MODEL OF INFECTION

Factors affecting the course and severity of transnasally induced Staphylococcus aureus pneumonia in mice

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In order to examine several factors that may affect the course and severity of transnasally induced Staphylococcus aureus pneumonia in mice, bacteria were prepared in a free suspension or bound to fetal mouse cells. Immunosuppression was induced in five strains of mice (ICR, C57BL/6, BALB/c, C3H/He and CBA/J) by injection of cyclophosphamide (200 mg/kg body weight), 2 days before infection. Impairment of mucociliary clearance was induced by intranasal instillation of formalin. Mice were then infected with various doses and strains of the organism. Although no significant differences were observed between either form of inoculum, pretreatment with formalin plus cyclophosphamide was associated with a significant increase in lung bacterial counts. In particular, cyclophosphamide treatment was associated with a high mortality in mice infected with several strains of S. aureus irrespective of their toxin production profiles. Histopathological examination demonstrated that in mice treated with formalin plus cyclophosphamide, clusters of bacteria were observed in lung parenchyma, associated with a mild accumulation of inflammatory cells at day 2 and extensive cell infiltration at day 7. CBA/J mice represented the most susceptible strain among those examined, with 10^4- and 10^2-fold higher bacterial counts in the lungs at days 3 and 5, respectively. These results indicate that neutropenia and impaired mucociliary clearance are major factors that influence the severity of S. aureus pneumonia in mice. Analysis of the role of genetic background in enhancement of vulnerability to infection is warranted in future studies.

Introduction

Staphylococcus aureus is a ubiquitous bacterial pathogen and a common cause of community- and hospital-acquired pneumonia [1]. In particular, immunocompromised hosts – such as neutropenic, intubated and debilitated patients – are highly susceptible to this agent and pneumonia is frequently life-threatening in those individuals [2]. Moreover, the increased frequency of strains exhibiting wide-spectrum resistance to antibiotics, such as methicillin-resistant S. aureus (MRSA), makes the problem more serious and makes it more difficult to provide appropriate management and effective antibiotic treatment [3].

Animal models of a particular disease whose features closely resemble those of the disease in man are necessary in order to understand the pathogenesis of that disease and to determine optimal treatment. Mice, rats and hamsters have been used in studies of S. aureus pneumonia, in which cyclophosphamide is usually used to induce neutropenia [4–7]. However, in these experimental animals, the exact factors that affect the course and severity of pneumonia – such as destruction of the airway and genetic background – have not been thoroughly examined. Recent studies established a murine model of pneumonia caused by non-typable Haemophilus influenzae with inocula containing cell-bound organisms [8]. There is ample evidence confirming that this organism can be internalised in epithelial and endothelial cells [9]. In this regard, the potency of different bacterial preparations in the induction of infection, e.g., inocula containing free bacteria in suspension or cell-bound bacteria, remains to be investigated.
The present study examined several factors that may influence the development, course and severity of pneumonia caused by *S. aureus* in mice. These factors include cyclophosphamide-induced neutropenia, airway destruction and mucociliary dysfunction by nasal instillation of formalin, the use of different strains of mice and different preparations of the challenging bacteria (free suspension or cell-bound organisms).

**Materials and methods**

**Bacterial strains**

Four strains of *S. aureus* (TUM, 6, 8, 12 and 21) were used in the study and were isolated at this hospital from patients with wound infection (TUM 6), eczema (TUM 8) or pneumonia (TUM 12 and 21). These strains were defined as methicillin resistant (MRSA) based on the results of routine bacteriological examination. The bacteria were maintained in Brain Heart Infusion Broth (BHB) (Difco Laboratories, Detroit, MI, USA) with glycerine 20% at -80°C until used.

**Animals**

ICR mice (Sankyo Labservice, Tokyo, Japan) and CBA/J, C57BL/6, BALB/c and C3H/He (Charles River Japan, Kanagawa, Japan) all male and aged 5–6 weeks, were used. All mice were housed in cages in groups of 10 and were provided with food and water *ad libitum*. The experimental protocol was approved by the Animal Ethics Review Committee of Toho University Medical School.

**Preparation of bacterial inocula**

Free bacterial suspension. Bacterial suspensions in BHIB were incubated for 3 h at 35°C, then harvested by centrifugation at 800 *g* for 10 min. The organisms were washed once with saline then diluted in saline to produce a final concentration of 10⁶ cfu/ml.

Cell-bound organisms (CBO). CBO were prepared as described recently by this laboratory [8]. Briefly, bacteria grown in BHIB were suspended in minimal Eagle's basal medium (MEM) with heat-inactivated fetal calf serum (10%) (Gibco Laboratories, Grand Island, NY, USA). Mouse fetal lung (MFL) cells were grown in flat-bottomed plastic flasks (25 cm², Corning Glass Works, Corning, NY, USA) at 35°C in an incubator in air with CO₂ 5%. On day 4 of incubation, the resulting highly subconfluent monolayer was washed three times with phosphate-buffered saline (PBS) and inoculated with bacterial suspension to produce a final concentration of 10⁶ cfu/ml. After incubation for 60 min at 35°C with gentle shaking, non-adherent bacteria were removed by washing three times with PBS. Then, cells with the adherent bacteria were isolated from the tissue-culture flask by a scraper and were suspended in 3 ml of MEM. These bacteria were designated CBO in the present study. To determine the number of viable bacteria in the cells, a portion of the CBO suspension was vigorously agitated with glass beads then plated on BHI agar plates after serial 10-fold dilutions.

**Induction of pneumonia and determination of bacterial counts in lungs**

Suspensions (0.05 ml) of free bacteria or CBO were inoculated nasally into mice, as described previously [10, 11]. On days 3, 5 and 7 after infection, mice were anaesthetised and the lungs were dissected carefully and removed under aseptic conditions. The lungs were homogenised in saline (2 ml) and bacterial numbers in the tissue were counted by plating serially diluted samples on to BHI agar. In some experiments, survival of mice was observed daily for 7 days after inoculation.

**Pretreatment of mice**

**Pretreatment with formalin**. In each mouse, 40 μl of formalin 1% was instilled nasally under ketaminexylazine anaesthesia 3 days before induction of infection, as described previously [8]. Under these conditions, the absence of inflammatory cells and the disappearance of the cilia in the bronchus was noted at the time of inoculation of challenging bacteria.

**Pretreatment with cyclophosphamide**. Neutropenia was induced by intraperitoneal injection of cyclophosphamide. (Shionogi Pharmaceutical, Osaka, Japan) 200 mg/kg, 2 days before bacterial inoculation, unless otherwise indicated. At this dose, cyclophosphamide reduced the neutrophil count between days 2 and 6, with a low point of 50–200/mm³ leucocytes recorded on day 4.

**Histopathological examination**

Lungs were fixed by intranasal instillation of 2 ml of a phosphate-buffered paraformaldehyde 4% solution and removed from mice. The tissue was processed for haematoxylin and eosin staining by a conventional paraffin-embedding procedure.

**Statistical analysis**

Differences in lung bacterial counts between the two groups were examined for statistical significance by the Student's *t*-test. A *p* value < 0.05 denoted the presence of a statistically significant difference.

**Results**

**Lung bacterial counts of different strains of *S. aureus***

The counts of the different bacterial strains were compared in lungs of CBA/J mice 3 days after...
intranasal challenge. While the four clinical strains showed different toxin-production profiles (Table 1), bacterial counts were not statistically different. As the count of strain TUM 8 was the highest in the group, this strain was used in subsequent experiments.

**Effects of pretreatment with cyclophosphamide and formalin on lung bacterial counts**

The effect of pretreatment of mice with cyclophosphamide or formalin, or both, on lung bacterial counts was examined. Pretreated or untreated CBA/J mice were challenged intranasally with bacterial suspension (6.8 × 10^5 cfu/mouse) or CBO (2.6 × 10^5 cfu/mouse), then the numbers of bacteria in the lungs were counted on days 3 and 5 (Table 2). Pretreatment with formalin or cyclophosphamide alone slightly increased bacterial counts compared with untreated mice, although the differences were not statistically significant. In contrast, combination treatment (formalin plus cyclophosphamide) resulted in a significant increase in bacterial counts, which was more profound on day 5. Although a greater number of bacteria was recovered from mice challenged with free bacteria than from those with CBO, these differences were considered to be due to the lower number of organisms used in the CBO method.

**Differences in lung bacterial counts in different strains of mice**

The next step of the study examined the susceptibility of several strains of mice to *S. aureus* TUM 8 (Fig. 1). ICR, C57BL/6, BALB/c, C3H/He and CBA/J mice

![Fig. 1. Comparison of bacterial counts in the lungs of different strains of mice.](image)

**Table 1.** Strain variation of *S. aureus* persisting in lungs of mice

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Coagulase type</th>
<th>TST-1</th>
<th>Enterotoxin type</th>
<th>Exfoliative toxin</th>
<th>Mean (SEM) number of organisms* (log_{10} cfu/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUM 6</td>
<td>II</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.63 (0.35)</td>
</tr>
<tr>
<td>TUM 8</td>
<td>II</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.50 (2.13)</td>
</tr>
<tr>
<td>TUM 12</td>
<td>II</td>
<td>+</td>
<td>C</td>
<td>–</td>
<td>1.82 (0.80)</td>
</tr>
<tr>
<td>TUM 21</td>
<td>II</td>
<td>+</td>
<td>C</td>
<td>–</td>
<td>2.40 (1.33)</td>
</tr>
</tbody>
</table>

*Bacterial numbers in lungs at 3 days after infection were compared in four clinical isolates.

**Table 2.** Effect of pretreatment on the number of bacteria in the lungs 3 and 5 days after infection

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>3 days</th>
<th>5 days</th>
<th>3 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.52 (0.50)</td>
<td>1.43 (0.32)</td>
<td>1.30</td>
<td>1.91 (1.05)</td>
</tr>
<tr>
<td>Formalin</td>
<td>1.44 (0.31)</td>
<td>1.62 (0.55)</td>
<td>1.81 (0.64)</td>
<td>2.53 (1.74)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>1.53 (0.37)</td>
<td>3.82 (2.75)</td>
<td>1.79 (0.85)</td>
<td>2.21 (0.31)</td>
</tr>
<tr>
<td>Formalin and</td>
<td>4.64 (1.24)</td>
<td>6.35 (1.37)</td>
<td>3.83 (1.35)</td>
<td>4.08 (1.88)</td>
</tr>
<tr>
<td>cyclophosphamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Challenge dose: bacterial suspension 5.83 × 10^5 cfu/mouse; cell-bound organism 2.62 × 10^5 cfu/mouse.
were pretreated with both formalin and cyclophosphamide as described above, then challenged intranasally with CBO. The results clearly indicated that CBA/J mice were the most vulnerable of the strains examined. More than $10^4$- and $10^2$-fold higher bacterial counts on days 3 and 5, respectively, were detected in this strain compared with those of other strains on the corresponding days.

Effects of pretreatment with cyclophosphamide and formalin on survival rates

To examine the effects of pretreatment with formalin or cyclophosphamide, or both, on the lethal effects of *S. aureus* in CBA/J mice, four bacterial strains prepared in free suspensions were instilled intranasally and the survival rate was observed daily for 7 days after infection (Fig. 2). In untreated mice, pneumonia caused by each of the bacterial strains was not lethal. In contrast, pretreatment caused a more severe infection, which culminated in the death of mice at various stages of the observation period. Specifically, in formalin-treated mice, the survival rate decreased to between 50 and 75% in mice infected with three of four strains 7 days after infection (Fig. 2b). On the other hand, pretreatment with cyclophosphamide resulted in a more dramatic decrease in survival rate: 0% in mice infected by three of four strains and 30% in mice infected with the fourth strain (TUM 12; Fig. 2c). Reflecting these results, pretreatment with formalin plus cyclophosphamide resulted in the death of all mice within 3 days after infection, irrespective of the bacterial strain.

Effects of timing of pretreatment with cyclophosphamide on the course of *S. aureus* infection

As the above results indicated that pretreatment with cyclophosphamide had a major impact on the severity of *S. aureus* pneumonia, the study examined the effect of timing of cyclophosphamide administration on survival rates in infected mice. Pretreatment with cyclophosphamide 4 days before infection resulted in the death of all test mice within 3 days, whereas 10 and 36% of mice died when they were pretreated on the day of and 2 days before bacterial inoculation, respectively. The study also examined the number of viable bacteria in the lungs of these mice 7 days after infection. The largest number of bacteria was detected in mice pretreated with cyclophosphamide 2 days before infection, which was followed by mice treated on the day of, 2 days after, and 4 days after infection.

Histopathological changes

The histopathological changes in lungs of mice infected with free bacterial suspension of *S. aureus* were examined. Clusters of bacteria were observed in the lung parenchyma of mice treated with formalin and cyclophosphamide both 2 and 7 days after infection. Histopathologically, inflammatory cells were sparsely scattered throughout the lungs at day 2, but marked cell infiltration was present at day 7 (Fig. 3a, b and c).

Discussion

This study demonstrated that neutropenia (induced by treatment with cyclophosphamide) and impairment of mucociliary clearance (induced by intranasal instillation of formalin) may be major predisposing factors for *S. aureus* pneumonia in mice. The data also suggested that genetic background is another critical component of vulnerability of mice to this organism, although the exact mechanisms were not investigated.

According to the surveillance data from the Centers for Disease Prevention and Control, USA, *S. aureus* is the second most common pathogen responsible for lower respiratory infection, especially in elderly individuals [12]. As this organism is carried persistently in the nasal cavity or oropharynx, or both, in 20–35% of individuals and transiently present in another 50% [13], it is likely that bacteria may enter the lung via aspiration preceded by oropharyngeal colonisation by the organism in the majority of cases. In addition, it has been reported that *S. aureus* can be internalised in epithelial and endothelial cells [9]. In this regard, it is plausible that bacteria that adhere to epithelial cells may play a role in the development of pneumonia. A new mouse model of non-typable *H. influenzae* pneumonia, in which enhanced virulence was specifically observed in cell-bound organisms, but not in suspension of free bacteria, was established recently [8]. However, in the *S. aureus* pneumonia model, the present study showed no apparent differences in bacterial counts in lungs of mice infected with free bacterial suspension or cell-bound organisms. Although the mechanism of this discrepancy was not explored, the data suggested a difference in the pathological process of pneumonia for *H. influenzae* and *S. aureus* infections.

The results of the present study indicated that combination treatment with cyclophosphamide and formalin synergically increased bacterial counts in the lungs, compared with each compound alone and the untreated control group. These results were consistent with epidemiological data on *S. aureus* pneumonia in man, in which elderly persons with destructive lung disease and immunocompromised hosts (such as neutropenic, intubated and debilitated patients), are reported as high risk groups for severe *S. aureus* pneumonia [1, 2]. Although this mouse model is slightly complicated and laborious, it may be useful for increasing understanding of the pathogenesis of staphylococcal pneumonia and the optimal treatment in these individuals.

Of the mouse strains examined in the present study,
Fig. 2. Effects of pretreatment with formalin, cyclophosphamide or both on survival of mice infected with different strains of *S. aureus*. CBA/J mice were challenged intranasally with 10⁸ cfu of bacteria mouse and survival was checked daily for 7 days after infection. (a) control mice; (b) formalin-treated mice; (c) cyclophosphamide-treated mice; (d) formalin plus cyclophosphamide-treated mice. ○, TUM 6; ●, TUM 8; □, TUM 12; ■, TUM 21. There were five mice in each group.

CBA/J mice were the most susceptible to *S. aureus* TUM 8 pneumonia when they were examined for lung bacterial counts and morbidity after nasal inoculation of this organism. Generally, CBA/J mice have a normal host defence system against invading microorganisms, and are usually used as counterparts of CBA/N mice, which have a defective antibody production [14]. In this regard, Iizawa et al. [15] reported specific responses of CBA/J mice in *Klebsiella pneumoniae* pneumonia, in which chronic pulmonary infection was observed after intranasal challenge of organisms. A recently study demonstrated that CBA/J mice, but not CBA/N, C3H/HeN, C3H/HeJ, C57BL/6 or ICR mice, were susceptible
to intranasal challenge with penicillin-resistant *Streptococcus pneumoniae* [10]. Furthermore, it has been shown that infection in this mouse strain closely resembles that of disease in man with regard to the course of infection and pathological findings [11]. Taken together, these findings suggest that the antibacterial host defence mechanisms against these organisms are weaker in CBA/J mice than in other strains of mice. Although a slightly higher susceptibility of neutrophils to cyclophosphamide was observed in CBA/J mice (data not shown), additional factors such as the killing activity of neutrophils and biological responses to bacterial components may be involved in the overall vulnerability of CBA/J mice. Further investigation of the genetic predisposition of mice is warranted for a better understanding of *S. aureus* pneumonia.

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**References**