Experimental Chronic Pulmonary Infection in Mice Caused by Klebsiella pneumoniae

Yuji IIZAWA,* Takeshi NISHI, Masahiro KONDO, and Akira IMADA

Biology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532

(Accepted for publication, June 15, 1988)

Abstract Examination of mouse strain differences in susceptibility to experimental respiratory tract infection with Klebsiella pneumoniae 27 revealed that a chronic pulmonary infection model could be established using CBA/J mice. After $6 \times 10^5$ colony-forming units of K. pneumoniae 27 were inoculated into the lung, the bacterial counts in the lungs changed with time showing four different phases: initial decrease, regrowth, steady-state, and final increase leading to death. Throughout the course of the infection, the challenge bacteria were isolated mainly from the respiratory organs. Pulmonary gross lesions appeared on day 2 after infection and persisted thereafter. Lobar consolidation was the primary lesion and occurred mainly in the anterior and middle lobes of the right lung, and the median lobe. Mice began to die from 4 weeks after aerosol exposure. This model may be useful for investigating the pathogenesis of chronic pulmonary infection by Klebsiella and its therapy.

In recent years, Klebsiella pneumoniae has frequently been reported as the cause of nosocomial infections, particularly in immunocompromised patients (7, 9, 10, 14, 15, 20). Respiratory K. pneumoniae infection in humans results mainly in lobar pneumonia, which is often difficult to control (11, 19).

Since Sale and Wood (17) reported the induction of experimental pneumonia with K. pneumoniae in rats using the technique of intratracheal instillation, there have been numerous reports on the production and characteristics of experimental respiratory tract infection with K. pneumoniae, using mice (1, 3, 8, 13), rats (2, 5), or squirrel monkeys (3, 4). However, these models are mostly concerned with acute infection. There have been only two rat models concerned with chronic infection: bronchopneumonia established by Berendt et al (2) and lobar pneumonia by Domenico et al (5). There have been no reports of chronic pulmonary infection in mice caused by K. pneumoniae.

Previous investigations in our laboratory have shown that there is a mouse strain difference in susceptibility to experimental respiratory tract infection with K. pneumoniae DT-S (13). Since K. pneumoniae DT-S is a highly virulent strain, it enabled us to establish the acute infection model using ICR mice (13). It seems that such investigations on the mouse strain difference in susceptibility to experimental infection are useful not only for evaluating the mechanisms of host defense.
to infection but also for providing a new infection model.

In the studies reported here, we examined the mouse strain difference in susceptibility to experimental respiratory tract infection with a weakly virulent strain, _K. pneumoniae_ 27. A marked strain difference in susceptibility to the infection was observed. The results also suggested that the organism used might cause a chronic pulmonary infection in CBA/J mice. Therefore, we examined the conditions for establishing the chronic pulmonary infection.

**MATERIALS AND METHODS**

_Bacteria._ _K. pneumoniae_ 27 (biotype aerogenes, capsular type 1) grown overnight in brain heart infusion broth (BHI; Difco Labs., Detroit, Mich., U.S.A.) at 37°C was harvested by centrifugation at 12,000 × g for 20 min at 20°C, suspended in a 3% skim milk solution containing 5% glucose, and stored at −80°C until use. The stock culture was thawed and first transferred onto a Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md., U.S.A.) slant and incubated for 8 hr at 37°C. Organisms from this culture were transferred to BHI broth, which was then incubated overnight at 37°C. This broth culture was centrifuged at 12,000 × g for 20 min at 20°C, and the cell pellet was washed with phosphate-buffered saline [PBS: Dulbecco’s formula (modified) without magnesium and calcium; Flow Labs., U.K.], and suspended in appropriate amounts of PBS.

_Mice._ Four- to five-week-old female DBA/2, BALB/cAn, CBA/J, C3H/He, C57BL/6, and CD-1 mice were obtained from Charles River Japan, Inc., Japan. Five- to six-week-old female Slc: ICR mice were obtained from Shizuoka Agricultural Cooperative Associations for Laboratory Animals, Japan. Five- to six-week-old female CF#1/K mice were maintained at the Kyoto Herbal Garden, Takeda Chemical Industries, Ltd., Japan. All the mice were caged in groups of 10 and given food and water _ad libitum._

_Respiratory tract infection procedure._ Mice were infected by aerosol exposure as described previously (13). In brief, mice were accommodated in an exposure chamber, and the bacterial suspension, charged in a nebulizer (Vaponefrin Pocket Nebulizer; USV Pharmaceutical Co., Tuckahoe, N.Y., U.S.A., was aerosolized at a pressure of 1.2 kg/cm² for 40 to 60 min.

_Bacterial examination._ Quantitative assessments of bacterial counts in the lungs, trachea, nasal washing, and blood were made as described previously (13). Briefly, mice were killed with ether and bled from the axillary artery and vein. The lung and trachea were homogenized in 4 and 2 ml of sterile distilled water, respectively, using Teflon tissue homogenizers. The nasal cavity was washed with 1 ml of sterile distilled water infused into the nasal passage from the choana and drained from the external nares. These homogenates, washings, and blood were serially diluted 10-fold with Trypticase soy broth (TSB; BBL Microbiology Systems), and 0.1-ml volumes of the various dilutions were inoculated onto TSA plates and incubated at 37°C for 20 hr. Colonies were counted and expressed as the log number of CFU per organ, or per milliliter of nasal washing or blood.
Agglutination test. Serum agglutinating antibody against the infected organism was examined by the microtiter method (12). The antigen was a suspension of Formalin-killed *K. pneumoniae* 27 in PBS that was adjusted to a density of 1.0 read at 660 nm by a Coleman Junior II Spectrophotometer (Coleman Instruments, Oak Brook, Ill., U.S.A.). The antigen (0.025 ml) was added to two-fold serial dilutions of the test serum (0.025 ml), and agglutinin titers were read after 2-hr incubation at 37 C and 18-hr incubation at 4 C.

Examination of the course of infection. The course of infection, other than bacterial quantitation described above, was observed using several variables: the course of death after aerosol exposure; gross lesions in the lungs, liver, spleen, and kidneys; and the distribution of infected bacteria to liver, spleen, and kidneys. Mice were killed with ether and bled from the axillary artery and vein. Then, the abdominal and thoracic cavities were opened and selected organs were aseptically removed. After gross lesions were observed, the central cut surfaces of liver, spleen, and kidneys, or the cut surfaces of the lesional sections, when a gross lesion existed, were imprinted onto TSA plates, which were incubated overnight at 37 C.

Histopathological examination. Samples of the lung were also taken for histopathological examinations. Tissues were fixed in 10% buffered neutral Formalin and stained with hematoxylin and eosin, or toluidine blue, and examined under light microscopy. In addition, the localization of the *Klebsiella* antigen in the lungs was examined with hyperimmune serum to Formalin-killed *K. pneumoniae* 27 in rabbits and a soluble horseradish peroxidase-antihorseradish peroxidase complex (18).

RESULTS

Mouse Strain Differences in Susceptibility to the Infection

The susceptibility of several strains of mice to infection with *K. pneumoniae* 27 was examined (Table 1). After about $1 \times 10^5$ CFU of bacteria were inoculated into the lungs, mortality of the mice was recorded and gross lesions in the lungs of mice that survived until 7 days after aerosol exposure were observed. Deaths were observed in 4 strains of mice (CF#1/K, DBA/2, ICR, and BALB/cAn). CF#1/K mice were most susceptible, and all mice of this strain died around day 2 after aerosol exposure. Though some of the DBA/2 and ICR mice, and most of the BALB/cAn mice survived until 7 days after, some of these animals had no gross lung lesions. CBA/J, C3H/He, CD-1, and C57BL/6 mice did not die until 7 days after aerosol exposure. However, the positive rate of lung lesions was different in these 4 strains. No lung lesions were observed in C57BL/6 mice; gross pulmonary lesions occurred in all CBA/J mice; and C3H/He and CD-1 mice showed intermediate responses. These results suggested that a chronic pulmonary infection model could be established using CBA/J mice, and we examined the possibility in the following experiments.
Effect of Challenge Dose

The effect of challenge doses on the respiratory tract infection in CBA/J mice was examined (Table 2). With a challenge dose of $3 \times 10^4$ CFU per lung, signs of infection were scarce until 14 days after the challenge. With a challenge dose of $6 \times 10^5$, all 10 mice survived with positive gross lesions and bacterial recovery. When the challenge dose was increased to $3 \times 10^6$, all the mice died. Thus there exists an optimal challenge dose for developing the chronic pulmonary infection. In the following experiments, the infection was initiated using a challenge dose of about $6 \times 10^5$ CFU per lung.

Course of Death

Deaths of the infected mice were followed by extending the observation period to 12 weeks after aerosol exposure using a challenge dose of about $6 \times 10^5$ CFU per lung (Fig. 1). No deaths occurred until 3 weeks after. Around 4 weeks after, however, some mice began to die, and by 12 weeks, 85% (231/272) of the animals

Table 1. Susceptibilities of various strains of mice to aerosol infection with *K. pneumoniae* 27a)

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Mortalityb) (death/total)</th>
<th>Timed) (days; mean±SD)</th>
<th>Gross lesionc) (positive/survival)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF#1/K</td>
<td>40/40</td>
<td>2.2±0.4</td>
<td>8/9</td>
</tr>
<tr>
<td>DBA/2</td>
<td>21/30</td>
<td>2.7±0.6</td>
<td>8/15</td>
</tr>
<tr>
<td>Slc::ICR</td>
<td>5/20</td>
<td>2.8±0.4</td>
<td>14/19</td>
</tr>
<tr>
<td>BALB/cAn</td>
<td>1/20</td>
<td>7.0</td>
<td>20/20</td>
</tr>
<tr>
<td>CBA/J</td>
<td>0/20</td>
<td></td>
<td>15/20</td>
</tr>
<tr>
<td>C3H/He</td>
<td>0/20</td>
<td></td>
<td>15/20</td>
</tr>
<tr>
<td>CD-1</td>
<td>0/20</td>
<td></td>
<td>0/30</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0/30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Bacterial suspension (about $5 \times 10^9$ CFU/ml) was nebulized at a pressure of 1.2 kg/cm² for 40 min. This procedure causes a deposition of about $1 \times 10^5$ CFU of the bacteria in the lung.

b) Mortality on day 7 after aerosol exposure.

c) Time to death was calculated for fatal cases only.

d) Animals that survived were killed on day 7 after aerosol exposure and examined for gross pulmonary lesions.

Table 2. Effect of challenge dose on respiratory tract infection in CBA/J micea)

<table>
<thead>
<tr>
<th>Challenge dose (CFU/lung)</th>
<th>Mortalityb) (death/total)</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gross lesion (positive/survival)</td>
</tr>
<tr>
<td>$3 \times 10^4$</td>
<td>0/5</td>
<td>1/5</td>
</tr>
<tr>
<td>$6 \times 10^5$</td>
<td>0/10</td>
<td>10/10</td>
</tr>
<tr>
<td>$3 \times 10^6$</td>
<td>10/10</td>
<td></td>
</tr>
</tbody>
</table>

a) Bacterial suspension ($5.20 \times 10^7$ to $6.25 \times 10^{10}$ CFU/ml) was nebulized at a pressure of 1.2 kg/cm² for 40 to 60 min.

b) Results on day 14 after aerosol exposure.
had died. The infected mice showed no apparent signs of illness until a few days before death, when they became inactive and slightly lost body weight.

Bacterial Examination

Changes in the bacterial populations in the respiratory tract were examined (Fig. 2). In the lungs, the bacterial counts changed with time and showed four different phases. In the first phase, the bacterial counts decreased to about one hundredth the initial level in 4 days. In the second, the bacterial counts started to increase from day 4, and reached the initial level 1 week after aerosol exposure. The third lasted from 1 to 4 weeks, during which period the bacterial counts stayed relatively unchanged. The fourth phase started at 4 weeks, and during it the bac-
terial counts gradually increased. In the trachea and nasal washing, the bacterial counts were about 1 to 2 logarithms lower than those in the lungs throughout the course of the infection. Until 4 weeks after aerosol exposure, no bacteria were recovered from organs other than respiratory tract or from blood. From 4 weeks after, some surviving mice were bacteremic (data not shown).

**Kinetics of Agglutinating Antibody**

Serum agglutinin titers in infected mice were examined (Fig. 3). Antibody titers began to rise on day 3 after aerosol exposure and gradually increased thereafter. Sera of most mice tested showed titers of 1:4 or more from day 6 after, and all the sera tested showed high titers (1:8 to 1:256) from 8 weeks onward.

![Fig. 3. Kinetics of agglutinin titers in sera of CBA/J mice infected with *K. pneumoniae* 27 by aerosol method. Symbols represent agglutinin titers of individual mice.](image)

![Fig. 4. Pulmonary gross lesion of CBA/J mice infected with *K. pneumoniae* 27 by aerosol method. (A) Lung, 24 hr after aerosol exposure: obscure congestion can be seen in the center of anterior lobe of right lung. (B) Lung at 3 weeks: consolidation can be seen in anterior and middle lobes of right lung.](image)
Gross Lesion

Gross lesions were limited to the lung throughout the test period, except for a small portion of mice at 8 and 12 weeks after aerosol exposure. In the lungs, diffuse congestions were observed on day 1 after exposure (Fig. 4A). Patchy consolidations became visible from day 2, and the consolidated area expanded on day 3 after exposure. Thereafter, lobar consolidations were formed in the lungs of most mice (Fig. 4B), and continued until 12 weeks. The anterior and middle lobes of the right lung and the median lobe were frequently affected. In organs other than the lung, abscess formation was observed in the livers of a small portion of mice at 8 and 12 weeks after aerosol exposure (data not shown).

Fig. 5. Histological changes in the lungs of CBA/J mice infected with K. pneumoniae 27 by aerosol method. Stained with hematoxylin and eosin. (A) Normal lung. ×307. (B) Neutrophilic leukocyte and macrophage infiltration in bronchi and alveoli 24 hr after aerosol exposure. ×307. (C) Slight lymphocyte infiltration around the vessel at 2 days. ×77. (D) Marked hyperplasia of lymphoid tissue around bronchi at 3 weeks. ×77.
Histological Changes

Histologically, the bronchial and alveolar spaces in the affected area were filled with an intense cellular infiltrate composed mainly of neutrophils and macrophages from day 1 after aerosol exposure (Fig. 5B); thereafter, similar lesions were observed throughout the course of the infection (Fig. 5, B–D). In addition to an intense infiltration of phagocytic cells, a lymphocyte infiltration around the vessel could be seen from day 1 (Fig. 5C), and a marked hyperplasia of lymphoid tissue around the bronchus could be seen from 2 weeks (Fig. 5D). Since infected organisms could not be observed in these samples stained with either hematoxylin and eosin, or with toluidine blue, the localization of Klebsiella antigen in the lung was examined.

Fig. 6. Localization of Klebsiella antigen in the lungs of CBA/J mice infected with K. pneumoniae 27 by aerosol method. (A) A small amount of antigen was observed in the alveolar spaces immediately after aerosol exposure. $\times 138$. (B) Antigen increased on day 1. $\times 138$. (C) The quantity of antigen became minimum on day 4. $\times 138$. (D) Antigen was present also in the lumen of the bronchus on day 7. $\times 138$. 
A small amount of antigen was observed in the alveolar spaces immediately after the infection began (Fig. 6A), and increased by day 1, when most of the antigen was separated from the phagocytic cells (Fig. 6B). The antigen decreased from day 2. On day 4 its quantity became minimum, when most of the antigen was intracellular (Fig. 6C). The antigen increased again from day 5. On day 7 the antigen was present also in the lumen of the bronchus, where it could be seen among an intense cellular infiltrate (Fig. 6D). The kinetics of the antigen was similar to that of counts of viable bacteria in the lungs: the antigen decreased in 4 days after aerosol exposure, increased from day 4, and stayed unchanged from 1 to 4 weeks.

**DISCUSSION**

Although there are many reports on pulmonary infection models with *K. pneumoniae*, they are mostly concerned with acute infection. There are only two reports concerned with chronic infection, using rats. In the bronchopneumonia model produced by Berendt et al (2), the animals died in the early phase of infection and there was a great range in the bacterial concentrations found in the lungs of individual rats. Moreover, bacteria were also found in blood, spleen, and liver tissue. The lobar pneumonia model produced by Domenico et al (5) showed a relatively uniform course of infection. No animals had succumbed by day 14 post-inoculation; however, death occurred thereafter, and the range in the bacterial concentrations found in the lungs of individual rats increased. In the chronic pulmonary infection model, which we have elaborated in this paper, mice rarely die 4 weeks after aerosol exposure, and the challenge bacteria are isolated almost exclusively from the respiratory organs. In addition, the bacterial counts in the lungs are kept within a small range until 4 weeks after aerosol exposure. This is the first report on chronic pulmonary infection with *K. pneumoniae* in mice.

We indicated that there is a marked strain difference in susceptibility to an experimental respiratory tract infection with *K. pneumoniae* 27. The CF#1/K mice were most susceptible to the challenge of $10^5$ CFU per lung of bacteria, and all mice of this strain died within 7 days after aerosol exposure. The C57BL/6 mouse strain was most resistant. No animals died and none had signs of any lung lesions. One of the reasons for this marked difference in susceptibility to the *Klebsiella* infection between CF#1/K and C57BL/6 mouse is supposed to be due to the number of polymorphonuclear leukocytes (PMNs) recruited into the lungs within 8 hr of infection being established (unpublished data). The CBA/J mouse strain showed an intermediate response. Although no animals died until 7 days after aerosol exposure, all the mice had gross pulmonary lesions at autopsy. Though several other strains of mice also showed an intermediate response, the conditions of individual mice varied: some died and some of those that survived had no gross lung lesions. Therefore, these results suggested that a chronic pulmonary infection model could be most suitably established using CBA/J mice, and we have examined this possibility.

After the aerosol inoculation of $6 \times 10^5$ CFU of *K. pneumoniae* 27 in CBA/J
mice, the bacterial counts in the lungs changed with time showing four different phases: initial decrease (1st phase), regrowth (2nd phase), steady-state (3rd phase), and final increase leading to death (4th phase). This kinetics of the bacterial counts in the lungs signifies the establishment of a chronic pulmonary infection. Especially, the steady-state of bacterial counts in the lungs lasted for 3 weeks in the third phase. The following two factors are considered important for the establishment of the chronic pneumonia. First, the initial decrease of bacterial counts in the lungs (1st phase) cannot proceed below a certain level. The surviving bacteria turn to regrow in the second phase. Though the mechanism is not clear, it is speculated that the environment around the bacteria in which the host-defense mechanism cannot act on the infected organisms resulted from the formation of the pulmonary lesion, because, contrary to the decrease of bacterial counts in the lungs, gross pulmonary lesions developed with time and lobular consolidations were observed in most of mice early in the second phase. The second factor is that there is a limit to the increase of bacterial counts in the second phase and the bacterial counts in the lungs keep the settled level in the third phase. A specific antibody against the infected bacteria was produced from day 3 after aerosol exposure and gradually increased thereafter. Accordingly, it is considered that this specific antibody suppressed the bacterial proliferation in the lungs and protected the shift to bacteremia. Fukutome et al (6) showed that both PMNs and specific antibody play important roles in protecting against Klebsiella infection. Besides, the injection of cyclophosphamide into the mice in the third phase induced a proliferation of bacteria in the lungs (unpublished data). Therefore, it is likely that PMNs also suppress the bacterial proliferation in the third phase. The reason why the bacterial counts in the lungs did not decrease remains to be clarified.

The fact that the bacterial counts in the lungs gradually increased and mice began to die in the fourth phase indicates that the equilibrium achieved between the mice and the infecting organisms began to collapse at this time. Pollack (16) demonstrated that the presence of detectable capsular polysaccharide in the serum of patients infected with K. pneumoniae appeared to correlate with the severity of infection. Therefore, it is speculated that a capsular polysaccharide released from the infected organism increased in the blood, interfered with the function of PMNs, and induced a collapse of the host-defense mechanism in the fourth phase. We are now analyzing the subtle host-parasite relation which occurs in the course of chronic pulmonary infection caused by K. pneumoniae 27 in CBA/J mice.

The lobular consolidations occurred primarily in the anterior and middle lobes of right lung and median lobe. This closely resembles the localization seen in the human form of the disease; the pulmonary lesion in humans is most frequent in the right upper lobe (11, 20). The intense cellular infiltration that we observed histologically progressed abreast of the formation of the pulmonary gross lesion. On the other hand, a marked hyperplasia of lymphoid tissue around the bronchus was observed from 1 to 2 weeks after aerosol exposure. This hyperplasia of bronchus-associated lymphoid tissue (BALT) is closely related with the kinetics of the bacterial counts in the lungs and antibody response. In fact, the Klebsiella antigen which had
spread throughout the lung immediately after aerosol exposure was present in large quantity in the lumen of the bronchus on day 7. Accordingly, it is considered that bacterial regrowth in the second phase mainly occurred in the bronchus and correspondingly the hyperplasia of BALT occurred.

The chronic pulmonary infection model established in this study may be useful for investigating the pathogenesis of chronic *Klebsiella* pneumonia and its therapy.

We thank Dr. M. Nomura for his assistance in the histopathological examinations.

REFERENCES


(Received for publication, March 4, 1988)