not immunized and given pneumococcal injections), six of the six animals in group 6 (immunized with Freund's adjuvant alone and given pneumococcal injections), and six of the six animals in group 7 (immunized with Freund's adjuvant plus pneumolysin and given pneumococcal injections). No bacteria were isolated from the blood cultures of animals in the remaining four groups. Animals in groups 1, 2, 4, and 7, which were immunized with pneumolysin, had good (1,000 U) levels of neutralizing antibody to pneumolysin (13). The animals in the other groups had negligible (10 U) levels of antibody.

The results of illness of rats observed for 1 week following the experimental procedure are shown in Table 1. Only the animals injected with pneumococci into their apical lobe bronchi became sufficiently ill to require sacrifice. The mean postoperative day of illness requiring sacrifice was increased from 2.8 days to 5.7 days for animals immunized with Freund's adjuvant plus pneumolysin ($P < 0.05$). The mean postoperative day of illness requiring sacrifice was later for animals stimulated with Freund's adjuvant alone (4.5 days) but not significantly later than that for nonimmunized animals.

The results of histologic assessment of overall tissue responses and pneumonitis at 24 h are shown in Table 2. Injections of either pneumolysin (group 3) or pneumococci (group 5) into the apical lobe bronchi of nonimmunized animals produced significant lobar pneumonia compared with that of the control groups. Following immunization with Freund's adjuvant and pneumolysin, pneumolysin injections (group 4) no longer produced a significant inflammatory response in the lung compared with that produced by PBS injection (group 2), but neither the overall tissue response nor the pneumonitis score of group 4 was statistically different from those of group 3 (not immunized but given pneumolysin injections), so no benefit from immunization was shown. Stimulation with Freund's adjuvant alone (group 6) and immunization with Freund's adjuvant plus pneumolysin (group 7) both significantly reduced the pneumonitis ($P < 0.05$ and 0.01, respectively) following a pneumococcal injection, which was no longer significantly different from the inflammation induced by PBS alone. With respect to the overall tissue response, the reduction in inflammation was significant only for group 7 (immunized with Freund's adjuvant and pneumolysin). Neither the overall tissue responses nor the pneumonitis scores for group 7 (immunization with Freund's adjuvant plus pneumolysin) and group 6 (stimulation with Freund's adjuvant alone) were significantly different from each other.

Immunization with Freund's adjuvant and pneumolysin together did not affect the incidence of bacteremia following pneumococcal infection, but it did delay illness severe enough to require sacrifice of such immunized animals compared with that of nonimmunized controls. Paton et al.

TABLE 2. Mean histologic scores for overall tissue responses and pneumonitis 24 h after experimental protocol[a]

Group (treatment)[b]	No. of animals	Overall tissue response	Pneumonitis
1 (immunization with FR and PL; no operation)	9	3.6 (0.7)	0.1 (0.1)
2 (immunization with FR and PL; PBS injection)	8	7.0 (1.1)	1.0 (0.7)
3 (no immunization; PL injection)	8	13.0[c] (1.8)	4.5[d] (0.9)
4 (immunization with FR and PL; PL injection)	9	7.2 (1.2)	1.9 (0.7)
5 (no immunization; pneumo injection)	11	14.5[e] (1.7)	4.8[f] (1.0)
6 (immunization with FR; pneumo injection)	9	8.9 (1.0)	1.6[g] (0.5)
7 (immunization with FR and PL; pneumo injection)	8	6.4[h] (1.7)	1.4[i] (0.8)

[a] For details about the determinations of overall tissue responses and pneumonitis, see the text. Standard errors are in parentheses.
[b] FR, Freund's adjuvant; PL, pneumolysin; pneumo, *S. pneumoniae* GB05.
[c] $P < 0.001$ versus group 1.
[d] $P < 0.001$ versus group 1; $P < 0.01$ versus group 2.
[e] $P < 0.001$ versus group 1; $P < 0.01$ versus group 2.
[f] $P < 0.001$ versus groups 1 and 2.
[g] $P < 0.05$ versus group 5.
[h] $P < 0.001$ versus group 5.
[i] $P < 0.01$ versus group 5.

(18) noted a similarly improved rate of survival in a mouse model of pneumococcal pulmonary infection and, like us, also found that pneumococcal bacteremia was unaffected by immunization. Immunization with Freund's adjuvant and pneumolysin reduced the severity of the lobar pneumonia produced by injections of live bacteria. This improvement occurred despite the known inflammatory effects of other components of the pneumococcus, such as its cell wall (20, 21).

A reduction in the inflammation produced by pneumococcal infections was also seen with animals stimulated with Freund's adjuvant alone, and the incidence of bacteremia was again unaffected. This may indicate that immunization with Freund's adjuvant affects pneumococcal growth in the rat lung, the release or effect of bacterial products, or a rat's immune response to the infection. A similar effect with complete Freund's adjuvant has previously been described. Granulocytopenic mice were partially protected against the lethal consequences of subsequent infections with *Staphylococcus aureus* by prior immunization (29 days earlier) with complete Freund's adjuvant (6). In our experiments, the complete Freund's adjuvant may have nonspecifically activated pulmonary macrophages, leading to enhanced clearance of injected pneumococci and/or particulate proinflammatory mediators, such as cell wall components (20, 21). We speculate that this immunization protocol might also in some way modify the early immune response to pneumococcal infection, which could then influence the final histologic picture. For example, the release of cytokines by local T-cell populations might have been influenced by immunization with Freund's adjuvant, which could in turn influence the neutrophil response. Variable T-lymphocyte cytokine responses have been previously described for other biological systems exposed to different antigens (10).

Recently, a genetically engineered, pneumolysin-negative pneumococcus was shown to be more rapidly cleared from the bloodstream of the mouse and was significantly less virulent than its pneumolysin-positive wild-type parent. The virulence of the mutant strain was restored by reinstatement of its ability to produce the toxin (4). Therefore, although immunization with pneumolysin did not influence the incidence of bacteremia, it might affect clearance of pneumococci from the bloodstream or neutralize the adverse effects of pneumolysin released at this site.

Paton et al. (18) found that immunization with Freund's adjuvant together with pneumolysin prolonged the survival of mice infected with *S. pneumoniae*. Histological examinations during their studies revealed evidence of pneumonic consolidation in both control and immunized mice, but their animals were examined only at the times of their death, and any beneficial effects of immunization causing delays in the development of pneumonia would have been missed by this investigation. A group of nonimmunized animals was not included in the experiments reported by Paton et al. (18), and thus the beneficial effects of immunization with Freund's adjuvant alone on the survival of animals could not be assessed. Our study shows that the decrease in the severity of the pneumonia in immunized animals is attributable not to the specific immune response elicited by pneumolysin but to nonspecific stimulation by Freund's adjuvant.

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