MODELS OF INFECTION

Establishment of a model of penicillin-resistant *Streptococcus pneumoniae* pneumonia in healthy CBA/J mice

K. TAKASHIMA, K. TATEDA, T. MATSUMOTO, T. ITO*, Y. IIZAWA†, M. NAKAO† and K. YAMAGUCHI

Department of Microbiology, Toho University School of Medicine, Tokyo, *Drug Safety Research Laboratories and †Pharmaceutical Research Laboratories III, Takeda Chemical Industries Ltd, Osaka, Japan

Examination of strain differences in the susceptibility of mice to experimental respiratory tract infection with penicillin-resistant *Streptococcus pneumoniae* TUM19 revealed that a fatal infection model could be induced in immunocompetent CBA/J mice, but not in C3H/HeN, C57BL/6 or ICR mice. After intranasal instillation of *c.* 10^6 cfu of *S. pneumoniae*, the bacterial counts in the lungs of CBA/J mice increased from 10^5 to 10^7 cfu after 3–5 days, and gradually increased thereafter. The challenge organisms localised mainly in the lungs until 14 days after infection. Mice began to die *c.* 7 days after infection, and by 3 weeks most of the mice had died. Histopathologically, infiltration of neutrophils and lymphocytes around bronchi was observed from 1 day after infection, and fibrin deposition was seen in alveolar and bronchial spaces from 5 days. This model may be useful for investigating therapy of respiratory tract infection caused by penicillin-resistant *S. pneumoniae* because its pathological features resemble those observed in the human disease.

Introduction

Streptococcus pneumoniae is a common cause of community-acquired pneumonia [1, 2]. Because this organism was originally highly susceptible to penicillin (MIC < 0.1 mg/L), penicillin was the drug of choice for treatment. However, penicillin-resistant strains have been isolated with increasing frequency in virtually every part of the world in recent years [3], and successful treatment with penicillin is no longer guaranteed. In addition, penicillin-resistant strains may be resistant to a wide range of β -lactam and macrolide antibiotics [4, 5] and new strategies for treating these infections should be investigated.

A suitable infection model that closely resembles the features of human disease is necessary to investigate the optimal treatment of infections caused by penicillin-resistant strains. Azoulay-Dupuis *et al.* [6] and Moine *et al.* [7] described a murine model of

pneumonia caused by penicillin-resistant *S. pneumoniae* in leucopenic mice, but the strain was avirulent in immunocompetent mice. Their model represented acute infection, with a high incidence of bacteraemia in the early phase of infection, and most mice died within 2 days. However, *S. pneumoniae* frequently causes community-acquired pneumonia in healthy individuals and only 20–30% of pneumococcal pneumonia is accompanied by bacteraemia [8]. There have been no reports of experimental pneumonia caused by penicillin-resistant *S. pneumoniae* in healthy immunocompetent mice.

Strains of mice differ in susceptibility to pneumococcal infection [9] and these differences might be useful for establishing a new infection model with penicillin-resistant *S. pneumoniae*. Therefore, the comparative susceptibility of various strains of mice to experimental respiratory tract infection with penicillin-resistant *S. pneumoniae* strain TUM19 was examined.

Materials and methods

Mice

Five-to-seven-week-old female CBA/J, C57BL/6 and ICR mice were obtained from Charles River Japan Inc., Kanagawa, Japan. Six-to-seven-week-old female C3H/

Received 5 Jan. 1996; revised version accepted 27 Feb. 1996.

Corresponding author: Dr K. Takashima. Present address: Pharmaceutical Research Laboratories III, Takeda Chemical Industries Ltd, 2-17-85 Jusohonmachi, Yodogawa-ku, Osaka 532, Japan.

HeN mice were obtained from SLC Japan Inc., Shizuoka, Japan. Mice were caged in groups of five-to-ten and given food and water *ad libitum*.

Organisms

A clinical isolate of *S. pneumoniae* serotype 19 (strain TUM19), maintained as a stock culture in the Department of Microbiology, Toho University School of Medicine, Tokyo, Japan, was used. The MIC of benzylpenicillin (Meiji Seika Kaisha Ltd, Tokyo, Japan) against the organism, determined by the broth microdilution method according to the reference procedure recommended by the Japanese Society for Chemotherapy [10] was 2 mg/L. The organisms were incubated at 37°C for 20 h on Mueller-Hinton Agar (MHA; Difco Laboratories) supplemented with defibrinated horse blood 5% suspended in sterile physiological saline.

Respiratory tract infection procedure

Mice were anaesthetised by intraperitoneal injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, ILL, USA) 50 mg/kg of body weight and challenged intranasally by instillation of 40 µl of bacterial suspension.

Bacteriological examination

Mice were killed by bleeding from the axillary artery and vein under ether anaesthesia. The lungs were removed aseptically and homogenised in 2 ml of sterile physiological saline with a Teflon tissue homogeniser. The homogenates and blood were serially diluted 10-fold with sterile physiological saline; 0.1 ml of the various dilutions was inoculated on MHA plates supplemented with defibrinated horse blood 5% and incubated at 37°C for 20 h. Colonies were counted and the results were expressed as the log₁₀ cfu/lung and cfu/ml of blood.

Histopathological examination

The lungs of the mice were fixed in neutral buffered formalin 10% and embedded in paraffin. Sections were stained with haematoxylin and eosin, phosphotungstic acid haematoxylin solution, Gram's stain and azan stain, and examined by light microscopy.

Results

Susceptibility of various strains of mice to infection

After intranasal instillation of 2×10^7 cfu of *S. pneumoniae* TUM19, eight of 11 CBA/J mice died within 7 days, whereas the all other strains of mice (C3H/HeN, C57BL/6 and ICR) survived (Table 1). Most of the mice that survived had no gross pulmonary lesions and eliminated the organisms from their lungs. Therefore, CBA/J mice were chosen for the following experiments.

Effect of challenge dose

With challenge doses of 5.6×10^6 and 1.1×10^6 cfu/mouse, most mice died *c.* 7 days and 11 days after infection, respectively (data not shown). With a challenge dose of 2.2×10^5 cfu/mouse, all five mice survived for 14 days after infection; bacteria were not recovered from the lungs of two of these mice. Thus, $\geq 10^6$ cfu/mouse were required to cause uniform infection.

Bacteriological examination

After challenge with *c.* 10^6 cfu/mouse, the bacterial counts in the lungs increased slowly from 10^4 to

Fig. 1. Bacterial counts in the lungs (●) and blood (○) of CBA/J mice infected with *S. pneumoniae* TUM19 by intranasal instillation. Each point represents the value for a mouse.

Table 1. Susceptibility of various strains of mice to intranasal instillation with *S. pneumoniae* TUM19*

Mouse strain	Mortality at 7 days (death/total)	Gross pulmonary lesion (positive/total)	Bacterial recovery from lung (positive/total)
CBA/J	8/11	NT	NT
C3H/HeN	0/11	3/11	1/11
C57BL/6	0/5	0/5	0/5
ICR	0/5	0/5	0/5

NT, not tested.

**c.* 2×10^7 cfu/mouse.

10^5 cfu/lung by 3 days after infection and increased rapidly to 10^7 cfu at 5 days. The number of bacteria in the lungs increased gradually thereafter (Fig. 1). Some mice developed bacteraemia when the bacterial counts in the lungs reached $>10^8$ cfu from 7 days after infection. Mice began to die *c.* 7 days after infection, and by 3 weeks 83% (48 of 58) had died.

Histopathological changes

Histopathological examination of lung from infected mice revealed the existence of bronchopneumonia. Infiltration of neutrophils and lymphocytes around bronchi and hyperplasia of alveolar epithelia were observed at 1 day (Fig. 2A); haemorrhage and necrosis of alveolar epithelia were seen from 3 days after

Fig. 2. Histopathological changes in the lungs of CBA/J mice infected with *S. pneumoniae* TUM19 by intranasal instillation. Stained with haematoxylin and eosin. **A**, Neutrophil and lymphocyte infiltration around bronchi and hyperplasia of alveolar epithelia 1 day after infection ($\times 400$). **B**, Haemorrhage and necrosis of alveolar epithelia 3 days after infection ($\times 400$). **C**, Fibrin deposition (arrow) in the bronchial and alveolar spaces 5 days after infection ($\times 400$). **D**, Proliferation of fibroblasts (arrow head) and infiltration of foam cells (arrow) 14 days after infection ($\times 400$).

infection (Fig. 2B). Similar lesions extended throughout the lungs thereafter (Fig. 2C, D). In addition to these inflammatory responses, fibrin deposition in the bronchial and alveolar spaces was found at 5 days (Fig. 2C), and fibrosis accompanied by proliferation of fibroblasts and infiltration of foam cells was observed at 14 days (Fig. 2D). Fibrin and collagen fibres were clearly identified by phosphotungstic acid, haematoxylin stain and azan stain, respectively. The infecting organisms in the bronchial and alveolar spaces were detected by Gram's stain after 5 days of infection (data not shown).

Discussion

It has been thought difficult to establish an experimental respiratory tract infection model with penicillin-resistant *S. pneumoniae* in immunocompetent mice. Clinical isolates of penicillin-resistant *S. pneumoniae* predominantly belong to serotypes 6, 14, 19 and 23 [3, 4], which are naturally avirulent for mice [11]. Several investigators have failed to induce pneumonia with any penicillin-resistant strains belonging to these serotypes in immunocompetent C57BL/6 and Swiss mice [6, 7]. This study also failed to induce pneumonia in C3H/HeN, C57BL/6, and ICR mice by intranasal challenge with serotype 19 strain (TUM19). However, CBA/J mice showed high susceptibility to the infection: most mice died after intranasal challenge with $\geq 10^6$ cfu of the organism. Genetic defects in the host defences against bacterial infection have not been reported in CBA/J mice and it is unclear why they are susceptible to pneumococcal infection.

Intranasal inoculation of CBA/J mice with 10^6 cfu of *S. pneumoniae* TUM19 induced a fatal infection. The challenge organisms localised mainly in the lungs without causing bacteraemia, at least in the first week of infection, while the bacterial counts in the lungs increased from 10^4 to 10^7 cfu. Histopathological features such as intense infiltration of inflammatory cells and fibrin deposition in alveolar spaces resembled the features of pneumococcal pneumonia in man [12].

As the course of the infection is long and lung lesions develop gradually, this model might be useful for examining the effect of antibiotics at the various stages of pneumonia caused by penicillin-resistant *S. pneumoniae*. Furthermore, it is also possible to investigate methods for the suppression of inflammation induced by pneumococcal infection.

References

1. Woodhead MA, Macfarlane JT, McCracken JS, Rose DH, Finch RG. Prospective study of the aetiology and outcome of pneumonia in the community. *Lancet* 1987; **1**: 671–674.
2. Pachon J, Prados MD, Capote F, Cuello JA, Garnacho J, Verano A. Severe community-acquired pneumonia. Etiology, prognosis, and treatment. *Am Rev Respir Dis* 1990; **142**: 369–373.
3. Appelbaum PC. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. *Clin Infect Dis* 1992; **15**: 77–83.
4. Baquero F, Martínez-Beltrán J, Loza E. A review of antibiotic resistance patterns of *Streptococcus pneumoniae* in Europe. *J Antimicrob Chemother* 1991; **28** Suppl C: 31–38.
5. Liñares J, Alonso T, Pérez JL *et al.* Decreased susceptibility of penicillin-resistant pneumococci to twenty-four β -lactam antibiotics. *J Antimicrob Chemother* 1992; **30**: 279–288.
6. Azoulay-Dupuis E, Vallée E, Veber B, Bédos JP, Bauchet J, Pocidalo J-J. In vivo efficacy of a new fluoroquinolone, sparfloxacin, against penicillin-susceptible and -resistant and multiresistant strains of *Streptococcus pneumoniae* in a mouse model of pneumonia. *Antimicrob Agents Chemother* 1992; **36**: 2698–2703.
7. Moine P, Vallée E, Azoulay-Dupuis E *et al.* In vivo efficacy of a broad-spectrum cephalosporin, ceftriaxone, against penicillin-susceptible and -resistant strains of *Streptococcus pneumoniae* in a mouse pneumonia model. *Antimicrob Agents Chemother* 1994; **38**: 1953–1958.
8. Boulnois GJ. Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*. *J Gen Microbiol* 1992; **138**: 249–259.
9. Briles DE, Horowitz J, McDaniel LS *et al.* Genetic control of the susceptibility to pneumococcal infection. *Curr Top Microbiol Immunol* 1986; **124**: 103–120.
10. Japanese Society for Chemotherapy. Determination of minimum inhibitory concentration (MIC) by the micro-dilution method. *Chemotherapy* 1990; **38**: 102–105.
11. Briles DE, Crain MJ, Gray BM, Forman C, Yother J. Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. *Infect Immun* 1992; **60**: 111–116.
12. Johnston RB. Pathogenesis of pneumococcal pneumonia. *Rev Infect Dis* 1991; **13** Suppl 6: S509–S517.