Mycoplasma pulmonis 感染の感受性におけるマウス系統差

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Strain Difference of Mouse in Susceptibility to *Mycoplasma pulmonis* Infection

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Abstract. Strain difference in susceptibility to *M. pulmonis* infection was studied in mice of 5 strains by cage-mating with previously infected ones. The organisms were isolated abundantly from the upper respiratory tracts of all mice examined within one week after contact, and did not disappear throughout the observation period of 11 to 16 weeks. However, a remarkable strain difference was demonstrated in development of gross pneumonic lesions by indication of the fact that the lesions appeared in 40 to 70% of ICR mice after 3 weeks of contact, while less than 10% of ddY mice. In NIH, CF#1 and C3H/He mice, the lesions were detected at various rates less than 40%. Growth of the organisms in the trachea was not significantly different between ICR and ddY mice, in contrast with numbers of the organisms in the lung which were less than 10^3 CFU/g in many of ddY mice but 10^{5-10} CFU/g in 24 of 40 ICR mice examined on the 3rd week of contact and thereafter. All lungs with pneumonic lesions harbored more than 10^5 CFU/g organisms, while grossly normal lungs less than 10^5 CFU/g. CF antibody appeared in almost all mice during the first and second weeks of contact, and reached to a peak of titer (1:64 to 1:1,024) on the 3rd week in both ddY and ICR mice. Titers of the antibody decreased from the 8th week in ddY mice, but persisted in ICR mice till the 10th week.

According to investigations published hitherto, it is obvious that *Mycoplasma pulmonis* is one of important causal agents of the pneumonia in mice [1, 4, 5, 15, 16]. However, natural infection of the organisms did not always resulted in production of the lesions but frequently retained in latent infection [9, 12, 21]. In mice experimentally infected with the organisms, the pneumonia was detected at various rates according to investigators or methods used for inoculation. For example, Lutsky and Organick [16] found pneumonic lesions in 50 of 73 gnotobiotic mice (68.5%) by intranasally inoculating *M. pulmonis*, and Atobe and Ogata [1] reported that pneumonic lesions were produced at maximum 90% of mice when inoculated intranasally with broth culture of *M. pulmonis*, but no gross lesions in mice cage-mated with previously infected ones.

Development of mycoplasmal pneumonia may be influenced by some factors such as virulence of mycoplasmal strains, inoculating dose of the organisms [15], susceptibility of mouse strains [3, 19], concentration of gaseous ammonia in environments [6, 23] and microbes other than mycoplasmas in the respiratory tract of animals [2, 5, 16].

The present study was performed to see the susceptibility of mice to *M. pulmonis* infection with a special reference to differ-

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ence between mouse strains.

Materials and Methods

Mice: Four-week-old mice (both sexes) of non-inbred ddY, ICR, NIH, CF#1 and C3H/He strains were used. All the mice were produced in a barriered breeding room in our institute and proved by bacteriological and serological [11] checking to be free from *Salmonella* spp., *Pasteurella pneumotropica, Escherichia coli* O115a, c:K(B) [17], *Corynebacterium kutscheri,* Tyzzer's organisms, *Mycoplasma* spp., Sendai virus, mouse hepatitis virus, reovirus type 3 and mouse adenovirus.

M. pulmonis: A fresh isolate of *M. pulmonis*, strain 925T [22], from the pneumonia of a naturally infected mouse was used for experimental infection. This strain showed film spot on agar plate, turbid growth in liquid medium and positive reduction of tetrazolium and grew in both aerobic and anaerobic conditions by the methods described by Ogata et al. [20]. In growth inhibition test [8], the strain reacted strongly with hyperimmune rabbit serum against *M. pulmonis* PG-22, but did not with those against *M. arthritidis* PG-6 and *M. neurolyticum* PG-28, the 3 reference strains had been kindly supplied by Prof. M. Ogata, University of Tokyo.

Media: Culture media used for *M. pulmonis* were solid and liquid ones described by Chanock et al. [7], consisting of 70 m*l* of 3.4% PPLO agar (Difco) or 2.1% PPLO broth without crystal violet (Difco), 20 m*l* of unheated horse serum, 10 m*l* of 25% w/w fresh yeast extract, 1 m*l* of 100,000 units/m*l* penicillin G and 1 m*l* of 2.5% thallium acetate which was excluded for liquid medium. Horse blood agar was also used for routine bacteriological examinations on lung samples.

Experimental infection: Contact infection was conducted by cage-mating with infectors which had been previously infected by the following procedures. ICR mice were inhaled with 3-day-culture of strain 925T in the liquid medium by dipping their noses in the culture fluid for about one second, and then they were maintained in a vinyl isolator for 4 to 6 weeks prior to use as infectors. For cage-mating, 10 each of intact mice were housed with 2 infectors in a 32×22×11 cm metal cage with wood shaving bedding sterilized by autoclaving. To prevent infections of any other pathogens than *M. pulmonis,* all the mice were maintained in 8 large vinyl isolators (140×50×170 cm), distributing same number of mice of each strain to every isolator. Gamma-irradiated pellets (Funabashi Farm) and non-sterilized tap water were given ad libitum. The bedding was changed twice a week.

Isolation and identification of *M. pumonis*: Blood was taken from the heart of mice anesthetized with chloroform and sera were stored at −20°C without preservative.

Samples of the nasal cavity and trachea were taken by inserting a fine cotton swab moistened with PPLO broth without crystal violet and direct culture was made on the solid medium. A 10% suspension of the lung was made in a sterile broth by use of a glass homogenizer, and a loopful of the suspension was streaked on the solid medium. All cultures were incubated aerobically at 37°C for 7 days in a tightly closed vinyl bag to keep humid condition.

Isolates were identified by growth inhibition test using hyperimmune rabbit sera against the 925T and 3 reference strains described above. Preparation of the immune sera and the growth inhibition test were performed according to Ogata et al. [20] and Clyde [8].

Quantitative cultivation of *M. pulmonis*: Trachea and lung tissues of ddY and ICR mice were submitted to quantitative cultivation of *M. pulmonis*. After making 1 or 10% suspensions of the tissues in PPLO broth by use of glass homogenizers, serial 10-fold dilutions were made with the same broth and 0.1 m*l* each of appropriate dilutions was spotted on plates of the solid medium. After incubation at 37°C for 7 days, an average count of CFU (colony forming unit) per 1 g tissue was calculated from the number of colonies developed on 3 plates which were used for each dilution.

Complement fixation (CF) test: Antigen was prepared according to Atobe and Ogata [1]. Strain 925T was cultured stationally in 3,000 m*l* of the liquid medium at 37°C for 4 days, and the culture was centrifuged at 20,000×G for 30 min. The resulted sediment was washed 3 times with phosphate buffered saline of pH 7.2 (PBS), resuspended in 30 m*l* of PBS and treated for 5 min by 10 KC of ultrasonic vibration. The antigen was stored at −20°C before use.

The CF test (Kolmer's method) was performed by a microtiter method [1], using Veronal buffer saline (VBS) as diluent. In U-shaped microtiter plates, 0.025 m*l* each of serial 2-fold dilutions of test sera heated at 56°C for 30 min, 0.025 m*l* of 4 unit-antigen and 0.05 m*l* of 2 unit-complement were mixed, and then the mixtures were incubated at 4°C for 18 hr. After that, a hemolytic system consisting of 6% sheep red cells and 3 unit-hemolysin in equal volumes was added. Reading was made after 30 min-incubation at 37°C, and the highest dilution showing 75% or greater inhibition of hemolysis was recorded as serum titer.

Patholgy: Lungs were examined for macroscopic lesions such as abscess formation and consolidation at autopsy.

Results

1. Isolation of *M. pulmonis* and detection of pneumonia

In order to examine for the presence of *M. pulmonis* and lung lesions, 20 each of mice were sacrificed at 1 week intervals after cage-mating with infectors during 11 weeks for ICR mice and 16 weeks for other strains of mice. For detection of *M. pulmonis*, cultivation was made on the nasal cavities and tracheas of all mice, which had been shown to be the favorite sites of the organisms [22], all the lungs with lesions and many grossly normal lungs.

M. pulmonis was isolated in a large number from both the nasal cavities and tracheas of all mice from 1st to 10th week of contact, but thereafter one of the sites became negative in a few cases, though individual isolation rates were always 100%. No strain difference of mice was observed in isolation of the organisms from these two sites.

On the other hand, detection of pneumonia was remarkably different according to strains as shown in Table 1. In ICR strains, pneumonic lesions appeared in 3 of 20 mice (15%) on the 2nd week of contact, developed most extensively in 14 to 15 of 20 mice (70 to 75%) on the 4th to 6th week and remained thereafter in 35 to 60% of mice examined till the end of experiment. On the contrary, detection rates of pneumonia in ddY mice were 10% (2 of 20 mice) at the highest, but usually several percentages during 16 weeks. The pneumonia was also detected in other strains of mice at various percentages between those of ddY and ICR, but development of the lesions was usually slower than in ICR mice appearing in later stage of the experiment. If calculated from the animal samples

Table 1. Development of gross pneumonic lesions in 5 strains of mice infected with *M. pulmonis*

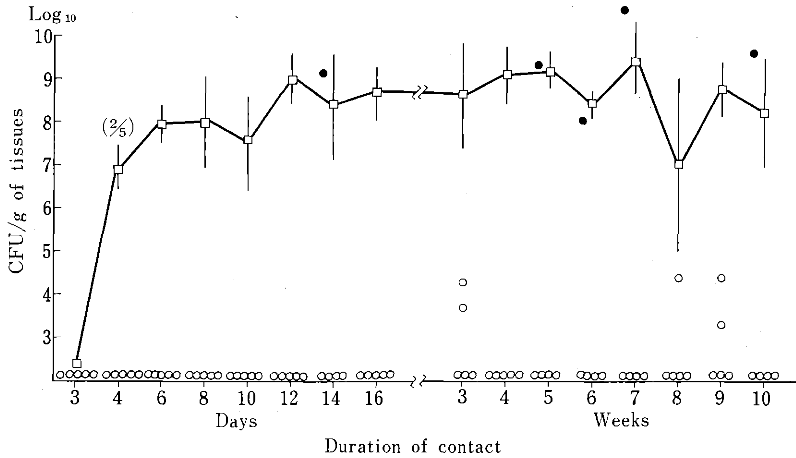
Weeks after contact	Mouse strains				
	ddY	C3H/He	CF#1	NIH	ICR
2	0/20*	0/20	0/20	0/20	3/20 (15.0)
3	0/20	0/20	0/20	0/20	8/20 (40.0)
4	0/20	0/20	0/20	1/20 (5.0)	15/20 (75.0)
5	0/20	0/20	0/20	5/20 (25.0)	14/20 (70.0)
6	0/20	0/20	0/20	3/20 (15.0)	14/20 (70.0)
7	0/20	0/20	0/20	2/20 (10.0)	9/20 (45.0)
8	1/20 (5.0)	2/20 (10.0)	2/20 (10.0)	6/20 (30.0)	12/20 (60.0)
9	2/20 (10.0)	6/20 (30.0)	3/20 (15.0)	8/20 (40.0)	12/20 (60.0)
10	1/20 (5.0)	2/20 (10.0)	2/20 (10.0)	2/20 (10.0)	9/20 (45.0)
11	1/20 (5.0)	4/20 (20.0)	4/20 (20.0)	3/20 (15.0)	7/20 (35.0)
12	0/20	0/20	2/20 (10.0)	1/20 (5.0)	•
13	1/20 (5.0)	2/20 (10.0)	2/20 (10.0)	4/20 (20.0)	•
14	1/20 (5.0)	5/20 (25.0)	6/20 (30.0)	3/20 (15.0)	•
15	0/20	2/20 (10.0)	2/20 (10.0)	3/20 (15.0)	•
16	1/20 (5.0)	1/20 (5.0)	2/20 (10.0)	4/20 (20.0)	•
Total	8/300 (2.7)	24/300 (8.0)	25/300 (8.3)	45/300 (15.0)	103/200 (51.5)

Remarks.

* : No. positive/No. examined. Parentheses : Per cent.

• : Not tested.

Fig. 1. Growth of *M. pulmonis* in the respiratory tract of ddY mice



Remarks.
Solid line: Trachea. Black dot: Lung with pneumonic lesion.
White dot: Lung without pneumonic lesion.
Parentheses: No. positive/No. examined.

collected on or after the 2nd week of contact, which was regarded as latent period for development of gross pneumonic lesions, average detection rates of the lesions was 51.5% (103 of 200 mice) in ICR, 15.0% (45 of 300 mice) in NIH, 8.3% (25 of 300 mice) in CF#1, 8.0% (24 of 300 mice) in C3H/He and 2.7% (8 of 300 mice) in ddY strains, respectively. However, no particular relation was observed macroscopically between extent of the lesions and strains of mice.

M. pulmonis was abundantly isolated from all the lungs with lesions, regardless of mouse strains. On the contrary, isolation rates of the organisms from grossly normal lungs were remarkably low, being 6% (15 of 238 mice) in ddY, 6% (9 of 149 mice) in NIH, 7% (8 of 134 mice) in CF#1, 14% (16 of 109 mice) in C3H/He and 19% (16 of 83 mice) in ICR, respectively, and the organisms recovered from the samples were usually small in number.

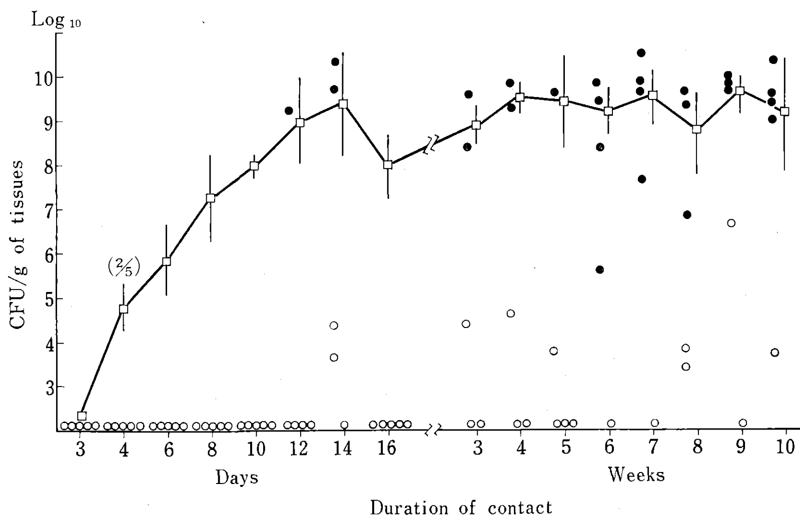
From these results, it was apparent that the sensitivity to *M. pulmonis* infection was comparatively high in ICR mice, but low in ddY mice, and therefore, these two mouse strains were used in the following experiments.

2. Growth of *M. pulmonis* in ICR and ddY mice

In order to see a relationship between the susceptibility of mice to *M. pulmonis* infection and growth of the organisms in the respiratory tract, quantitative cultivation was made on the tracheas and lungs of 5 mice each which were sacrificed periodically after cage-mating with infectors.

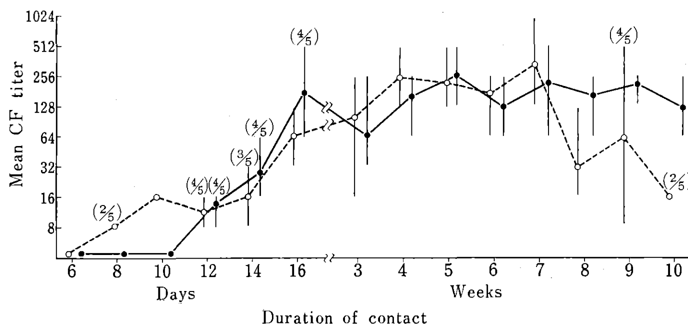
As shown in Figs. 1 and 2, there observed no significant difference in growth of the organisms in the tracheas of ddY and ICR mice. Namely, in the both strains, the organisms were first recovered from the tracheas of 2 of 5 mice sacrificed on the 4th day of contact, and became positive in all mice on the 6th day, reaching around 10^{6-8} CFU/g in number. Thereafter a gradual increase in number of the organisms continued up to the end of observation

Fig. 2. Growth of *M. pulmonis* in the respiratory tract of ICR mice



Remarks.
Solid line : Trachea. Black dot : Lung with pneumonic lesion.
White dot : Lung without pneumonic lesion.
Parentheses : No. positive/No. examined.

Fig. 3. CF antibody in ddY and ICR mice infected with *M. pulmonis*



Remarks.
Solid line : ICR mice. Broken line : ddY mice.
Parentheses : No. positive/No. examined.

period, 10 weeks, fluctuating between 10^7 and 10^{10} CFU/g. In some mice, so many as $1\text{–}5\times10^{10}$ CFU/g organisms were recovered from their tracheas.

On the other hand, great differences were observed in growth of *M. pulmonis* in the lung and in formation of the pneumonia between ICR and ddY mice as shown in Figs. 1 and 2. Although the number of the organisms remained less than 10^3 CFU/g in the both strains of mice till the 10th day of contact, one each of 5 ICR and ddY mice examined on the 12th day and 14th day yielded first more than 10^9 CFU/g organisms, respectively. After 3 weeks of contact, however, the number of organisms was $10^{5\text{–}10}$ CFU/g in 24 of 40 ICR mice, but less than 10^3 CFU/g in the most of ddY

mice. Regardless of the mouse strains, the pneumonic lesions developed in all of 32 mice harboring 10^5 CFU/g or more in their lungs, except for one which had grossly normal lung in spite of yielding 10^6 CFU/g, and no abnormality was observed in all lungs containing less than 10^5 CFU/g organisms.

Production of serum CF antibody is shown in Fig. 3. In ddY mice, the antibody was detected in some animals 8 days after contact, followed by increase in positive percentage and titer, and reached to maximum titers ranging from 1:64 to 1:1,024 during the 4th to 7th week. However, the antibody decreased gradually in titer thereafter. Similar antibody response was also observed in ICR mice, except for that a rise of the antibody was somewhat later than in ddY mice, and no decrease in titer was observed even on the 10th week.

No particular correlation was obtained between the antibody titer and bacterial numbers in the trachea and lung or formation of the pneumonia in both ddY and ICR mice.

Discussion

Experimental infections of *M. pulmonis* in mice and rats have been performed by many investigators using 2 different methods, one of which was intranasal inoculation of bacterial culture and the other was cage-mating with previously infected animals. The former method had the virtue of being adjustable in inoculum number of bacteria and usually resulted in higher production of the pneumonia than the latter. However, the results obtained by the former method might be different from attitudes in natural infection owing to simultaneous inoculation of culture medium with the organisms. For example, Edward [9] and Lutsky and Organick [16] reported that intranasal instillation of sterile broth to conventional mice produced the pneumonia at significantly higher rates than that of non-treated mice of same quality, and also Atobe and Ogata [1] reported that the pneumonia was produced in so many as 90% of ICR mice intranasally inoculated with broth culture of *M. pulmonis*, but in none of the mice cage-mated with infectors. In our experiment, the cage-mating method was adopted to see the susceptibility of mice to *M. pulmonis* infection under conditions similar with natural infection.

The results indicated that when housed 10 contact mice and 2 infectors in a cage, the organisms became recovered from the trachea of all contact mice after considerably uniform period as 3 to 7 days, regardless of mouse strains, and the pneumonic lesions were formed in more than a half of ICR mice but rarely in ddY mice. According to the report by Atobe and Ogata [1], contact infection was established in only 60% of mice used 3 to 6 weeks after cage-mating in combination with 4 susceptible mice and 10 infectors, but no gross pneumonic lesions was observed even at 10 weeks of contact, in spite of use of ICR mice as in our case. On the other hand, Nelson [18] failed to isolate *M. pulmonis* from the respiratory tracts of 5 mice which had been housed with 5 infectors in a cage, though 13% of the mice harbored the organisms in the middle ear. Such discrepancies between our results and those by other investigators might be caused by difference in pathogenicity of *M. pulmonis* strain or in susceptibility of mouse strain employed. Although we used vinyl isolators for maintenance of animals throughout experiment, environmental condition in the isolators seemed to give no effect on susceptibility of mice, because almost the same results as

those described above were obtained from 20 mice each of ICR and ddY strains which were subjected to contact infection for 6 weeks without isolators (unpublished observation).

As regards to strain difference of mice in susceptibility to *M. pulmonis*, Nikaido and Katagiri [19] reported that the growth of the organisms in the lung was more active in ICR mice than in DSf mice, and Lane-Petter et al. [13] also suggested possible occurrence of the same phenomenon in different rat strains. In our experiment in which 5 mouse strains were used for comparison, remarkable differences were observed in detection rate of the pneumonia due to *M. pulmonis* according to strains of mice, particularly between high susceptible ICR and low susceptible ddY strains, while there was no strain difference in infection rate. On the other hand, numbers of the organisms in pneumonic lungs were demonstrated to be more than 10^5 CFU/g (usually more than 10^9 CFU/g), except for one case, being significantly different from those in grossly normal lungs. These results indicated that the strain difference of mice was the reflection of difference in growth of the organisms in the lung. Brennan and Feinstein [3], who used mice of wild type and acatalasemic mutant for *M. pulmonis* infection, suggested that normal catalase levels of host tissue might stimulate growth and prolong survival of the organisms in vivo. This phenomenon, however, might not fully explain the strain difference of mice reported here, because similar growth curves of *M. pulmonis* were obtained in the tracheas of both high susceptible ICR and low susceptible ddY mice.

For description of extent of lung lesions or severity of the pneumonia, Whittlestone et al. [24] and Atobe and Ogata [1] applied a scoring system in which definite points were given to pulmonary lobes with lesions. Although such a scoring system was not used in our experiment, macroscopical observation revealed no particular relation between mouse strains and extent of lung lesions when occurred.

In experimental infection of *M. pulmonis* to CFW mice, Lindsey and Cassell [15] indicated that formation of the pneumonia depended on numbers of the organisms inoculated. Although number of the organisms inoculated was not able to count in our experiment, the strain difference in development of the pneumonia did not seem to be reflected directly to the inoculum number of the organisms, because there observed no difference in bacterial counts in the tracheas of ICR and ddY mice, and in addition no different results were obtained in development of the pneumonia when used ICR or ddY mice as infectors (unpublished observation).

Atobe and Ogata [1] reported that CF antibody appeared in some of mice with pneumonic lesions 10 days after infection with *M. pulmonis* and became positive in all mice with or without lung lesions on the 30th day, showing the highest titer of 1:320 on the 3rd month, followed by decrease in titer thereafter, and average titer of the antibody was higher in mice with lung lesions than in those without the lesions. Such a correlation between CF antibody titer and the presence of pneumonic lesions was also noticed by Lemcke [14]. On the other hand, production of CF antibody was shown in mice which had no lung lesions but harbored the organisms in the upper respiratory tract [1, 10]. In our experiment, the following two phenomena were compatible with those described by the above investigators; CF antibody was detectable on the 7th to 10th days of contact and demonstrated in many of mice

without pneumonic lesions. However, a special relationship was not observed between the antibody titer and the formation of pneumonia, while CF antibody persisted longer in ICR mice than in ddY mice, suggesting long persistence of the antibody in pneumonic cases.

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