Synergistic Effect of Sendai Virus on *Mycoplasma pulmonis* Infection in Mice

Manabu SAITO, Masaro NAKAGAWA, Eiko SUZUKI,
Kuniaki KINOSHITA and Kiyoshi IMAIZUMI

Department of Veterinary Science, National Institute of Health,
10-35 Kamiosaki 2-chome, Shinagawa-ku, Tokyo 141

(Received for publication July 2, 1980)

Abstract. The synergistic effect of Sendai virus on *Mycoplasma pulmonis* infection was investigated in ddY mice infected with the two agents. As the results, growth of *M. pulmonis* was remarkably enhanced in the lung by superinfection of Sendai virus, although not significantly affected in the trachea. The enhanced growth of *M. pulmonis* was most evident when the both organisms infected at the same time, and the less enhancement of the growth was observed in the longer time difference between infections of the two agents. Detection rates of pneumonia in mice infected with both *M. pulmonis* and Sendai virus were 80 to 100 per cent, remarkably higher than 12 per cent of control mice infected with *M. pulmonis* alone, and comparable to 80 per cent of control mice infected with Sendai virus alone. However, the lesions in the dually infected mice were more severe and persisted longer than those in the mice infected with Sendai virus alone. CF antibody against *M. pulmonis* persisted longer in the dually infected mice, as compared with that in control mice infected with *M. pulmonis* alone. Production of HI antibody to Sendai virus was considerably inhibited by combined infection with *M. pulmonis*.

It is well known that combined infections of viral and bacterial agents in the respiratory tracts of man and animals interact in the pathogenesis of the disease, resulting in increases of symptoms and mortality of the hosts. Jakob et al. [10] also reported that intrapulmonary killing of *Pasteurella pneumotropica* was delayed in mice previously infected with Sendai virus.

In previous reports [19, 20], we indicated that *Mycoplasma pulmonis* was frequently and abundantly isolated from pneumonic lesions naturally occurred in ddY strain of mice. The same lesions were rarely produced by experimental infection of the organisms to the same strain of mice. We also suggested the importance of combined infections of *M. pulmonis* and Sendai virus in naturally occurring pneumonic lesions.

In the present study, synergistic effect of

Sendai virus on *M. pulmonis* infection was investigated in ddY mice experimentally infected with the two agents.

Materials and Methods

Mice: Four-week-old mice of outbred ddY strain, both sexes, were used in this study. They were obtained from a barrier-sustained breeding colony free of any noticeable pathogens.

***Mycoplasma pulmonis* and culture media:** Strain 925T of *M. pulmonis* which had been isolated from the pneumonia of a mouse [20] was used. Culture media were solid and liquid ones described by Chanock et al. [5].

Quantitative cultivation of *M. pulmonis*: Quantitative cultivation was made on tracheal and lung samples of mice. After making 1% tracheal and 10% lung suspensions in PPLO broth (Difco), serial 10-fold dilutions were made in the same broth and then 0.1 ml each of appropriate dilutions were dropped on 3 plates of the solid medium. The inoculated plates were put in vinyl bags to keep humidified atmosphere and incubated at 37°C for 6 days.

An average colony forming units (CFU) in one gram tissue was calculated.

Titration of *M. pulmonis* antibody: Complement fixation (CF) test was performed by a microtiter method, using ultrasonicate of strain 925T culture as antigen. In the CF test, 4 unit-antigen, 2 unit-complement and a hemolytic system consisting of 6% sheep red cells and 3 unit-hemolysin were used. Details of the antigen preparation and the CF test were described previously [20]. The highest serum dilution showing 75% or greater inhibition of hemolysis was recorded as serum titer.

Sendai virus: Strain M-73 [14] isolated from the trachea of a naturally infected mice was used. Chorioallantoic fluid of 10-day-old chick embryos inoculated with the strain was used as challenge virus in experimental infection. The challenge virus suspension contained $10^{8.5}$ EID₅₀ (50% egg infecting dose)/ml and 256 hemagglutinin units/ml.

Titration of Sendai virus antibody: Hemagglutination inhibition (HI) test was performed for titration of the antibody, using formolized and glycerin added chorioallantoic fluid of 10-day-old chick embryos inoculated with the virus strain as antigen. Mouse sera tested were prepared by heating at 56°C for 30 min and then eliminating natural hemagglutinins with packed chick red cells. Methods for the antigen preparation and the HI test were reported previously [14]. The highest serum dilution showing hemagglutination inhibition was recorded as serum titer.

Experimental infection: Contact infection was made by housing intact mice and infectors together in cages. Methods of the infector preparation, contact infection and care of mice were the same as described in our previous reports [14, 20].

Sampling: Five mice each were periodically autopsied for observation of pneumonic lesions and for taking specimens after bleeding by cardiac puncture under chloroform anesthesia. The tracheal and lung specimens were submitted to quantitative cultivation of *M. pulmonis* and individual serum to titration of antibodies against *M. pulmonis* and Sendai virus.

Scoring of pneumonic lesions: The extent of gross pneumonic lesions was recorded according to the scoring system of Whittlestone et al. [23].

Design of experiment: Five experimental groups were designed as follows.

Group 1: Both *M. pulmonis* and Sendai virus infectors were introduced at the same time into mouse cages having intact mice.

Groups 2 and 3: Sendai virus infectors were introduced 2 and 6 weeks after introduction of *M. pulmonis* infectors, respectively.

Groups 4 and 5: Introduction of *M. pulmonis* infectors were preceded by Sendai virus infectors at 2- and 6-week-intervals.

Mouse groups infected with *M. pulmonis* and Sendai virus alone were also prepared as control, and designated as control groups M and S, respectively.

Results

1. Growth of *M. pulmonis* in the respiratory tracts

Growth of *M. pulmonis* in the trachea and lung of mice were periodically examined by sacrificing 5 mice each at 2-week-interval after cage-mating with infectors. Results are shown in Fig. 1.

There observed no significant effect of Sendai virus infection on the growth of *M. pulmonis* in the trachea. Almost maximum growth, 10^{8-9} CFU/g tissue in number, was obtained 2 weeks after contact with *M. pulmonis* infectors and persisted to the termination of the experiment in all the experimental mouse groups superinfected with Sendai virus, likewise in control mice infected with *M. pulmonis* alone.

However, growth of *M. pulmonis* in the lung remarkably differed not only between experimental and control mouse groups, but also among experimental groups. Although the numbers of the organisms in the lungs of almost all control mice were 10^3 CFU/g tissue or less, 37 of 40 mice of experimental group 1 in which *M. pulmonis* and Sendai virus infectors were introduced simultaneously harbored so many organisms as more than 10^7 CFU/g in their lungs. Comparing with control mice, enhanced growth of the organisms was also observed in groups 2 to 5 mice which were infected with the two agents at 2- or 6-week-interval, although not so remarkable as in group 1 mice. Such an enhancement of the growth was more evident in groups 2 and 4 mice than in groups 3 and 5 ones, indicating that

 Fig. 1. Growth of *M. pulmonis* in the trachea and lung.

—○—, Trachea (mean),
 ●, Lung (individual).
 ↓ M : Contacted with *M. pulmonis* infectors.
 ↓ S : Contacted with Sendai virus infectors.

synergistic effect of Sendai virus to growth of *M. pulmonis* in the lung was stronger when the both agents infected at shorter intervals.

2. Development of pneumonia

Detection of pneumonia and its extent are summarized in Table 1. In contrast to control group M in which only 4 of 40 mice were suffered from the pneumonia caused by *M. pulmonis* alone, the lesions were frequently detected in mice of experimental groups infected with *M. pulmonis* and Sendai virus, especially in group 1 in which

the lesions appeared in all mice with exceptional one examined 6 weeks after contact with infectors. However, mice of another control group S infected with Sendai virus alone were also frequently suffered from pneumonia. In mice of groups 2 and 3, no lesion was detected until they were superinfected with Sendai virus after primary infection of *M. pulmonis* alone. Contrarily in mice of groups 4 and 5, the pneumonia was produced in almost all mice by infection with Sendai virus alone before superinfection of *M. pulmonis*. Thus the

Table 1. Detection of pneumonic lesions and their scores of severity

Mouse group		Weeks after contact with <i>M. pulmonis</i> and/or Sendai virus infectors							
		2	4	6	8	10	12	14	16
Experimental group	1	5/5* (24.0)	5/5 (44.2)	4/5 (42.0)	5/5 (49.6)	5/5 (49.6)	5/5 (53.2)	5/5 (52.8)	5/5 (50.0)
	2	0/5 (0)	5/5 (15.8)	4/5 (12.0)	2/5 (19.0)	5/5 (11.0)	4/5 (19.3)	4/5 (20.8)	3/5 (28.0)
	3	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (20.0)	4/5 (13.0)	4/5 (7.3)	2/5 (5.0)	4/5 (19.0)
	4	5/5 (20.0)	5/5 (9.2)	5/5 (12.4)	5/5 (9.4)	5/5 (12.6)	5/5 (12.4)	4/5 (10.8)	4/5 (14.5)
	5	5/5 (17.8)	5/5 (15.6)	4/5 (5.3)	5/5 (7.0)	4/5 (21.8)	4/5 (8.0)	4/5 (28.5)	2/5 (24.5)
Control	M	1/5 (76.0)	0/5 (0)	1/5 (58.0)	0/5 (0)	1/5 (68.0)	0/5 (0)	0/5 (0)	1/5 (38.0)
	S	5/5 (16.8)	5/5 (14.4)	3/5 (4.7)	3/5 (6.3)	3/5 (4.3)	3/5 (4.0)	3/5 (3.7)	4/5 (6.3)

* Positive/Examined. (): Mean score of pneumonic lesions.

M: Infected with *M. pulmonis* alone.

S: Infected with Sendai virus alone.

high incidence of the lesions in experimental group mice could not be attributed to synergistic effect of the two infectious agents but was supposed to be mainly resulted by Sendai virus infection.

Nevertheless, a significant difference was observed in extents of the lesions between experimental group and control group S mice as expressed by scoring the lesions. Mean scores of the lung lesions in the control mice of Sendai virus single infection were always lower than 20 points, decreasing to lower than 10 points after 6 weeks of experiment. In group 1 mice, however, mean scores were always higher than 40 points after 4 weeks of contact and persisted at high levels to the end of the experiment, showing no tendency of decrease with the time course of infection. Similarly, mean scores in mice of other experimental groups were usually higher than those in the control mice, but not so significant as in group 1 mice. However, in control mice of *M. pulmonis* single infection, extents of the lesions were wide and

Fig. 2. Mean titers of CF antibody to *M. pulmonis* in each mouse group.

severe as shown by scores of 76, 58, 68 and 38 points, although the lesions rarely developed.

3. Production of antibodies against *M. pulmonis* and Sendai virus

Production of serum antibody against *M. pulmonis* is shown in Fig. 2. In control group M mice infected with *M. pulmonis*

Fig. 3. Mean titers of HI antibody to Sendai virus in each mouse group.

alone, the antibodies were detected in all mice 2 weeks after contact with infectors, followed by increases in titers and reached the peak at the 4th week. Thereafter the antibody titers decreased rapidly and became 1:16 or lower in most cases at the 12th week and later. In group 1 mice, however, antibody titers increased somewhat more slowly than in control group M mice, reaching the peak ranging from 1:64 to 1:512 during the 6th to 8th weeks, and the titers persisted till the 16th week when the experiment was terminated. Similar antibody responses were also observed in group 4 mice. In other experimental groups, antibody titers were comparatively low after sequential infection of the two agents but not decreased during the observation period, except for group 5 mice which showed similar antibody responses as the control mice.

Productions of HI antibody to Sendai virus are shown in Fig. 3. In the control group S mice infected with Sendai virus alone, antibody titers were always 1:256 or higher throughout the experimental period after the 2nd week. However, in-

hibitory effect on the antibody titers was resulted by superinfection of *M. pulmonis*. In the experimental groups, antibody titers were generally lower than those of control mice and decreases of titers were usually observed at the late stage of the infection, especially remarkable in groups 2 and 3.

Discussion

In our previous study, it was already reported that in ddY mice infected with strain 925T of *M. pulmonis*, the organisms grew always in the trachea but rarely in the lung, producing pneumonia in only a few of the infected mice [19]. However, in the present study in which ddY mice were exposed to both *M. pulmonis* and Sendai virus, the growth of *M. pulmonis* in the lung was found to be remarkably enhanced by superinfection of Sendai virus, increasing severities of the pneumonic lesions developed.

It was generally said that the synergistic phenomenon appeared only when viral infection preceded bacterial infection or they occurred at the same time [7, 10, 11, 17]. In our experiment, the enhanced growth of *M. pulmonis* in the mouse lung was most evident when Sendai virus infected at the same time. The longer time difference between infections of the two agents resulted in the less enhancement of the growth, confirming the phenomenon reported by the previous investigators. However, the same phenomenon was also observed to some degrees in mice contacted with *M. pulmonis* infectors 2 or 6 weeks before contact with Sendai virus infectors, suggesting that viral infection was not always necessary to precede bacterial infection for appearance of the synergistic effect.

Although no special investigation was made in our study, the following mechanisms are supposed to be concerned on the synergistic phenomenon: a decreased

clearance ability of the respiratory tract owing to destructive damage of the epithelial cells with cilia [1, 6, 15, 18], a decreased phagocytic ability of pulmonary macrophages [9, 21] and/or enhancement of *M. pulmonis* infection by selecting pathogenic form of the organisms by viral infection [3, 4]. Further studies are needed to make clear the true mechanisms of this phenomenon.

Pneumonia developed in 80 to 100 per cent of mice infected with both *M. pulmonis* and Sendai virus. However, the pneumonia was also detected in many control mice infected with Sendai virus alone, and thus the lesions appeared in the dually infected mice could not be always attributed to the effects of combined infections. Nevertheless, severities of the lesions observed in the dually infected mice were remarkably stronger than those in control mice of Sendai virus single infection. In addition, although the extents of the pneumonia produced by Sendai virus infection usually decreased and localized around the bronchioles from 2 to 3 weeks after infection [14], the lung lesions appeared in the dually infected mice seemed to become more severe according to time course of the infection. Therefore, it was considered that severities of the lesions were enhanced by remarkable growth of *M. pulmonis* in the lung, especially at the late stage of combined infections. This consideration was supported by the facts that 96%, 36%, 20%, 44% and 28% of groups 1 to 5 mice harbored *M. pulmonis* of more than 10^5 CFU/g tissue in their lungs, respectively, which was the enough number of the organisms to produce pneumonia in mice [20].

CF antibody titers against *M. pulmonis* in control mice were at maximum 4 weeks after infection and then decreased rapidly, but such a rapid decrease in titers was not

observed in experimental groups 1, 2 and 4 in which both *M. pulmonis* and Sendai virus infectors were introduced simultaneously or in a short time difference. Such a persistence of the antibody titers in superinfected mice might be reflected by persistent infection of *M. pulmonis* in the mouse lung [2, 13, 19].

On the other hand, HI antibody titers against Sendai virus in dually infected mice were usually lower than those in control mice infected with the virus alone. This phenomenon was especially evident in mice of groups 2 and 3, in which Sendai virus infection was preceded by *M. pulmonis* infection, suggesting that growth of Sendai virus was somewhat inhibited by *M. pulmonis* previously grown over the epithelial cells of respiratory tract [12, 16, 22] or antibody production to the virus was interfered by *M. pulmonis* infection [8]. To investigate the causes of this phenomenon, further studies are needed with special reference to the growth of Sendai virus in the respiratory tract.

Acknowledgments. The authors express their gratitude to Miss K. Nakayama and Mrs. J. Matsubara for technical assistance.

This work was supported by a grant from the Science and Technology Agency and by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References

- [1] Appell, L. H., Kovatch, R. M., Reddeclift, J. M., and Gerone, P. J. (1971). Pathogenesis of Sendai virus infection in mice. *Am. J. Vet. Res.* **32**, 1835-1841.
- [2] Atobe, H., and Ogata, M. (1974). Pneumonitis in mice inoculated with *Mycoplasma pulmonis*: Production of pulmonary lesions and persistence of organisms and antibodies. *Jpn. J. Vet. Sci.* **36**, 495-503.
- [3] Buddingh, G. J. (1956). Experimental combined viral and bacterial infection (influenza C and *Hemophilus influenzae* type B) in embryonated eggs. *J. Exp. Med.* **104**, 947-958.

- [4] Buddingh, G. J., Al-Talib, A. M., and Pipes, F. J. (1966). Combined viral and bacterial infection. An *in vitro* analysis of the population dynamics and factors influencing the enhancement of virulence of *Hemophilus influenzae* in combined infection with influenza virus in embryonated eggs. *Am. J. Pathol.* **49**, 353–363.
- [5] Chanock, R. M., Hayflick, L., and Barile, M. F. (1962). Growth on artificial medium of an agent associated with atypical pneumonia and its identification. *Proc. Nat. Acad. Sci. Wash.* **48**, 41–49.
- [6] Corstvet, R. E., and Sadler, W. W. (1966). A comparative study of single and multiple respiratory infections in the chickens: Multiple infections (with *Mycoplasma gallisepticum*, Newcastle disease virus and infectious bronchitis virus). *Am. J. Vet. Res.* **27**, 1703–1720.
- [7] Degre, M., and Glasgow, L. A. (1968). Synergistic effect in viral-bacterial infection I. Combined infection of the respiratory tract in mice with parainfluenza virus and *Hemophilus influenzae*. *J. Infect. Dis.* **118**, 449–462.
- [8] Gerone, P. J., Ward, T. G., and Chappell, W. A. (1957). Combined infections in mice with influenza virus and *Diplococcus pneumoniae*. *Am. J. Hyg.* **66**, 331–341.
- [9] Harford, C. G., and Hara, M. (1950). Pulmonary edema in influenzal pneumonia of the mouse and the relation of fluid in the lung to the inception of pneumococcal pneumonia. *J. Exp. Med.* **91**, 245–263.
- [10] Jakob, G. J., and Dick, E. C. (1973). Synergistic effect in viral-bacterial infection: Combined infection of the murine respiratory tract with Sendai virus and *Pasteurella pneumotropica*. *Infect. Immun.* **8**, 762–768.
- [11] Janssen, R. J., Chappell, W. A., and Gerone, P. J. (1963). Synergistic activity between PR8 influenza virus and *Staphylococcus aureus* in the guinea pig. *Am. J. Hyg.* **78**, 275–284.
- [12] Kohn, D. F. (1971). Bronchiectasis in rats infected with *Mycoplasma pulmonis*: An electron microscopy study. *Lab. Anim. Sci.* **21**, 856–861.
- [13] Lemcke, R. M. (1961). Association of PPLO infection and antibody response in rats and mice. *J. Hyg. (Camb.)* **59**, 402–412.
- [14] Nakagawa, M., Saito, M., Kinoshita, K., Suzuki, E., and Imaizumi, K. (1980). Pathogenicity of Sendai virus in mice cage-mated with infectors and their offsprings. *Jpn. J. Vet. Sci.* **42**, 337–344.
- [15] Nonomura, I., Sato, S., Shoya, S., Shimizu, F., and Horiuchi, T. (1971). Multiplication of *Mycoplasma gallisepticum* and Newcastle disease virus B1 strain in the respiratory tract of chickens. *Natl. Inst. Anim. Health Q. (Jpn.)* **11**, 1–10.
- [16] Organick, A. B., Siegesmund, K. A., and Lutsky, I. I. (1966). Pneumonia due to *Mycoplasma* in gnotobiotic mice II. Localization of *Mycoplasma pulmonis* in the lung of infected gnotobiotic mice by electron microscopy. *J. Bacteriol.* **92**, 1164–1176.
- [17] Reed, S. E. (1971). The interaction of mycoplasma and influenza viruses in tracheal organ cultures. *J. Infect. Dis.* **124**, 18–25.
- [18] Robinson, T. W., Cureton, R. J. R., and Heath, R. B. (1968). The pathogenesis of Sendai virus infection in the mouse lung. *J. Med. Microbiol.* **1**, 89–95.
- [19] Saito, M., Nakagawa, M., Kinoshita, K., and Imaizumi, K. (1978). Etiological studies on natural outbreaks of pneumonia in mice. *Jpn. J. Vet. Sci.* **40**, 283–290.
- [20] Saito, M., Nakagawa, M., Muto, T., and Imaizumi, K. (1978). Strain difference of mouse in susceptibility to *Mycoplasma pulmonis* infection. *Jpn. J. Vet. Sci.* **40**, 697–705.
- [21] Sawyer, W. D. (1969). Interaction of influenza virus with leukocytes and its effect on phagocytosis. *J. Infect. Dis.* **119**, 541–556.
- [22] Ventura, J., and Domaradzki, M. (1967). Pathogenesis of experimental bronchiectasis in laboratory rats. *Arch. Pathol.* **83**, 80–85.
- [23] Whittlestone, P., Lemcke, R. M., and Olds, R. J. (1972). Respiratory disease in a colony of rats II. Isolation of *Mycoplasma pulmonis* from the natural disease, and the experimental disease induced with cloned culture of this organisms. *J. Hyg. (Camb.)* **70**, 378–407.

要 約

マウスの *Mycoplasma pulmonis* 感染に及ぼすセンダイウイルス重複感染の影響: 齊藤 学・中川 雅郎・鈴木映子・木下邦明・今泉 清 (国立予防衛生研究所獣疫部) ——ddY 系マウスを用いて *Mycoplasma pulmonis* 感染に及ぼすセンダイウイルス重複感染の影響を調べた。肺における *M. pulmonis* の菌数は、単独感染例では 40 匹中 35 匹が 10^3 CFU/g 以下であったのに対して、*M. pulmonis* とセンダイウイルスの同時感染例では 40 匹中 37 匹が 10^7 CFU/g 以上であった。両病原体のいずれかを 2~6 週間先に感染させても *M. pulmonis* の増殖促進効果は認められ、両者の感染の時間差が短いほどその効果は大きかった。肺病変は重複感染例で 80~100% 発現し、*M. pulmonis* 単独感染例の 12% より高率であった。センダイウイルス単独感染例でも 80% が肺病変を形成したが、重複感染例の方が病変は強く、長期間持続した。重複感染例における *M. pulmonis* の CF 抗体持続期間は単独感染例のそれより長かった。また、センダイウイルスの抗体産生は *M. pulmonis* の重複感染によってやや抑制された。