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## Development of a model of focal pneumococcal pneumonia in young rats

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### Abstract

**Background:** A recently licensed pneumococcal conjugate vaccine has been shown to be highly effective in the prevention of bacteremia in immunized children but the degree of protection against pneumonia has been difficult to determine.

**Methods:** We sought to develop a model of *Streptococcus pneumoniae* pneumonia in Sprague-Dawley rats. We challenged three-week old Sprague-Dawley pups via intrapulmonary injection of *S. pneumoniae* serotypes 3 and 6B. Outcomes included bacteremia, mortality as well histologic sections of the lungs.

**Results:** Pneumonia was reliably produced in animals receiving either 10 or 100 cfu of type 3 pneumococci, with 30% and 50% mortality respectively. Similarly, with type 6B, the likelihood of pneumonia increased with the inoculum, as did the mortality rate. Prophylactic administration of a preparation of high-titered anticapsular antibody prevented the development of type 3 pneumonia and death.

**Conclusion:** We propose that this model may be useful for the evaluation of vaccines for the prevention of pneumococcal pneumonia.

### Background

*Streptococcus pneumoniae* is the leading cause of bacterial pneumonia in children and adults in both developing and developed countries. In the United States, *S. pneumoniae* accounts for about 500,000 cases of pneumonia each year [1]. The recent dramatic rise in the prevalence of clinical isolates that are multi-drug resistant raises the possibility that antibiotic therapy may become less effective in treating pneumococcal disease. At the same time, the institu-

tion of universal immunization with polysaccharide-protein conjugates in the United States offers the promise of significant reduction in the number of cases of invasive pneumococcal disease [2]. The extent to which conjugate vaccines will have an impact on mucosal and respiratory pneumococcal disease, however, is less certain. Data from the Kaiser Permanente Northern California vaccine trials and phase IV studies suggest a significant reduction in the frequency of clinically-diagnosed as well as radiologically-

confirmed pneumonia [2,3]. Due to the difficulties inherent in the diagnosis of pneumonia, however, these data must be interpreted with caution.

In addition, because the distribution of serotypes responsible for pneumococcal pneumonia is not as well characterized as for bacteremic disease, the spectrum of coverage provided by conjugate vaccines may be narrower for non-bacteremic pneumonia than for bacteremic illness. This is particularly relevant in the developing world, where pneumococcal serotypes responsible for both invasive and mucosal disease differs from that in industrialized countries [4].

Current animal models of pneumococcal disease have several limitations. Not all serotypes are reliably pathogenic in mice and most models require very high inocula to cause disease. In addition, existing animal models of invasive pneumococcal disease are highly virulent and depend on outcomes such as bacteremia, sepsis and mortality [5-8]. These models, with the exception of the chinchilla otitis media model [9], therefore may not be appropriate for the evaluation of vaccines for the prevention of nonbacteremic or mucosal pneumococcal disease.

In this study we sought to develop a model of focal pneumococcal pneumonia in young rats. In addition, we hypothesized that pretreatment with anticapsular pneumococcal antibody would prevent pulmonary pathology in this model.

## Methods

### Bacteriologic methods

Strains of *Streptococcus pneumoniae* were originally obtained from the collections of Drs. George Siber (Wyeth-Lederle Vaccine and Pediatrics, Pearl River, NY) and David Briles (University of Alabama, Birmingham) and passaged through rats via intraperitoneal challenge as described previously [7]. Passaged strains were stored in either skim milk or Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, MI) and 20% glycerol at -70°C, and fresh subcultures were used for all experiments. Inocula for animal challenge were prepared by growing *Streptococcus pneumoniae* to mid-log phase (approximately 10<sup>7</sup> CFU/ml) in 10 ml of Todd-Hewitt broth supplemented with 0.5% yeast extract. The suspension was diluted in 0.5% low melting-point agarose (as an adjuvant [7]) to a desired inoculum concentration. The number of cfus delivered in the inoculation was calculated the following day based on the dilutions made from the mid-log phase culture.

### Animal model

Outbred virus-free Sprague-Dawley rats were obtained from Charles River Laboratories, Wilmington, MA. Preg-

nant female rats were quarantined 4 to 5 days prior to delivery of a litter. On day 4 post delivery, infant pups from all litters were randomly redistributed so that each mother had 10–12 pups. Animals weaned at about three weeks of life, after which the dam was removed and the litter rats were distributed in cages of six animals each.

Intrathoracic inoculations were performed in the following fashion. The right chest of each 3-week-old rat was prepared with alcohol, and a 0.05 ml inoculum was injected transthoracically into the mid-right lung via a 29-gauge needle on an insulin syringe. The depth of the intrathoracic injection was controlled by a small hemostat clipped at the base of the needle. Following the injection, animals were observed for the presence of any distress that may signify the development of a pneumothorax. Animals that appeared ill immediately after the injection were sacrificed.

In a second series of experiments, animals were randomly assigned to receive either 1 cc of bacterial polysaccharide immune globulin (BPIG) or normal saline intraperitoneally, administered 24 hours prior to bacterial challenge. BPIG is a hyperimmune serum obtained from adults immunized with 23-valent pneumococcal vaccine, *Haemophilus influenzae* type b conjugate vaccine and *Neisseria meningitidis* polysaccharide vaccine and consists predominantly of IgG, with trace amounts of IgA and IgM. Outcomes following intrathoracic injection were compared between the two groups (see below).

### Outcomes

Mortality was assessed for 7 days after inoculation. Bacteremia was assessed on days 1 and 4 after inoculation. The distal dorsal tail vein of each unanesthetized pup was cleansed with 70% alcohol and punctured with a sterile lancet and 0.01 ml of blood was spread on 5% sheep's blood agar. Plates were incubated overnight at 37°C, and colonies were counted the following morning. The lower limit of detection of bacteremia was 100 cfu/ml.

Randomly selected animals were sacrificed on days 2 and 4 following challenge for lung culture and assessment of lung histopathology. Lung microbiology and histopathology specimens were obtained from randomly selected animals sacrificed on day 2 and 4 following intrathoracic challenge. Lung cultures were obtained using sterile techniques. Lungs were dissected en bloc from the thorax, transported in sterile vials, and then homogenized using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Lung cultures were performed on blood agar plates supplemented with gentamicin (2.5 mg/L) to suppress the growth of normal oral flora. Lung specimens were also obtained for histologic examination. Formalin (10%) was instilled via tracheal instillation via a 20-gauge

**Table 1: Effect of serotype and inoculum size on the occurrence of pneumonia, bacteremia, and mortality following intrathoracic challenge in rats**

| Serotype | Inoculum (cfu)  | N  | % pneumonia | % bacteremia | % mortality |
|----------|-----------------|----|-------------|--------------|-------------|
| 19       | 10 <sup>6</sup> | 10 | 100         | 50           | 50          |
| 6B       | 10 <sup>3</sup> | 5  | 40          | 0            | 0           |
|          | 10 <sup>4</sup> | 6  | 33          | 0            | 0           |
|          | 10 <sup>5</sup> | 6  | 50          | 0            | 0           |
|          | 10 <sup>6</sup> | 12 | 75          | 100          | 100         |
| 3        | 10              | 10 | 100         | ND           | 30          |
|          | 100             | 10 | 100         | ND           | 50          |

ND: not determined

intravenous catheter immediately upon dissection. An animal was considered as having had pneumonia if any area of polymorphonuclear infiltration or infiltrative consolidation of lung parenchyma was seen under 100X.

Experimental procedures for use with animals were reviewed and approved by the Children's Hospital Animal Care and Use Committee, and were in keeping with the guidelines of the National Institutes of Health.

## Results

### **Virulence is dependent on serotype and inoculum size (Table 1)**

In our initial experiments, we used a strain of *S. pneumoniae* serotype 3, which was found to be highly virulent in a previously published infant rat model of invasive pneumococcal disease [7]. An inoculum of 10 or 100 cfu reliably produced pneumonia in 100% of animals. This serotype was highly virulent; death occurred in 3/10 and 5/10 animals, with inocula of 10 and 100 cfu respectively. While we did not assay for bacteremia in this subset of animals, we found in pilot experiments that the presence of bacteremia was a highly reliable predictor of mortality in this model (data not shown).

Given the high virulence of type 3 in this model, we next studied a strain of serotype 6B. The aim of these experiments was to select a strain and inoculum size that would cause pneumonia without bacteremia or death. Using inocula ranging from 10<sup>3</sup> to 10<sup>6</sup> colony-forming units (cfu) per 0.05 cc (the volume of the intrathoracic injection), we then examined the frequency with which pneumonia developed. Table 1 demonstrates that the frequency of pneumonia increases with the inoculum size. This can also be seen with representative histopathological sections in Figure 1. Bacteremia was only detected in animals that received the highest inoculum (10<sup>6</sup> cfu/dose). Nonbacteremic animals looked clinically well up to seven days after inoculation. This remained true regard-

less of whether pneumonia was present on histopathological examination.

From these experiments, we concluded that a transthoracic inoculum of this strain of serotype 6B with 10<sup>5</sup> cfu would result in pneumonia in approximately 50% of animals, without causing bacteremia. Using a similar inoculum with a serotype 19F isolate (10<sup>6</sup> cfu), pneumonia was produced in all challenged animals, but was also associated with 50% bacteremia and mortality.

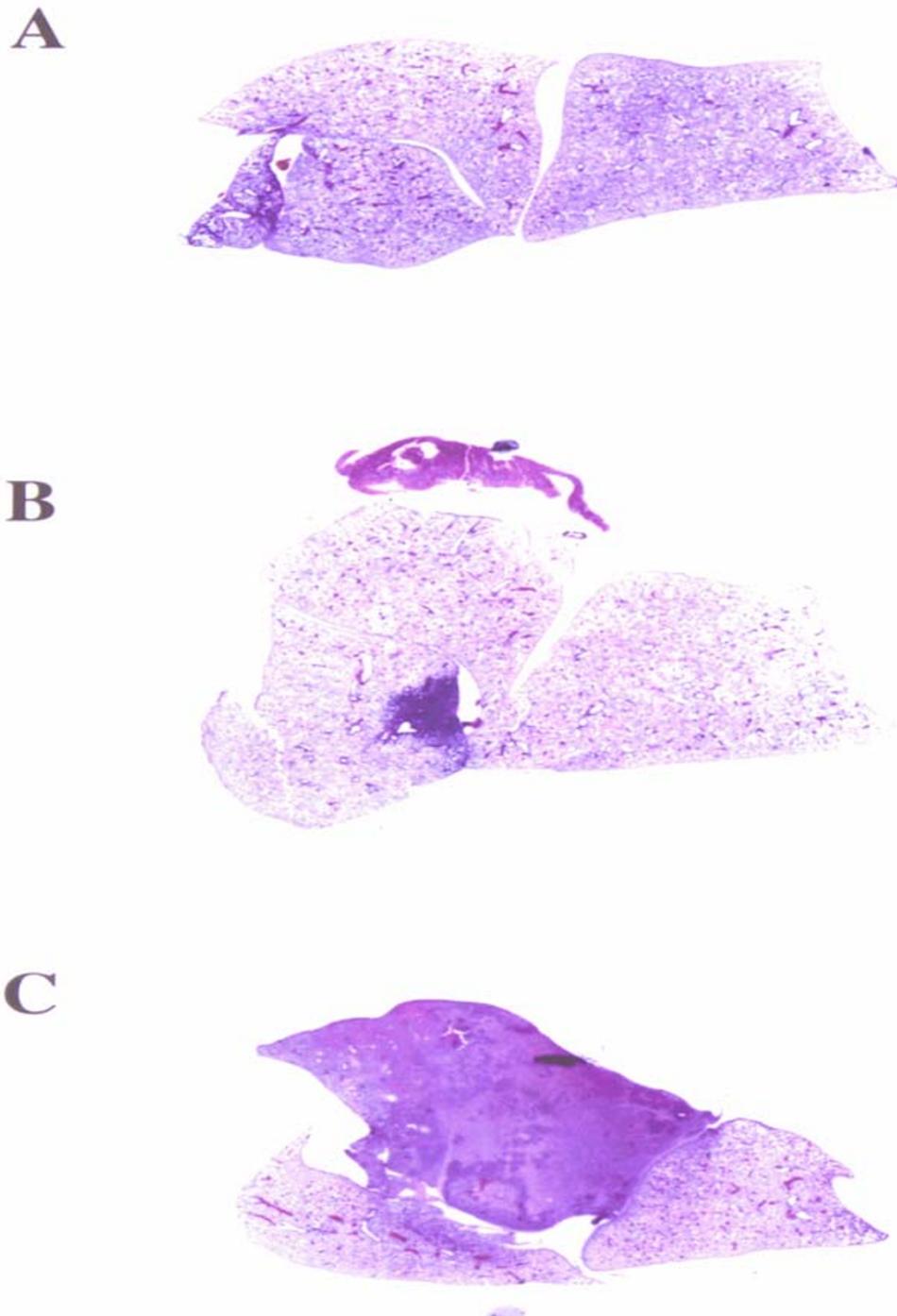
### **Pretreatment with bacterial polysaccharide immune globulin prevents pneumonia and death (Table 2)**

For the following experiments, animals were challenged intrathoracically with WU-2, a serotype 3 laboratory strain of *S. pneumoniae*. Animals that received prophylactic intraperitoneal administration of 1 ml BPIG were significantly less likely to develop pneumonia than animals that received saline (0/23 vs. 17/30 (57%),  $p < 0.0001$ ). Mortality was significantly reduced as well in pre-treated animals (2/30 vs. 14/30,  $p < 0.001$ ).

## Discussion

We have developed a model of focal pneumococcal pneumonia in young rats. As has been previously noted in mouse and infant rat models by different investigators, we found that the virulence of *Streptococcus pneumoniae* in our model is dependent on the serotype. In our model, the bacterial inoculum necessary to produce pneumonia in >50% of animals was 100 cfu for WU-2 (serotype 3 strain) and 10<sup>5</sup> cfu for a serotype 6B strain, a 1000-fold difference. By varying the serotype and the inoculum, the frequency of pneumonia and the mortality rate was correspondingly modified. Of interest, despite the high virulence of WU-2 in this model, pneumonia and mortality could still be abrogated by pre-administration of bacterial polysaccharide immune globulin.

Previously established animal models of pneumococcal invasive disease have several disadvantages. The most



**Figure 1**

Hematoxylin-Eosin stain preparation of lung sections (original magnification 100×) obtained from autopsied rats following injection with a low (100 cfu per injection, panel A), medium (1000 cfu per injection, panel B) and high (10,000 cfu per injection, panel C) inoculum of type 6B pneumococcus in 0.5% low melting-point agarose. As the size of the inoculum increases, there is a clear progression from normal-appearing lung, focal pneumonia and diffuse pneumonia. Shown are 3 slides from a representative experiment.

**Table 2: Pretreatment with bacterial polysaccharide immune globulin (BPIG) prevents pneumonia and death due to type 3 pneumococcus in rats**

| Serotype | Inoculum (cfu) | Pretreatment | N  | # animals with pneumonia (%) | mortality n, (%) |
|----------|----------------|--------------|----|------------------------------|------------------|
| 3        | 100            | Saline       | 30 | 17 (57)                      | 14 (47)          |
|          | 100            | BPIG         | 30 | 0 (0) *                      | 2 (7) **         |

\* P < 0.0001 and \*\* P < 0.001 by Fisher's Exact

commonly used model of pneumococcal disease has been the mouse model [5], in which very high inocula are required, particularly for higher numbered serotypes, which are less virulent in the mouse. Furthermore, these models require intraperitoneal or intravenous routes of inoculation, which are not representative of the human route of pulmonary infection. Conversely, we have previously published data from an infant rat model in which inocula of different serotypes ranging from 1 to 400 cfu caused overwhelming pneumonia and sepsis [7]. While this model has been useful for the determination of minimal protective concentrations of anticapsular antibodies (a range that was subsequently confirmed in the Kaiser Permanente heptavalent pneumococcal conjugate trial in California), a legitimate concern is that this model may result in an underestimation of the protective capacity of antibodies (whether capsular or other), by virtue of increased susceptibility of the infant rat to pneumococci. The data presented here may represent a more physiologically relevant model of pneumococcal pneumonia. Using a strain of serotype 6B, we show that at the highest inoculum of  $10^6$  cfu per injection, animals develop a fulminant pneumonia with 100% bacteremia and mortality. In contrast, lowering the inoculum (using a range between  $10^3$  and  $10^5$  cfu per injection), we were able to show that pneumonia can be reproduced reliably, without concomitant bacteremia, sepsis, or high mortality. In sum, we propose that this model may therefore be more applicable for the study of the pathophysiology and therapeutic interventions in nonbacteremic pneumococcal pneumonia than previously published models.

We previously showed that the onset of bacteremia and sepsis occurs later in rats challenged via the intrathoracic route compared to the intraperitoneal route [7]. We also demonstrated that rats challenged via the intrathoracic route reliably develop pneumococcal pneumonia, as demonstrated by an increase in the colony counts from whole lung tissue cultures. Together, these data suggest that the initial event leading to disease in these animals is the establishment of pneumococcal pneumonia, followed by seeding of the bloodstream and subsequent sepsis.

Recent data suggest that the expression of virulence genes is phase-variable [10]. Most recently, investigators have demonstrated that pneumococci grown in peritoneal fluid express significantly more pneumolysin, a known intracellular pulmonary toxin, than those cultured in vitro [11]. It is quite plausible that the expression of different virulence genes may vary depending on whether the organism is grown in the lung versus the bloodstream or peritoneum. Using our model of nonbacteremic pneumococcal pneumonia, an analysis of the virulence genes expressed during lung infection vs. peritoneal challenge may provide important information regarding the pathophysiology of pneumococcal lung disease and the factors which promote dissemination of pneumococci from the lung to the bloodstream.

### Conclusions

We have developed a model of nonbacteremic pneumococcal pneumonia in the Sprague-Dawley rat. The inocula in this model range from  $10^2$  and  $10^4$  cfu per intrathoracic injection, which are substantially lower than that required in mouse models of pneumococcal disease. We were able to utilize this model to demonstrate a protective effect of anticapsular antibody against pneumonia and death. In this light, we propose that this model may be useful for the evaluation of vaccines for the prevention of pneumonia as well as for the study of the pathophysiologic mechanisms that lead to the development of pneumonia and bacteremia.

### Competing interests

None declared.

### Authors' contributions

RM, AMS, CMT and RAS carried out the animal experiments, participated in the analysis and all contributed to the original drafts of the manuscript. RM and AMS reviewed the histological preparations. RNH and GRF participated in the design of the study, the interpretation of the results and in the statistical analysis. All authors read and approved the final manuscript.

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