Experimental Infection with *Mycoplasma pneumoniae* in the Young Hamster: Location of Ferritin-Labeled Antibody Binding to Infective Tissue

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HARA, K., IZUMIKAWA, K., KINOSHITA, I., OTA, M., IKEBE, A., KOIKE, M. and HAMADA, M. Experimental Infection with *Mycoplasma pneumoniae* in the Young Hamster: Location of Ferritin-Labeled Antibody Binding to Infective Tissue. Tohoku J. exp. Med., 1974, 114 (4), 315-337 — Microbiological and pathological examinations of the respiratory tract of young hamsters infected with *Mycoplasma pneumoniae* by inhalation of aerosol were carried out for up to a maximum of 98 days after infection. *Mycoplasma pneumoniae* organisms were found mainly in the pharynx and larynx for the first two weeks, then they continued to proliferate in the main bronchi or intrapulmonary bronchi for up to 98 days. On the third day after infection, inflammatory changes consisting mostly of infiltration with lymphocytes and monocytes appeared in the bronchial epithelium. These inflammatory changes proceeded to the peribronchial or interstitial tissues and reached a maximum on the 21st day after infection. After that, they showed a tendency to decrease and were replaced partially by atelectatic and emphysematous changes. These pathological processes seemed to be associated with the presence of *Mycoplasma pneumoniae*. On electron microscopic examination by the double staining and the ferritin-antibody method, *Mycoplasma pneumoniae* organisms were found among cilia and microvilli of the bronchial epithelium during the first two weeks. Structures resembling mycoplasmas, which bound ferritin were also located among the debris of exfoliated cells from epithelium. As the inflammatory process spread, exfoliation and desquamation of bronchial epithelium and increase of mucus-secreting cells, became more marked and eventually vacuolation in the remaining epithelial cells, with an increased number of basal cells was seen throughout the epithelium. In the terminal stage the ciliary epithelium was replaced partially by squamous epithelium. These studies, especially the identification of intact mycoplasma organisms at the surface of the epithelial cells, suggested that this may be the site of growth of this organism in the respiratory tract. —— *Mycoplasma pneumoniae*; electron microscope; ferritin-labeled antibody

Liu (1957) reported that *Mycoplasma pneumoniae* was an etiologic agent of primary atypical pneumonia and Maisel et al. (1967) stated that pathological findings in respiratory tracts of patients with *M. pneumoniae* infection were those of

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interstitial pneumonia and necrotizing bronchitis. Since fatal cases of mycoplasmal pneumonia are very rare, the pathological effects of *M. pneumoniae* infection have usually been studied in experimental animals such as ferret (Eaton and Meiklejohn 1944), cotton rat (Eaton and Meiklejohn 1944) and hamster (Eaton and Meiklejohn 1944; Dajani et al. 1965; Kawai 1968; Collier et al. 1971).

In this paper, the pathological changes in the respiratory tracts of young hamsters were followed for 98 days after infection. The changes have been paralleled to the presence of *M. pneumoniae* organisms in the tissue recognized by the fluorescent antibody method and by electron microscopy using ferritin-labeled antibody.

**MATERIALS AND METHODS**

*Infection of hamster.* The FH strain of *M. pneumoniae* grown in PPLO broth (Hayflick 1965) for three days at 35°C was used. This strain had been adapted to growth in vitro, but was passed twice in hamsters before use in this experiment. The mycoplasma titer of the suspension used for aerosol inoculation was 5.0×10^4 colony forming units (CFU) per ml in the first experiment and 4.2×10^7 CFU per ml in the second experiment. Syrian hamsters, three weeks old, were obtained from one farm. In the first experiment, 30 ml of PPLO broth containing *M. pneumoniae* as above described were used to inoculate sixty hamsters by inhalation of aerosol for 30 min. Each ten hamsters were enclosed in a sealed cylindrical box (29 cm in diameter and 34 cm in height) and an aerosol produced by means of a nebulizer of standard type was introduced. As the control, 12 hamsters were similarly exposed to an aerosol of PPLO broth not containing mycoplasmas. In the second experiment, fifty hamsters were inoculated by the same method.

At 1, 3, 5, 7, 10, 14, 21, 28, 35, 42, 56 and 98 days after infection, five hamsters inoculated with *M. pneumoniae* and one control animal inoculated with broth alone were sacrificed. In addition, two uninoculated animals were killed for histological examination, one at the start of the experiment, and one at its end. Blood samples were taken for immunological studies. Both lungs were removed aseptically; one was used for the isolation of *M. pneumoniae* and the other for pathological and immunopathological studies. The pharynx, larynx and trachea were also removed for identification of *M. pneumoniae*.

*Isolation of M. pneumoniae.* Throat swabs from the hamsters were inoculated on PPLO agar plates (Hayflick 1965) kept in 35°C incubator and these were observed microscopically for up to 14 days. This procedure was carried out every 2 to 7 days from one to 98 days after infection in all hamsters. The pharynx and trachea removed separately were placed into PPLO broth tubes.

For the isolation of mycoplasma, lungs were weighed, homogenized with PPLO broth and a w/v 10% suspension was prepared. This was clarified by centrifugation at 1,500 rpm for 15 min. An aliquot of 0.1 ml of suspension in ten-fold dilution was inoculated onto each of two PPLO agar plates. The numbers of *M. pneumoniae* in the lung were calculated as CFU/ml.

*Serological tests.* Blood collected just before sacrifice was defibrinated and the serum stored at -20°C.

*Complement fixation test (CF).* The micro-technique modified by the National Institute of Health in Japan was used. *M. pneumoniae* antigen prepared with PPLO broth was treated for 5 min at 100°C by the method of Kenny and Grayston (1965). Tests were performed using 32 units of the antigen and two-fold dilution of hamster serum.

*Growth inhibition test (GI).* 0.2 ml of serum diluted 1:2 was mixed with 0.2 ml of a dilution of mycoplasmal cultures containing 10^4 CFU/ml. The mixture was incubated
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for 60 min at 35°C and three separate drops of exactly 0.01 ml were dropped on PPLO agar in a Petri dish of 80 mm diameter. After 5 days incubation at 35°C, colony counts were made. Results were scored as positive if the number of colonies was under 10% than that of the control. Rabbit immune serum prepared against M. pneumoniae or human serum from a patient recovered from pneumonia due to M. pneumoniae (1: 256 GI titer) was simultaneously used as controls.

Histopathological studies. One lung with its bronchus removed from each sacrificed hamster was fixed for 18 hr in Van de Grift's solution. These specimens were washed with distilled water for 6 hr, dehydrated in alcohol and afterwards embedded in paraffin. The blocks were sectioned, and stained with hematoxylin-eosin.

Immunofluorescent studies. Pieces of the lung (2.5 mm³) were frozen quickly in O.C.T. compound (Ames Co., Elkhart, Indiana-Collier et al. 1971), subsequently were cut to ultra-thin sections in a microtome cryostat, and were fixed with cold acetone for 5 min on the glass. The sections were treated with human convalescent M. pneumoniae antiserum for 45 min followed by rabbit anti-human globulin conjugated with fluorescein isothiocyanate for 30 min at room temperature.

Preparation for electron microscopy. The lungs (1.0 mm³) were fixed under negative pressure in 1.5% glutaraldehyde in Millonig-buffer (pH 7.3) and were washed, post-fixed in 2% osmium tetroxide dissolved in Millonig-buffer (pH 7.2) for 2 hr, dehydrated in routine alcohol series and finally embedded in epon-araldite resin. The sections were cut on an ultramicrotome using a diamond knife and stained with uranyl acetate for 40 min and lead nitrate for 10 min (double staining). The sections were examined in a JEM-7A electron microscope.

Electron microscopic studies using ferritin-labeled antibody. Commercial horse spleen crystalline ferritin (Wako Co.) was recrystalized five times using the procedure of Granick (1942). The γ-globulin was prepared from anti-M. pneumoniae equine serum supplied by BBL Co. (CF titer 1:160) by alkaline ammonium sulfate fractionation. To 5.0 ml of anti-M. pneumoniae equine serum, a saturated solution of ammonium sulfate was added to a final saturation of 1: 3. The mixture was adjusted with 1 N sodium hydroxide to pH 7.8, stirred for 60 min, and centrifuged at 12,000 rpm for 30 min at 0°C. The precipitate was dissolved in 0.85% saline solution. Precipitation with ammonium sulfate was repeated three times. The final precipitate was dissolved in 0.85% saline and then dialyzed against 0.85% saline solution for 24 hr and centrifuged at 15,000 rpm for 30 min. The supernatant fluid, which constituted the γ-globulin, was conjugated to ferritin by a method of Sri Ram et al. (1963).

Lung tissue was sliced into very small pieces in phosphate buffer solution containing 5% formalin and then allowed to stand for 10 min in negative pressure to facilitate penetration of the fixative. After fixation and washing, tissue suspension was mixed with an equal volume of ferritin-labeled γ-globulin dissolved in 0.05M phosphate buffer (pH 7.5) and allowed to stand for 40 min at room temperature. The tissue was washed with cold Millonig-buffer (pH 7.3), post-fixed in 1.5% osmium tetroxide dissolved in Millonig-buffer (pH 7.2), dehydrated, embedded, sectioned and stained as before.

M. pneumoniae, M. orale and M. salivarium obtained from the centrifuged precipitate of PPLO broth cultures resuspended in veronal buffer (pH 7.4) and used to confirm the specific binding of ferritin to M. pneumoniae.

RESULTS

Growth of M. pneumoniae

Isolation from the throat. In the first experiment, M. pneumoniae was isolated from the throat in 60% of the infected hamsters at 3 days after infection.
The isolation rate rose rapidly and reached 100% by 10 days after infection. Then it fell gradually to 89% at 21st day and 43% at 24th day, and subsequently maintained the level of approximately 40% from 25th day to 42th day after infection. In the second experiment, the isolation rate was similar to the first experiment, although the peak recorded was only 70% at 7th day after infection.

Isolation from the larynx and trachea. Mycoplasma organisms were isolated from the larynx of all infected hamsters up to 14 days and were not isolated after the day 21. However, the positive isolation was obtained from tracheas of all inoculated animals up to the day 98, except for two negative instances on 3rd day.

Growth in the lungs. *M. pneumoniae* grew in the lungs of all hamsters. The average numbers of organisms isolated from the lungs of five hamsters during the period of observation are shown in Fig 1. Results were similar in the two experiments, except that the mycoplasma titers obtained from lungs of infected animals were generally $10^1$-$10^2$ CFU/ml lower in the second experiment.

![Growth curve of *M. pneumoniae* in the hamster lung.](image)

**Fig. 1.** Growth curve of *M. pneumoniae* in the hamster lung.

- - - , the first experiment (inoculation dose, $5.0 \times 10^6.0$ CFU/ml);
- - - , the second experiment (inoculation dose, $4.2 \times 10^7.0$ CFU/ml).

Serological response

In complement fixation test, only two of 60 hamsters in the first experimental group showed titers of 1: 16 on the day 28 and the day 42 after infection, three showed titers of 1: 8 and all remaining animals showed titers of under 1: 8. No hamsters showed detectable growth-inhibition antibody.

Histopathological studies

The histopathological specimens obtained from infected hamsters were compared with specimens from animals inoculated with PPLO broth alone, and with
TABLE 1. Histopathological findings in light microscopy

<table>
<thead>
<tr>
<th>Time after infection (day)</th>
<th>Pathological evidence in bronchial epithelium and intrabronchial spaces</th>
<th>Peribronchitis</th>
<th>Interstitial pneumonitis</th>
<th>Fibrosis</th>
<th>Other findings</th>
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<tr>
<td>3</td>
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<tr>
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<td>7</td>
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<td>4/5</td>
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<td>98</td>
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<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
<td>Emphysema (5)</td>
</tr>
</tbody>
</table>

* The very slight findings were excluded.
† In one case findings were not clear because of severe purulent changes.
‡ ( ) showed the positive results of each one control.

The two uninoculated animals. Five categories of microscopic abnormality were sought: (1) pathological evidence in bronchial epithelium and intrabronchial spaces, mainly edema, cellular infiltration, degeneration or presence of debris, (2) peribronchitis, (3) interstitial pneumonitis, (4) fibrosis, (5) other findings including bronchiectasis, atelectasis, edema in alveolar septa and local emphysema. The occurrence of these changes in five mycoplasma-inoculated hamsters and one broth-inoculated control animal sacrificed on each occasion is shown in Table 1.

At the end of one day or 3 days after infection, bronchial or peribronchial tissue was slightly congested and edematous. Afterwards these findings became prominent and were admixed with interstitial pneumonitis which apparently occurred at the stage of profuse mycoplasmal growth (Fig. 4). The peribronchial tissue was infiltrated with monocytic and lymphocytic cells, the ciliated epithelial cells were exfoliated into the bronchi, and interstitial tissue and alveolar septa became thick (Fig. 5). Evidence of bronchitis and peribronchitis was found in varying degrees and was more intensive in the main bronchi and bronchioles but less evident in the smaller bronchi. Interstitial pneumonitis occurred simultaneously with peribronchitis (Fig. 6). 35 days after infection, peribronchial and interstitial inflammatory changes were reduced to some degree. At this stage, fibrosis in interstitial tissues was prominent and was frequently associated with atelectasis and local emphysema in some specimens (Fig. 7).

Lungs of hamsters inoculated with PPLO broth alone (Fig. 3), when compared
with uninoculated hamsters (Fig. 2), showed slight edema in the interstitial tissues and cellular infiltration in bronchial or peribronchial tissues, but the changes persisted for only 2 weeks and were easily distinguished from changes seen in the infected hamsters (Table 1). It has been supposed that these alterations may occur as the chemical and/or physiological reaction due to PPLO broth medium.

**Immunofluorescent studies**

Every week for up to 28 days after infection, lung specimens were processed for immunofluorescent studies in order to confirm the site of the growth of *M. pneumoniae* in vivo. Specific fluorescence was identified along the epithelial surface of the bronchus, as demonstrated in Fig. 8 in the 14-day specimens. However, less or no specific fluorescence was observed in the 21-day and 28-day specimens. No specific staining was recognized in the lung tissues of normal hamsters.

**Electron microscopic studies using double staining and binding of ferritin-labeled γ-globulin**

**Identification of *M. pneumoniae* organisms.** Sections prepared from the pellets of centrifuged broth cultures of *M. pneumoniae*, *M. orale* and *M. salivarium* were treated by the ferritin antibody method as previously described. Ferritin represented by fine granules was absorbed to the limiting membrane of *M. pneumoniae*, FH strain (Fig. 9), but very little absorption occurred on the surface membrane of *M. orale* and *M. salivarium*.

Scanty rounded bodies thought to be *M. pneumoniae* organisms were found among cilia and microvilli of the bronchial epithelium in specimens examined by double staining (Fig. 10) or ferritin-labeling (Fig. 11) from 3 to 14 days after infection. The internal structure of these organisms was coarse granular, net-like or vacuolated. No intact mycoplasma organism was definitely identified in the bronchial submucosal layer or alveolar wall.

**Alteration in bronchial and alveolar epithelium.** The disintegration, vacuolization and protrusion of bronchial epithelial cells into the lumen commenced by the third day after infection. At that time, mucus-secreting cells had not increased greatly in whole parts. In double staining preparations, some high electron-dense substances were observed around cilia and microvilli, but it was not clearly associated with mycoplasma-like bodies. 7 days after infection, desquamation of the epithelial cells had begun.

From 14 to 98 days after infection the following pathological changes were mainly observed: (1) increase of mucus-secreting cells, (2) protrusion of epithelial cells into the bronchial lumen, (3) swelling of mitochondria, (4) intracellular vacuolization, especially vacuolization of clear cells, (5) desquamation and exfoliation of epithelial cells, which were partially replaced by squamous cells. The majority of epithelial cells were disintegrated and exfoliated by 28th day and were replaced by squamous cells up to 35 days after infection. However, in the ferritin-
labeled electron microscopic studies, ferritin was also distributed among desquamated or exfoliated epithelial cells (Fig. 12) and among the protruded epithelial tissues (Fig. 13).

Compared with the 3-day specimens, some specimens taken at the end of 14 days or later showed atelectasis which would be induced by the thickening with fibrosis of alveolar wall. No ferritin was found in any place of the alveolar wall.

**DISCUSSION**

Dajani et al. (1965) have indicated that *M. pneumoniae* in the lung of infected hamsters reached a maximum of $10^{5.5}$ CFU per ml at 7 days and then declined to $10^{3.8}$ CFU per ml at 24 days after infection. Our results are confined to the growth of *M. pneumoniae* in hamster lung and showed that the organisms persisted there for up to 98 days. In our first experiment, the number of *M. pneumoniae* in infected lungs reached a maximum of $10^{6.1}$ CFU per ml 14 days after infection, and maintained $10^5$ CFU per ml for up to 42 days. In the second experiment, however, it rose and then fell more slowly during the 98 days after infection. This indicates that *M. pneumoniae* organisms are multiplying in the respiratory tract for periods of weeks. Electron microscopic studies showed that the most likely sites of multiplication were the bronchi and bronchioles.

*M. pneumoniae* induced a variety of pathological lesions in the respiratory tract. The cytopathological changes in the hamster were indicated in preliminary studies by Eaton and Meiklejohn (1944), Dajani et al. (1965) and Collier et al. (1971). The results observed in our experiment corresponded with those of the previous workers, but we have added long term observations on the consequences of prolonged infection.

The ultrastructure of the respiratory organs in the infected hamster has been studied in tracheal organ culture by Collier et al. (1971) and in experimental inoculation by Kawai (1968). In our experiment an attempt was made to confirm the presence of *M. pneumoniae* on the respiratory tract by electron microscopy using binding of ferritin-labeled γ-globulin and double staining techniques.

In the first, it must be emphasized that mycoplasma organism could be exactly identified with ferritin in this experiment. Up to 14 days after infection, *M. pneumoniae* organism could be found among the cilia or microvilli of bronchial epithelium. At the same time, mycoplasma-organism-like bodies, deformed in shape, surrounded with ferritin, were seen in the debris in the areas where the bronchial epithelium was exfoliated. The earliest change on the bronchial surface accompanying infection was exfoliation of epithelial cells, and subsequently the increase of mucus-secreting cells. As proceeding changes, vacuolization in epithelial cells, propagation of basal cells, and finally appearance of squamous cells were observed. Furthermore, ferritin was also located within the epithelial cells. As above mentioned, the ferritin labeling method had also a great advantage in the confirmation of *M. pneumoniae* within the respiratory tract of the infected animal. The findings observed in these experiments, suggested that the epithelial
surface on which ferritin attached might be the site of infection of this organism in the respiratory tract and the invaded mycoplasmas would be propagated within the epithelial cells.

In the findings observed by Collier et al. (1971), there were complete loss of cilia and an appearance of numerous pleomorphic structure corresponding to mycoplasma organisms among the cilia. Furthermore, Kawai (1968) reported that *M. pneumoniae*-like organisms were recognized not only among the cilia but also within the alveolar lumen. These pathological changes were not always noticed in our study. In Collier's work (Collier and Clyde 1971), organ cultures were constantly in contact with high titers of mycoplasma organisms and were maintained in PPLO broth; conditions were therefore somewhat different from those in the intact animal. One reason for the apparent difference in pathological effects produced in these various studies might be due to the state of strains used.

In an attempt to correlate these findings to the pathological effects of *M. pneumoniae* in man, it may be noted that homogeneous or flocculated shadows which were supposed to be pulmonary parenchymal changes were observed in half or more (64 percent in our experience) of patients with mycoplasmal pneumonia (Hebert 1966). However, any evidence of the parenchymal inflammatory change could not be found in our experiments using hamsters.

### Acknowledgments

We wish to thank Dr. Kumato Mifune for helpful suggestion of the immunofluorescent technique, Mr. Tadashi Suematsu for preparing the specimens for electron microscopy and Miss Kayoko Kubo for technical assistance.

### References


Figs. 2-7. Histopathology of the respiratory tract of hamster.

Fig. 2. Normal lung.

Fig. 3. Control lung inhaled with PPLO broth alone.

Fig. 4. Inflammatory changes in the broncho-epithelial and interstitial tissues, 3 days after infection.

Fig. 5. Peribronchitis, thickening of interstitial tissue and alveolitis, 7 days after infection.
Fig. 6. Interstitial pneumonitis, edema and hyperplasia of alveolar wall, 28 days after infection.

Fig. 7. Local emphysema, 35 days after infection.

Fig. 8. Bronchial epithelium of hamster infected with *M. pneumoniae* (indirect immunofluorescent technique), 14 days after infection.
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Fig. 9. *M. pneumoniae* FH strain binded with ferritin-labeled γ-globulin. Ferritin distributed around the organism (×55,000).
Figs. 10–13. Electron micrographic pictures of the respiratory tract.

Fig. 10. Bronchial epithelium with *M. pneumoniae*, 3 days after infection (double stain, ×29,600).
Fig. 11. Bronchial epithelium with *M. pneumoniae*, 3 days after infection (ferritin-labeled, ×51,800).
Fig. 12. Distribution of ferritin among the epithelial cell (ferritin-labeled, $\times 51,800$).
Fig. 13. Distribution of ferritin in relation to a protruded epithelial cell (ferritin-labeled, $\times 37,000$).