# Immunohistopathological and Electron Microscopical Studies on Influenza Virus Multiplication in Hatching Egg and on Pathogenesis of Experimental Influenza Pneumonia

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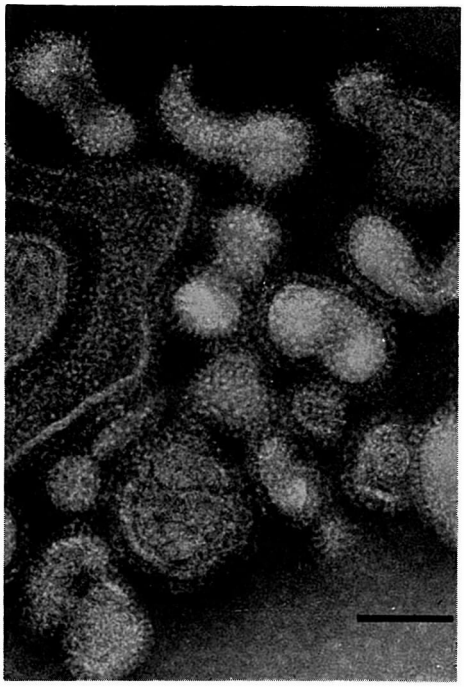
## INTRODUCTION

Influenza is an acute infectious respiratory disease caused by influenza virus and brings pandemic infection to human being. Since Rous and Murphy[1] used the chorioallantoic membrane (CAM) to produce experimental infection to the virus of the Rous sarcoma, CAM has been used to investigate virus multiplication as intermediate method between in vitro and in vivo. Watson and Coons[2] first described influenza virus antigen in infected cells on CAM by fluorescent antibody technique (FAT). Influenza virus is known to infect the ciliated epithelium of the respiratory tract of human being and some animals, such as ferret, chick, horse and pig. After succeeding adaptation of influenza virus to mice, it has become easy to investigate the experimental influenzal pneumonia, and many authors described the murine viral pneumonia caused by influenza virus in their literatures. Hers and Mulder[3,4] and Nayak et al[5] reported virus antigen in infected respiratory epithelial cells of mice.

Chorioallantoic cavity (CAC) of the hatching egg was investigated as the place of virus multiplication (Egg experiment) and in addition, early stage of murine viral pneumonia and its pathogenesis were discussed on relationship between distribution of virus antigen and histological appearance (Mouse experiment) in this paper.

## MATERIALS AND METHODS

Virus: The virus used in these experiments was influenza virus A/Tokyo/1/72 adapted to mouse through 22 passages of egg and 32 passages of mouse, which was kindly provided by Dr. Yamashita. The



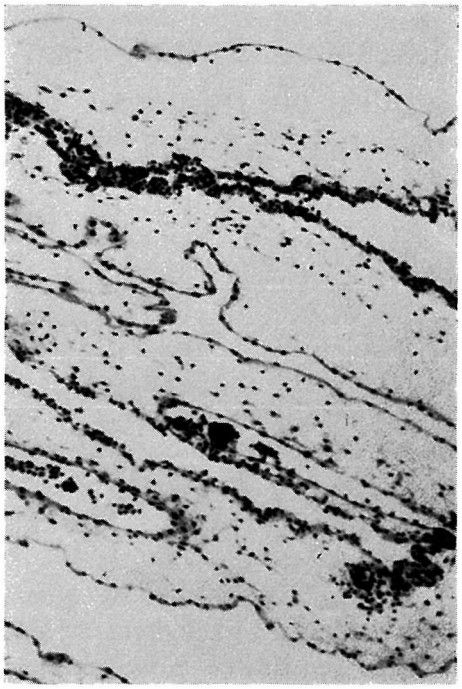


**Fig. 1.** **Fig. 2.**

**Fig. 1.** Inoculated influenza virus A/Tokyo/1/72 in negative staining. Pleomorphic spherical and filamentous figures are characteristic. Envelope is detected to project from the surface of the virus.

**Fig. 2.** Normal CAM of 11 days old hatching egg. Hematoxylin and eosin stain. ×100

virus showed pleomorphic feature in negative staining shown in (Fig. 1).

Egg: 11 days old hatching eggs were used. (Fig. 2).

Mouse: 4 weeks old dd-SPF mice were used.

Immune Serum: An antiserum to the influenza virus A/Tokyo/1/72 was prepared from immunized ferret with multiple abdominal injections of virus fluids.

Afterward, obtained gamma-globulin labeled with isothiocyanate was absorped with both normal chick embryo powder and mouse liver powder to remove nonspecific factors.

Inoculation to Eggs and Materials Obtained: Above all eggs were inoculated into CAC with 0.2 ml of a $10^{-3}$ dilution of the virus fluids ($10^{7.5}EID_{50}$) and incubated at 35°C. After 1 to 8 days incubation, 10 eggs were daily sacrificed, and the embryos, membranes and CAF were obtained. The embryos and membranes were thoroughly washed in

phosphate-buffered saline and quickly frosen in n-hexane cooled in a dry ice cabinet at −80°C. By cold microtomy, serial sections about 5 $\mu$ in thickness were made for FAT (direct method) and light microscopy. According to the standard method, one of them was fixed in aceton cooled at 4°C for 30 minutes and mounted with following fluorescein-labelled immune serum and observed under fluorescent microscope. And the other section was fixed in alcohol mixture (adultrated 95% alcohol with ethyl ether of the same quantity and mixed 9 parts of the mixture with 1 part of 10% formalin) and stained with hematoxylin and eosin. The specimens for electron microscopy were also obtained from the same egg and fixed in 4.15% glutaraldehyde and 1% osmium tetroxide, dehydrated in the graded ethanol and embedded in epoxy resin according to the method of Luft[6]. The hemagglutinin (HA) level and chicken red cell agglutination (CCA) level were performed by using the CAF. Furthermore, the all membranes and embryos were obtained and fixed in 10% formalin for paraffin embedding for conventional histological examination.

Inoculation to Mice and Materials Oatained: Under light chloroform anesthesia, each mouse was intranasally inoculated with 0.05 ml of a $10^{-1}$ to $10^{-5}$ dilution of virus fluids ($10^{7.5}$ $EID_{50}$). Seventy mice were devided into fourteen groups, and each group of mice were sacrificed after 20 and 60 minutes, and 3, 6, 9, 12 and 24 hours and thereafter every day for 10 days after inoculation. As a control, 0.05 ml of medium 199 was intranasally inoculated at the same time. After bleeding, tracheas and lungs were obtained from two mice and frozen rapidly by the same method in egg experiment. The specimens for electron microscopy were also obtained. The all remaining organs of the above two mice and all organs of the other three mice were obtained for light microscopy. In addition, five mice were intracerebrally inoculated with 0.03 ml of a $10^{-1}$ dilution of virus fluids and sacrificed at 1 day after inoculation, and the other five mice were inoculated with 0.02 ml of a $10^{-3}$ dilution of virus fluids into right pleural cavity and sacrificed at 4 days after inoculation. From these mice, the brains and bilateral lungs were obtained for immunofluorescent and light microscopy.

## RESULT

1. Egg Experiment

a) Macroscopic Finding: In these series of experiments remarkable macroscopic changes were not observed except for red colored small area injuried by needle on CAM. Macroscopic changes were not detected on the other membranes and embryo either.

b) Light Microscopic Finding: In any of the embryo, distinctive lesions were not detected, but in the ectodermal lining cells on CAM, histological changes were noticed remarkably day by day.

1) CAM: At 24 hours after inoculation, as the first response of infection to CAM, nodular formation or simple thickening of epithelium associated with slight infiltration of inflammatory cells composed of mononuclear cells were recognized in some areas in entoderm. The proliferated epithelial cells became more cuboidal with round pyknotic nuclei depending to intracellular edema or clear nucleus having one to two marked nucleoli. Beneath this lesion, close-packed cellular accumulation of mainly mononuclear cells and eosinophiles were recognized in the mesoderm, so that the mesoderm appeared to be thickened by moderate increasing of these cells. While, the lesion thickened by intercellular edema was also present in the mesoderm. A little blood cells were observed on the surface of the ectoderm. Nodular proliferation and slight simple thickening of the lining cells were detected in the ectoderm. Beneath this lesion, focal inflammatory cell accumulation to the mesoderm and primary lesion of the entoderm were observed. In some of those cells, especially in nodular proliferation, intracellular edema were present. The single layer of flattened ectodermal cells became hyperplastic extensively and had clear cytoplasm and pyknotic nuclei. Some limit of inflammatory cell accumulation was usually observed among the lining cells.

At 48 hours after inoculation, necrotic areas and their desquamation into the CAC, induced by virus infection, were as characteristic as simple thickening (Fig. 3) and intercellular edema. The edema became so severe that in some areas the nuclei appeared as a thin crescent at the periphery of the cells or disappeared. The ectoderm increased its thickness and some of its necrotic areas fell off the layer with accumulated inflammatory cells and formed the necrotic masses, leaving only few cell layers in the ectoderm. The histological findings in mesoderm and entoderm in this stage were similar to those mentioned at 24 hours after inoculation. The mesoderm showed moderate edema and a considerable amount of inflammatory cell accumulation, and in some areas, some epithelial nodules were observed deeply in the mesoderm. Few necrotic areas similar to those in the ectoderm were also present in the entoderm and likewise fell off into the CAC (Fig. 4). The proliferated capillaries were detected in the intermediate position of the ectodermal layer.

At 60 hours after inoculation, wide-spread intranuclear vacuoles were recognized in various degree in the ectodermal lining cells (Fig. 5). In some areas, the capillaries among the lining cells connected each other and took

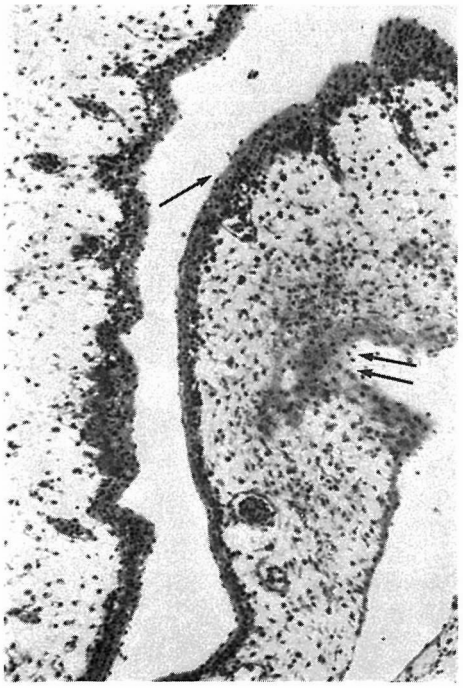


Fig. 3.

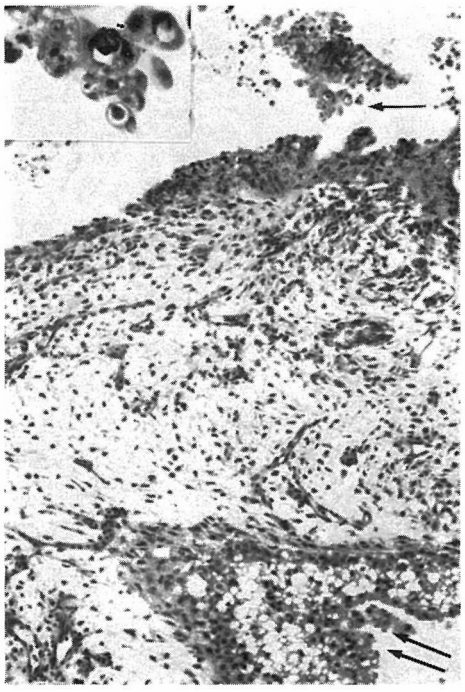


Fig. 4.

**Fig. 3.** CAM at 48 hours after inoculation. Simple thickening (↗) is detected in the ectoderm, the surface of which is slight necrotic. Inflammatory lesion is recognized in mesoderm (↗↗). Hematoxylin and eosin stain. ×100

**Fig. 4.** CAM at 48 hours after inoculation. Proliferated lining cells (↗) with atypism and different polarity are recognized in entoderm (upper left). Severe vacuolization is observed in ectoderm (↗↗). Hematoxylin and eosin stain. ×100, ×400

the intermediate position, separating the ectoderm into two layers. The necrotic masses desquamated from the ectoderm increased moreover. The degenerative changes of the other two layers were also recognized to slightly progress.

At 3 days after inoculation, the degenerative changes were also detected in three layers at a high degree. The capillary nets were noticed below the ectoderm.

2) The Other Membrane: Though, the virus was inoculated into CAC, the responses to virus infection were also observed in amnion and yolk sac.

Amnion: At 48 hours after inoculation, epithelial nodules similar to those of the CAM were detected in the ectoderm of amnion. The cells composing the nodules had clear cytoplasm and one to two clear nucleoli.

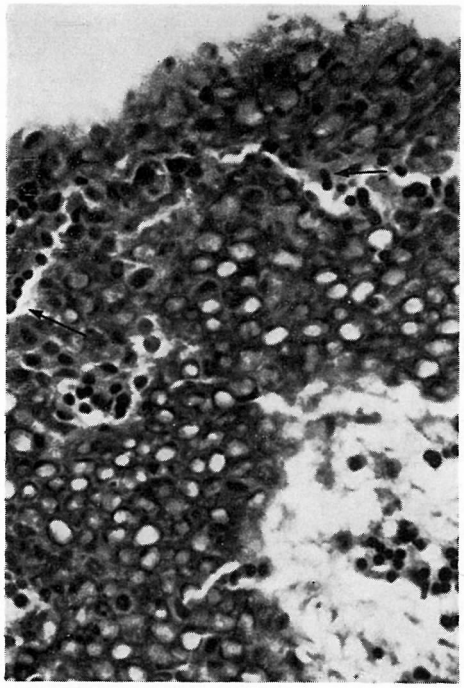


Fig. 5.

Fig. 6.

**Fig. 5.** Ectoderm of CAM at 60 hours after inoculation. Wide-spread intranuclear vacuoles are detected. The capillaries (↗) take the intermediate position and separated the ectoderm into two layers. Hematoxylin and eosin stain. ×400

**Fig. 6.** CAM at 1 day after inoculation. Strong cytoplasmic IF is detected in entodermal layer and some of mesodermal cells (↗). IF is not recognized in ectoderm. Immunofluorescent stain. ×100

Yolk Sac: At 48 hours after inoculation, the nuclei of the entodermal lining cells had large eosinophilic nucleoli, and some eosinophilic granules were recognized in cytoplasm, but proliferating figure as those observed in CAM could not be detected.

c) Fluorescent Microscopic Finding: Brilliant immunofluorescence (IF) was recognized in the entodermal lining cells in the CAM since at 1 day after inoculation.

1) CAM: At 1 day after inoculation, virus antigen was already detected. The cytoplasms of single layer of the entodermal cuboidal cells in contact with CAF, proliferated entodermal cells and some of mesodermal cells beneath the lesion were all IF strongly positive (Fig. 6). The IF was diffuse and granular in cytoplasm.

At 2 days after inoculation, IF was noticed also in cytoplasm of the

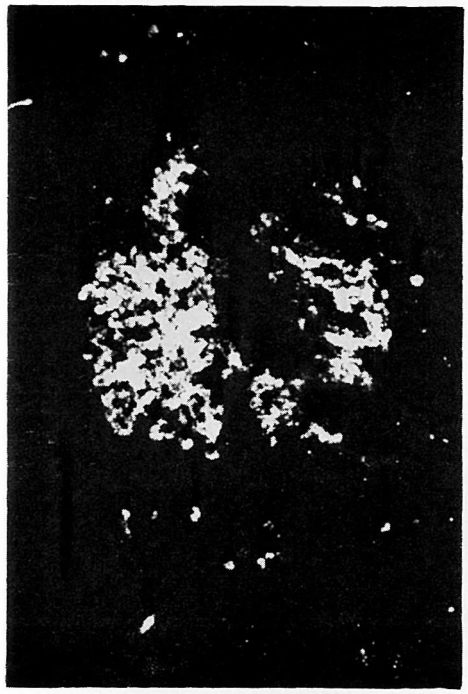


Fig. 7.

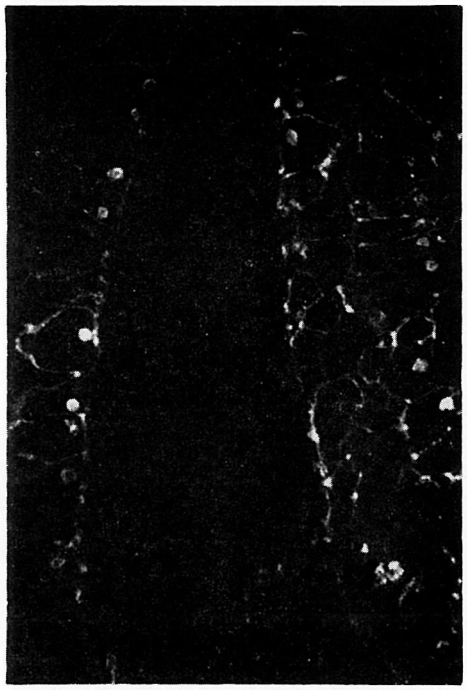


Fig. 8.

**Fig. 7.** CAM at 2 days after inoculation. Brilliant IF is detected in necrotic mass desquamated from proliferated entoderm. Immunofluorescent stain. ×100

**Fig. 8.** Yolk sac at 60 hours after inoculation. Strong nuclear IF and peripheral cytoplasmic IF are recognized in entoderm. Immunofluorescent stain. ×100

entoderm, mesoderm and necrotic masses derived from the entoderm (Fig. 7). The intensity of IF was as strong as that at 1 day after inoculation.

Since at 2 to 4 days after inoculation, IF positive parts and its density were not observed to progress.

Since at 5 days after inoculation, intensity of IF gradually decreased and at 7 days after inoculation, IF completely disappeared from the membranes.

2) The Other Membranes and Embryo: The virus was inoculated into CAC, but at 60 hours after inoculation, IF was also detected in epithelial cells of yolk sac, amnion and embryo. In the entoderm of yolk sac, IF was recognized strongly granular in nucleus and faintly in periphery of cytoplasm (Fig. 8).

In longitudinal and cross sections of embryo, the cytoplasmic IF was observed in the cells organizing arachnoidea and epithelial cells of trachea

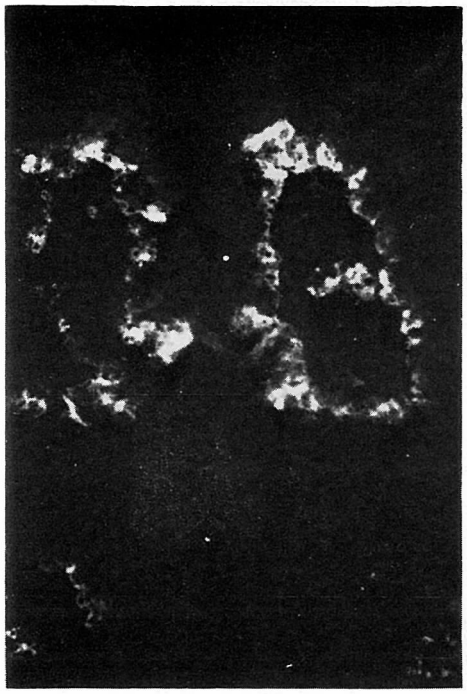


Fig. 9.

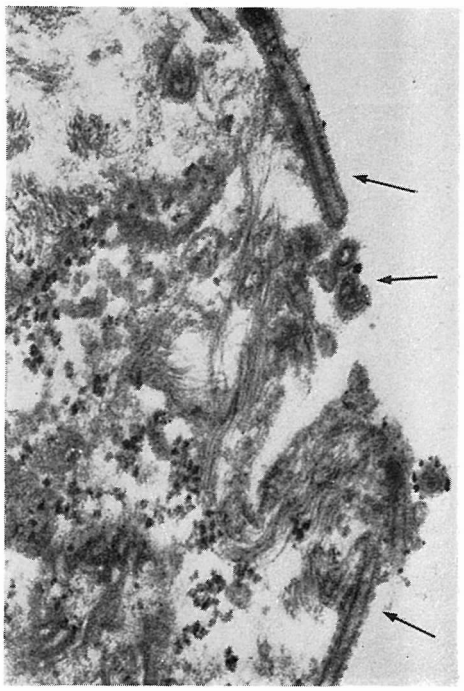


Fig. 10.

**Fig. 9.** Chicken embryo at 4 days after inoculation. Cytoplasmic IF is observed in bronchiolar epithelium. Immunofluorescent stain. ×400

**Fig. 10.** CAM at 2 days after inoculation. Spherical and filamentous virus particles (↗) are detected on the surface of degenerated ectoderm. Electron micrograph. ×12000

and bronchial tube (Fig. 9).

d) Electron Microscopic Finding: At 2 days after inoculation, infected cells in ectoderm in CAM had degenerated cytoplasm, in which many vacuoles were detected and cell membrane was destructed. The spherical and filamentous virus particles with apparent envelopes were noticed among the microvilli of infected ectodermal cells and tonofilaments in the degenerated cytoplasm (Fig. 10). Dourmashkin and Tyrrell[7] described virus particles in cytoplasmic vesicles (viropexis) of chick CAM, but viropexis could not be detected in this study.

e) Virus Multiplication in CAF: Infectious titer, HA level and CCA level in CAF are summarized in Fig. A. Infectious titer shows the highest level at 2 and 3 days after inoculation, and HA level and CCA level exhibits the highest level at 3 days after inoculation.

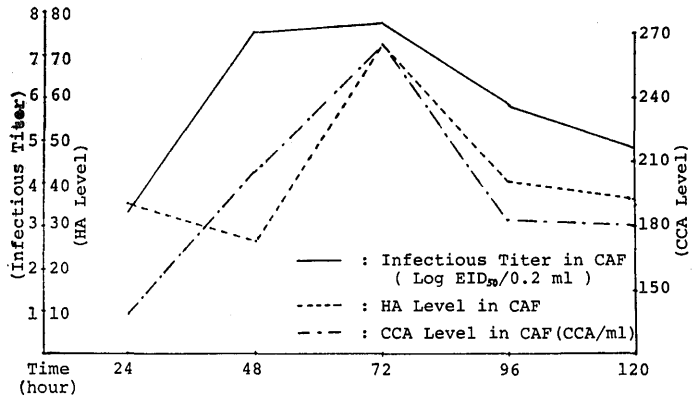



**Fig. A.** Virus Multiplication in CAF

2. Mouse Experiment

a) Macroscopic Finding: At the early stage, mice reduced their movement and gathered together. Some of the mice which were inoculated $10^{-1}$ dilution of virus fluids were dead at 3 days after inoculation. On macroscopic finding, dark red small patches were noticed on the visceral pleura, which patches at the next stage spread gradually, and finally formed dark red consolidation.

b) Light Microscopic Finding: At 20 minutes after inoculation, significant changes could not be detected.

At 60 minutes after inoculation, some cilia of tracheal epithelium disappeared and nuclear fragmentation were noticed in the tracheal epithelium.

At 3 hours after inoculation, the number of secretory vacuoles were increased in the tracheal epithelium, oppressing the nuclei in the basal side of the cells, so that nuclei appeared like crescents. Much exudate was observed in the tracheal lumen of the same mouse. Inflammatory cells could not be noticed in the tracheal lumen and bronchial tubes untill this stage.

At 6 hours after inoculation, some inflammatory cells infiltrated into the bronchial tubes of some mice, being mingled with few desduamated epithelial cells.

At 9 hours after inoculation, histological changes were recognized more extensively and many neutrophiles were noticed to surround the desquamated epithelial cells in bronchioles (Fig. 11). Amount of intracellular vacuolizations were observed in the tracheal epithelium.

At 12 hours after inoculation, mild inflammatory lesions were noticed

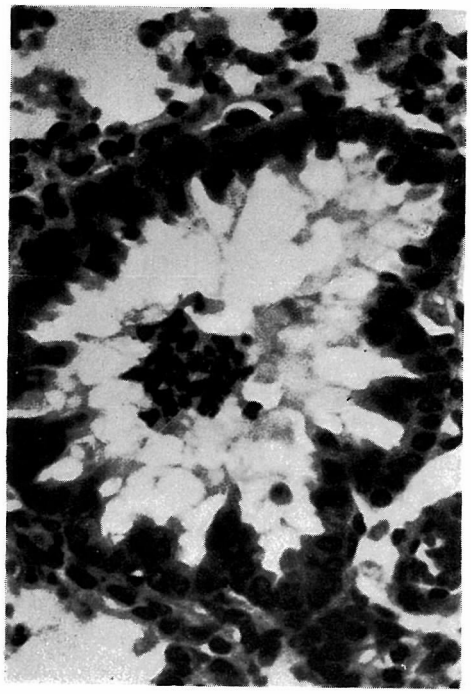


Fig. 11.

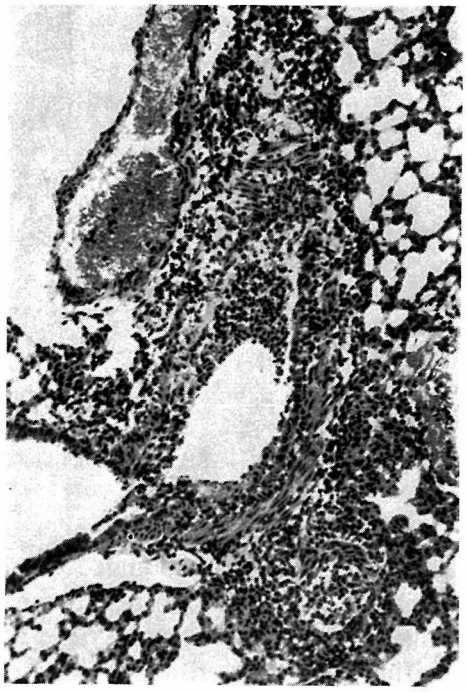


Fig. 12.

**Fig. 11.** Murine bronchiole at 9 hours after inoculation. Some desquamated bronchiolar epithelial cells are sorrounded by neutrophiles. Hematoxylin and eosin stain. ×400

**Fig. 12.** Murine bronchiole at 2 days after inoculation. Desquamated epithelial cells, neutrophiles and nuclear fragmentations are recognized in the lumen. Hematoxylin and eosin stain. ×100

interior and exterior of the bronchial tubes in some mice and nuclear fragmentations phagocytosed by ciliated epithelium were also observed. In some areas where bronchiolitis was detected, alveolar septum adjacent to the bronchiole was appeared to be partially thickened owing to inflammatory cell infiltration principally composed of neutrophiles and mononuclear cells.

At 1 day after inoculation, in some areas of trachea, many nuclei of epithelium were pyknotic and epithelial cells desquamated into the lumen, leaving only basal replacement cells for covering basement membrane on the tracheal wall. Necrotic masses composed of desquamated epithelial cells and some inflammatory cells were extensively observed in the tracheal lumen and bronchial tubes, accompanied with inflammatory lesion around them.

At 2 days after inoculation, the number of inflammatory cell increased

rapidly, forming severe inflammatory lesions in almost all over the lung (Fig. 12). But alveolar epithelium still almost remained. In the longitudinal sections of bronchioles, many neutrophiles and mononuclear cells were observed as a belt surrounding them. In some areas, focal interstitial pneumonia accompanied with some exudate in the alveolar space was noticed (Fig. 13). The alveolar lining cells were degenerated and had small round pyknotic nuclei. The histological findings of this stage were most impressive throughout this experiment.

From 3 to 4 days after inoculation, interstitial pneumonia slightly developed, and some perivascular regions were edematous accompanied with many inflammatory cells and nuclear fragmentations.

At 5 days after inoculation, atypism of bronchiolar epithelium was characteristic. The cells lining bronchiolar epithelium were possessed of

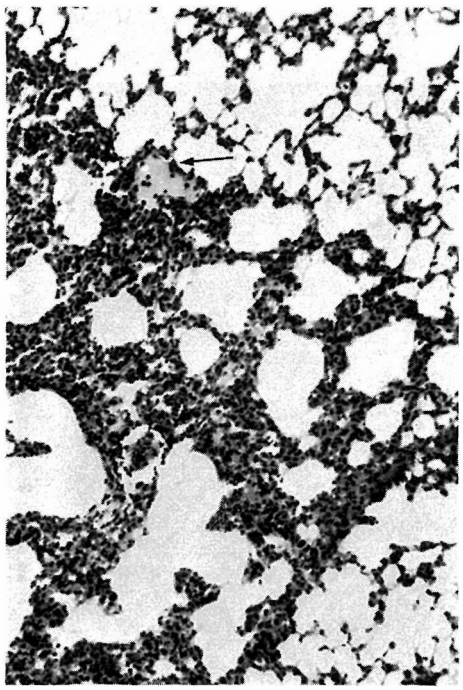


Fig. 13.

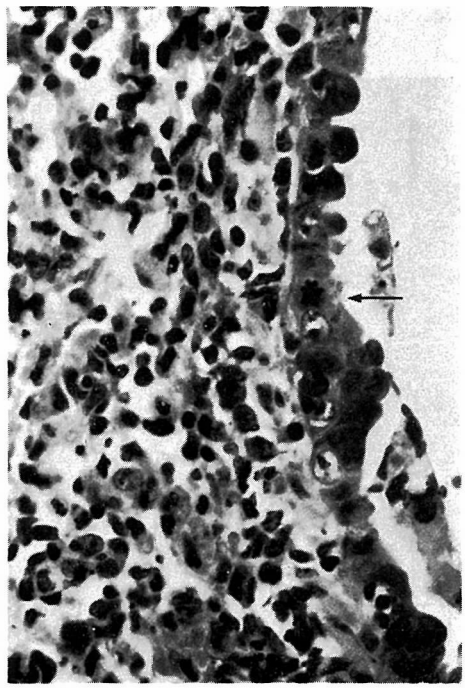


Fig. 14.

**Fig. 13.** Murine alveolar wall at 2 days after inoculation. Neutrophiles and mononuclear cells infiltrate to the alveolar wall and form focal interstitial pneumonia. Some exudate is recognized in the alveolar space (↗). Hematoxylin and eosin stain. ×100

**Fig. 14.** Murine alveolar wall and bronchiole at 5 days after inoculation. Bronchiolar epithelium is still remained but has atypism and mitotic figure (↗). Peribronchial atelectasis is detected. Hematoxylin and eosin stain. ×400

hyperchromatic nuclei with one to two clear nucleoli and some mitotic figures were also detected (Fig. 14). Stillmore, inflammatory cell infiltration to alveolar wall were observed with some degree of exudate in the alveolar space, and was detected to have a tendency to infiltrate along the pleura.

At 6 days after inoculation, the severity of inflammatory changes further progressed, and focal atelectasis were noticed in some mice. Atypism of bronchiolar epithelial cells became more distinctive.

At 10 days after inoculation, inflammatory changes began to disappear gradually, and granulation and fibrosis were recognized in some mice.

Significant changes were not observed in the other organs at any stage and the histological changes which indicated viremia could not be detected in this study.

In intracerebrally inoculated mice, inflammatory change could not be detected in the central nervous system. In addition, in intrapleurally

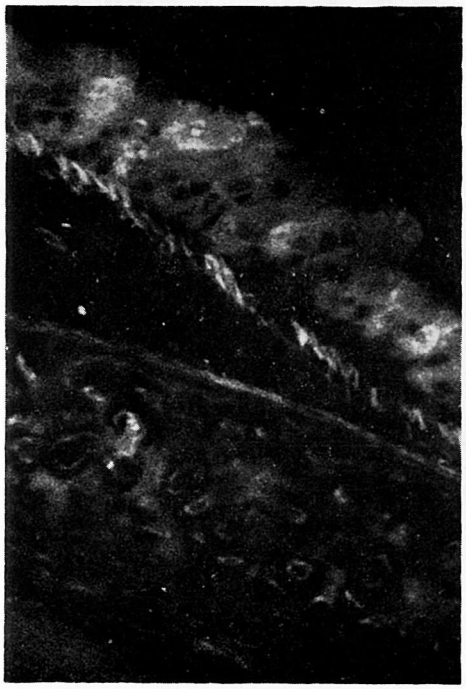


Fig. 15.

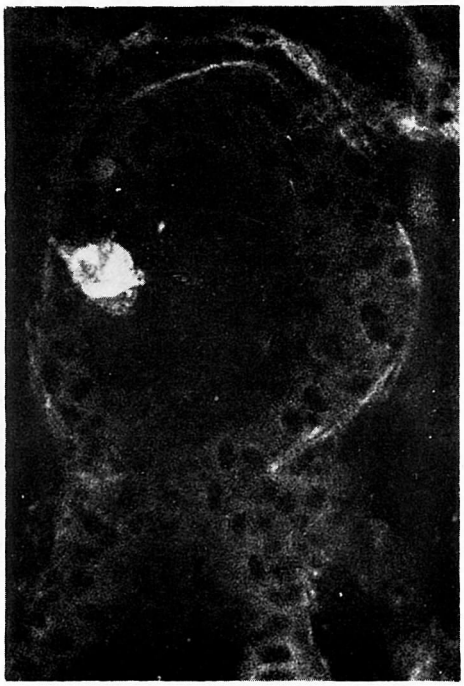


Fig. 16.

**Fig. 15.** Murine tracheal epithelium at 9 hours after inoculation. Granular IF is detected in the epithelial cells. Basement membrane shows nonspecific (blue) fluorescence intensely. Immunofluorescent stain. ×800

**Fig. 16.** Murine bronchiolar epithelium at 9 hours after inoculation. Only one group of cells are IF strongly positive. Immunofluorescent stain. ×400

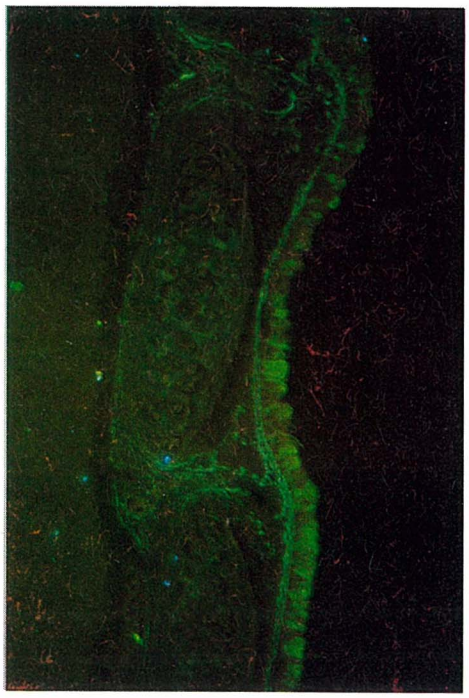


Fig. 17.

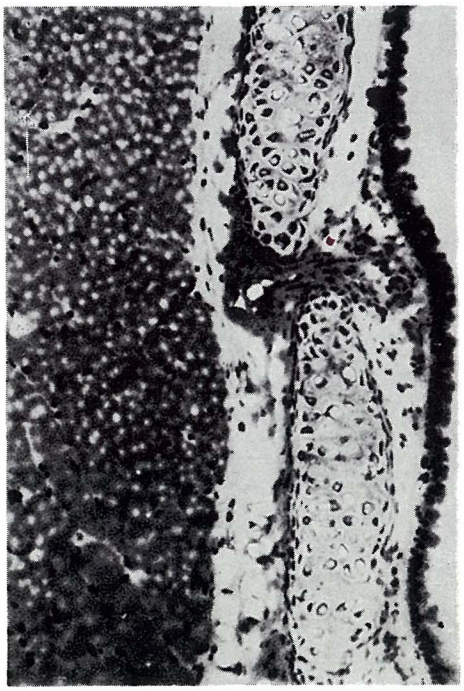


Fig. 18.

**Fig. 17.** Murine tracheal epithelium at 12 hours after inoculation. Discrete patches of cytoplasmic IF are recognized in the epithelium. Many blue fluorescences are detected in basement membrane. Immunofluorescent stain. ×100

**Fig. 18.** The same fields as in Fig. 17. in hematoxylin and eosin stain. ×100

inoculated mice, significant histological difference couldn ot be noticed between right and left visceral pleurae.

c) Fluorescent Microscopic Study: IF could not be detected in anywhere untill 9 hours after inoculation, when positive IF was recognized as discrete patches, a group of cells, in epithelium of trachea and bronchial tube. Many groups of lining cells were IF positive in the trachea (Fig. 15), while only one group of cells was IF positive in bronchiolar epithelium (Fig. 16). At this stage, IF was already intensely noticed in cytoplasm, and in some areas, IF was still granular. IF could not be noticed in alveolar wall.

At 12 hours after inoculation, intense IF was also noticed scatteringly in the epithelium of trachea and bronchial tube, and in some mice, IF developed extensively in almost all tracheal epithelial cells (Fig. 17, 18). The number of IF positive bronchioles increased and the cells desquamated into the lumen were also cytoplasmic positive. In some alveolar walls near the bronchiole, where IF was noticed as a cluster, IF was also observed

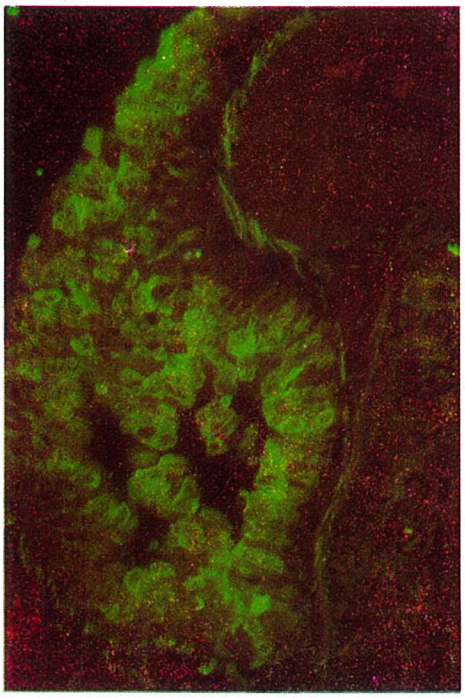


Fig. 19.

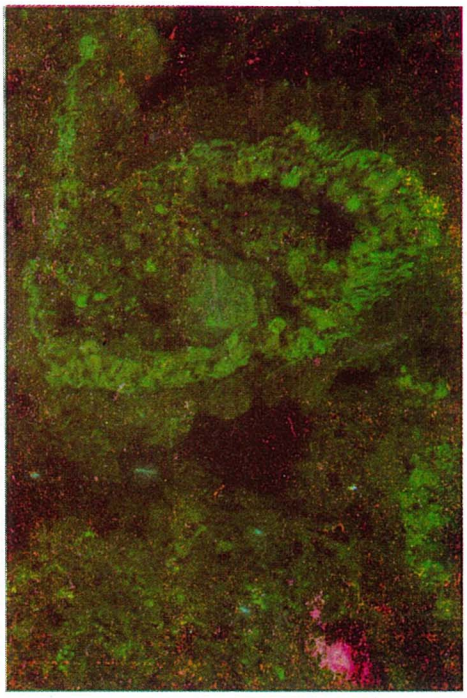


Fig. 20.

**Fig. 19.** Murine trachea at 1 day after inoculation. The number of IF positive cell increased extensively over the epithelium. Immunofluorescent stain. ×100

**Fig. 20.** Murine bronchiole at 2 days after inocuation. All epithelial cells are IF strongly positive and nuclear IF is observed in necrotic masses. Scattered IF is also recognized in alveolar wall (lower half). Immunofluorescent stain. ×200

scatteringly positive.

At 1 day after inoculation, IF was extensively positive in all over the tracheal epithelium and partially or entirely positive in the bronchial epithelium and necrotic masses in the lumen (Fig. 19). It was likely characteristic that the surface of the bronchial epithelium, probably cilia, was more strongly IF positive than cytoplasm. IF of nucleus was also positive in principally desquamated bronchial epithelium. IF positive parts of remained brochial epithelium were cilia, cytoplasm and nucleus in order to high intensity. Some of the alveolar composing cells were scatteringly IF cytoplasmic positive, but IF was not extensively observed in alveolar wall.

At 2 days after inoculation, all epithelial cells of trachea were IF strongly cytoplasmic positive and some of them desquamated into the lumen, so that necrotic mass was strongly positive in tracheal lumen. At this stage, in the desquamated cells, the IF in nucleus was stronger than

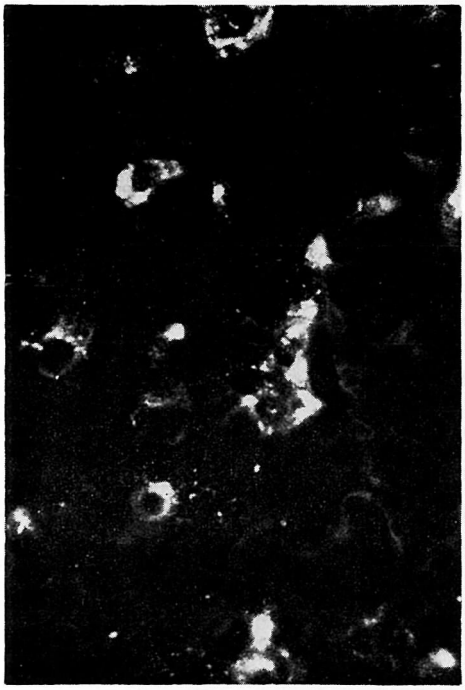


Fig. 21.

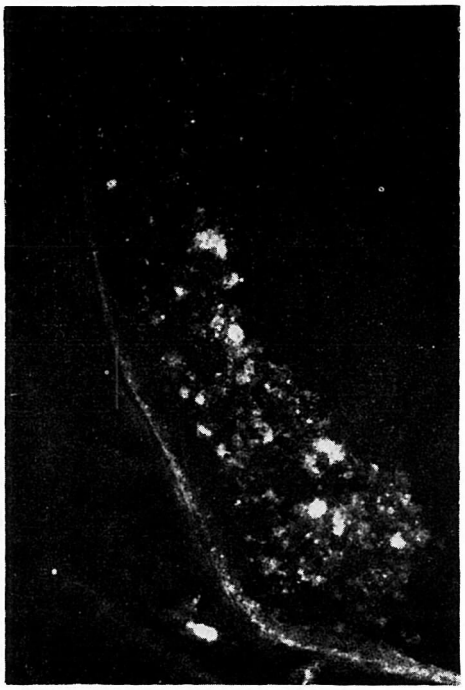


Fig. 22.

**Fig. 21.** Murine alveolar region at 2 days after inoculation. Scattering IF developed extensively. Many alveolar cells shows granular or diffuse IF in cytoplasm. Immunofluorescent stain. ×400

**Fig. 22.** Murine trachea at 4 days after inoculation. Relatively weak IF is observed in the necrotic mass. Immunofluorescent stain. ×200

that in cytoplasm (Fig. 20). But in remained bronchial epithelium, IF was still cytoplasmic. The distribution of scattering IF positive cells in alveolar wall developed extensively over the lung (Fig. 21). There were no IF positive cells in the alveolar space.

At 4 days after inoculation, almost all tracheal epithelium desquamated into the lumen and only nuclear fragmentations were IF positive (Fig. 22). The intensity of IF slightly decreased in all epithelium, and in alveolar wall, IF was faintly positive.

At 5 days after inoculation, IF was faintly positive even in bronchial epithelium,where IF was cytoplasmic and scattered.

At 7 days after inoculation, IF could not be detected in the lung tissue.

The time relationships between histological changes and IF intensity is summarized in Table A, Table B and Fig. B.

**Table A:** Histological Changes in Mouse Lungs

| Time after inoculation | 6H | 9H | 12H | 1D | 2D | 3D | 4D | 5D | 6D | 7D | 8D | 9D | 10D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trachea | +1 | +2 | +2 | +3 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +3 | +3 |
| Bronchial tube | +1 | +2 | +2 | +3 | +4 | +4 | +4 | +5 | +5 | +4 | +4 | +4 | +3 |
| Alveolar wall | − | − | +1 | +2 | +3 | +3 | +4 | +5 | +6 | +5 | +4 | +4 | +3 |

**Table B:** IF Intensity in Mouse Lungs

| Time after inoculation | 6H | 9H | 12H | 1D | 2D | 3D | 4D | 5D | 6D | 7D |
|---|---|---|---|---|---|---|---|---|---|---|
| Trachea | − | +2 | +3 | +4 | +4 | +3 | +2 | +2 | +1 | − |
| Bronchial tube | − | +1 | +3 | +3 | +4 | +4 | +3 | +2 | +1 | − |
| Alveolar wall | − | − | − | +1 | +2 | +2 | +1 | +1 | +1 | − |

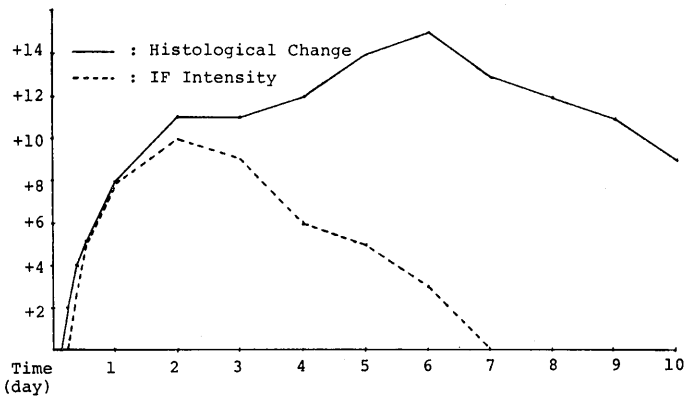



**Fig. B.** Time Relationship between Histological Change and IF Intensity

The histological changes exhibited severe feature at 2 to 10 days after inoculation, while IF intensity represented the highest level at 1 to 3 days after inoculation. Therefore, there is a difference in time between them.

In intracerebrally inoculated mice, brilliant IF was recognized in ependymal cells (Fig. 23), on the other hand, in intrapleurally inoculateed mice, IF could not be detected.

d) Electron Microscopic Study: At 1 day after inoculation, many virus particles were observed to attach the cilia. Some tracheal ciliated cells desquamated into the lumen and exposured basal replacement cells were detected. Virus particles were also noticed in the tracheal lumen being

Fig. 23.

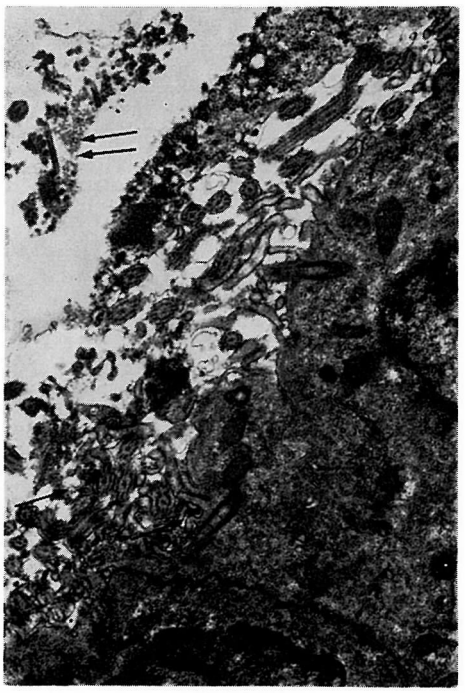


Fig. 24.

**Fig. 23.** Cross section of the mouse brain at 1 day after intracerebral inoculation. Specific IF is recognized in ependymal cells. Immunofluorescent stain. ×100

**Fig. 24.** Murine trachea at 1 day after inoculation. Pleomorphic virus particles (↗) are detected to attach the cilia. Virus particles are also recognized among the necrotic mass in the lumen (↗↗). Electron micrograph. ×4300

mingled with organellae of desquamated cells (Fig. 24, 25). The virus particle resembled to microvilli in prophile, but was smaller than microvilli and had characteristic structure with envelope. Remarkable changes were not detected in remained tracheal epithelium.

In the alveolar wall, numerous virus particles were principally noticed to attach the microvilli of infected type II pneumocyte, in which the number of osmiophilic inclusion increased and its density decreased (Fig. 26, 27). Furthermore, virus particles were occasionally observed to surround osmiophilic inclusion released from type II pneumocyte into alveolar space (Fig. 28).

Virus particles could not be detected in the other cells in alveolar wall in the present study.

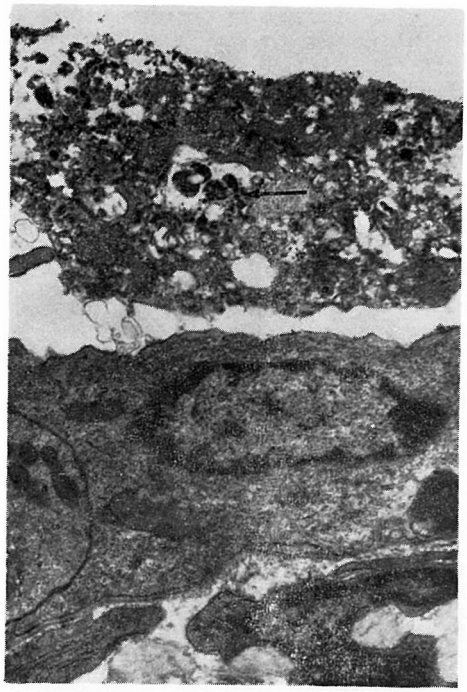


Fig. 25.

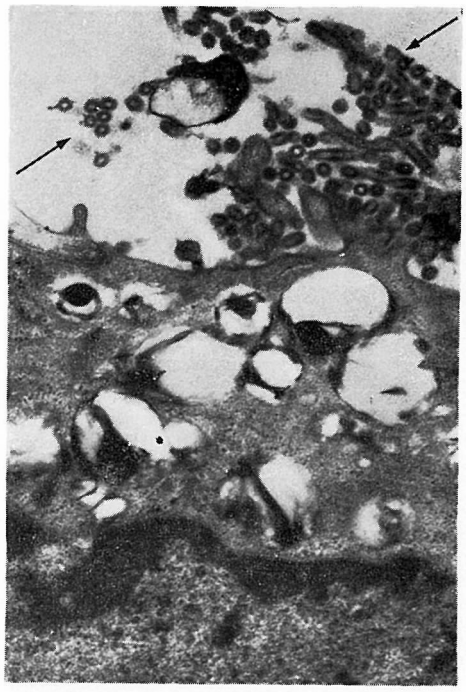


Fig .26.

**Fig. 25.** Another murine trachea at the same time as in Fig. 24. All of the tracheal epithelial cells are destructed and desquamated into the lumen, so that exposured basal replacement cells are detected. Virus particles (↗) are recognized among the organellae of destructed cells. Electron micrograph. ×4300

**Fig. 26.** Type II pneumocyte of mouse alveolar wall at 1 day after inoculation. Numerous virus particles (↗) are noticed to adhere the microvilli. Osmiophilic inclusions in the cytoplasm are characteristic. Electron micrograph. ×12000

## DISCUSSION

On immunofluorescent study, in the CAM inoculated with influenza virus adapted to mouse, virus distribution was detected in the layer of entoderm and in some of mesodermal cells beneath the entoderm at first, and later in desquamated cells from entoderm. This fact suggested that some of inoculated virus went through the mesoderm by cell to cell infecsion and reached to ectoderm, prompting extreme proliferation and cytonecrosis closely followed by desquamation of lining cells. Virtually, virus particles were detected on the surface of ectoderm by electron microscopic study. But virus multiplication was not detected in the ectoderm but in the

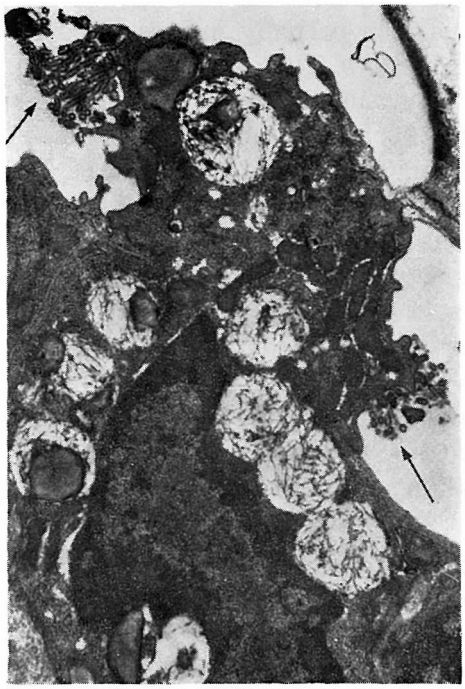


Fig. 27.

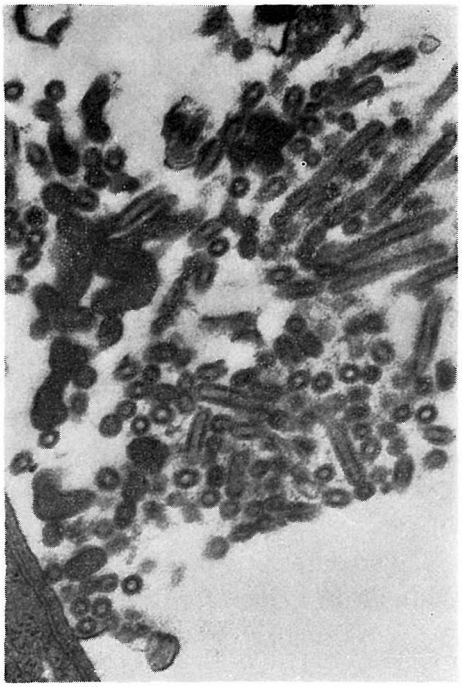


Fig. 28.

**Fig. 27.** Type II pneumocyte at the same time as in Fig. 26. The number of the osmiophilic inclusion is increased, but its density is decreased. Virus particles (↗) are detected to adhere the cell membrane. Electron micrograph. ×4300

**Fig. 28.** Murine alveolar space at the same time as in Fig. 26. Numerous pleomorphic virus particles are recognized. Electron micrograph. ×21000

entoderm and some of mesodermal cells on IF appearance. Nevertheless, the proliferation of entoderm induced by virus infection was so scanty that it could not compare with that of ectoderm. Woodruff and Goodpasture[8] held a debate on the susceptibility of CAM of the hatching egg to infection with fowl-pox virus by dropping the virus suspension on the injuried area of CAM, and arrived at a conclusion that ectoderm was more susceptible to fowl-pox virus than entoderm. Therefore, between their experiment and present study, there were differences of inoculating parts and kinds of virus, but in the respect of susceptibility to virus infection, the histological finding of the present study, though used influenza virus, correponded to their result. Namely, ectoderm might be so much susceptible to virus infection that it proliferated remarkably by only few virus.

Although, influenza virus was inoculated into CAC, IF study indicated

that it multiplied not only in entoderm but also partially in mesoderm of CAM, the other membranes and rarely in embryo. The reason why IF was detected in the embryo was that a part of inoculated virus might diffuse into amniotic sac and multiplied in it and accidentally swallowed by embryo. Because IF positive embryo was only one at 4 days after inoculation among all of the eggs. The reason why IF was recognized in the other membranes in almost all eggs came to a settlement when recalling the membrane system. As yolk sac and amniotic sac both hold in common mesodermal layer with CAM as shown in Fig. C, so if the virus was inoculated practically into CAC, it was considered that the virus could invade easily through the mesoderm into the other membranes and cavity. As above mentioned, influenza virus could be present in amniotic sac, it seemed certain that whether embryo would be IF positive or not depended upon the virus concentration and possibility of embryo to swallow the virus.

The intensity of IF in the egg experiment was stronger and more granular in cytoplasm than in nucleus except for yolk sac. This immunofl-

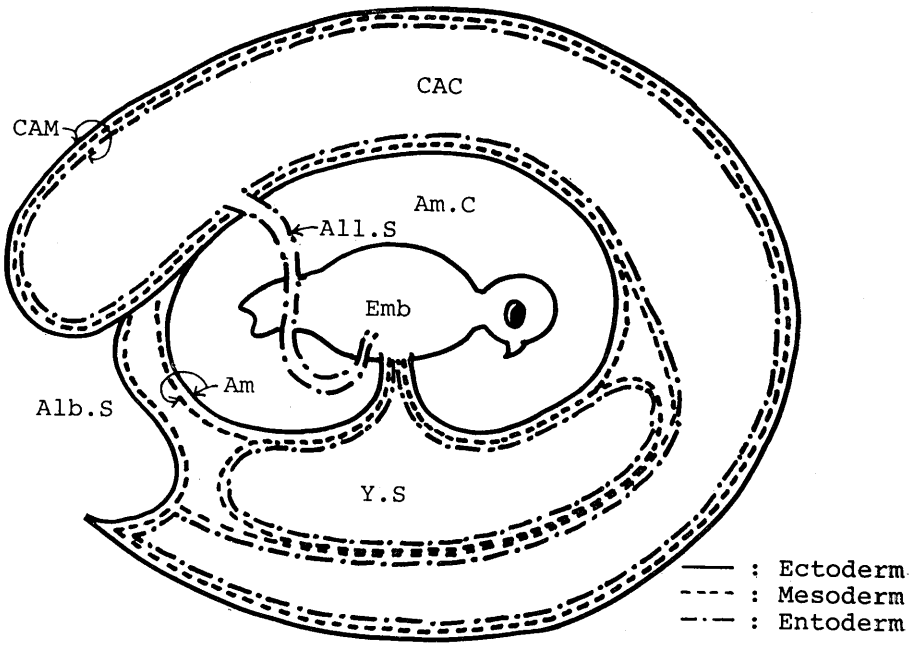



**Fig. C.** A diagram of the 11 days old hatching egg. Emb., Embryo. CAM., Chorioallantoic membrane. CAC., Chorioallantoic cavity. Ali. S., Allantoic stalk. Y. S., Yolk sac. Am., Amnion. Am. C., Amniotic cavity. Alb. S., Albumin sac.

uorescent feature of CAM coincided with that of amniotic sac in experiment by Watson and Coons[2]. They inoculated influenza virus into amniotic sac and detected with the virus antigen at first along the cell surface in contact with the CAF, later deeper in the nucleus. While in yolk sac, IF was observed in nucleus and in peripheral cytoplasm of entodermal lining cells since at 48 hours after inoculation. This fact suggested that, in yolk sac, the place of multiplication of virus in cytoplasm might be limited in periphery because of its a large quantity of lipid.

In the egg experiment, it was observed that the capillary net in CAM changed its distribution from surface to below of the ectoderm in 3 days after inoculation. With regards to pathogenesis of this change, a speculation of Woodruff and Goodpature[8] is quoted. They concluded that migration of capillaries rather than degeneration of epithelial cells had occured in the change of position of the respiratory net and that position of the capillary corroborated Danchakoff's theory[9] concerning the developement of the respiratory net of the allantois.

In mouse experiment, since at 1 day after inoculation, tracheal epithelial cells were detected to desquamate into the lumen and to leave some basal replacement cells on the tracheal wall. As some authors mentioned the changes of the ciliated epithelium into a stratified layer of cells in later stage[10-14], squamous metaplasia was observed at 10 days after inoculation in some mice in this study.

From the histological finding on the longitudinal section of bronchial tube at 2 days after inoculation, it was found that influenza virus invaded to pulmonary parenchym along the bronchial tube, and on immunofluorescent study, in spite of severe inflammatory lesion surrounding the bronchial tube, positive IF was recognized in only epithelium of bronchial tube. In that respect, Cesario et al[15] reported the susceptibility of chicken tracheal epithelium to virus infection. They described by using FAT that influenza virus could be found only in the epithelium. Westerberg et al[16] mentioned that the destructive effecs of the influenza virus was confined to the epithelial layer of the tracheal tissue. Furthermore, Finkelstein et al[17] described the tracheal resistence to Newcastle disease virus infection and reported amounts of detectable interferon.

At 1 day after intracerebral inoculation, IF was noticed in only ependymal cell without inflammatory changes around it. And at 4 days after intrapleural inoculation, IF was not noticed in the pleural mesothelium and significant difference was not detected between right and left pleural mesothelium. In addition, in egg experiment, the cytoplasmic IF was observed in the cells organizing arachnoidea and epithelium of trachea and

bronchial tube. These findings suggested that influenza virus might possess epitheliotropism in broad sense, especially respiratory epitheliotropism.

Throughout this experiment, no violent IF more than scattered was recognized in alveolar wall. Hers and Mulder[3,4] mentioned scattered IF in alveolar wall of human and murine influenzal pneumonia, and reported several fluorescent alveolar cells at 24 hours after inoculation and suggested that viral pneumonia was caused by viral attack on the alveolar cell lining. Nayak et al[5] emphasized that IF was more pronounced in the alveoli in specific area adjacent to fluorescent bronchioles at 4 days after inoculation, and that IF was limited to macrophage and epithelial cells of bronchi, alveoli and turbinates.

In alveolar wall, there are morphologically six kinds of cells[18] (Fig. D), they are type I pneumocyte, type II pneumocyte, two kinds of alveolar septal cells, endothelium and alveolar macrophage. Type I pneumocyte resembles to endothelium and type II pneumocute has characteristic osmiophilic inclusions in cytoplasm. But electron microscopic references concerning the cell types of IF positive alveolar lining cells were not made. Electron microscopy in this paper showed that, although they were both

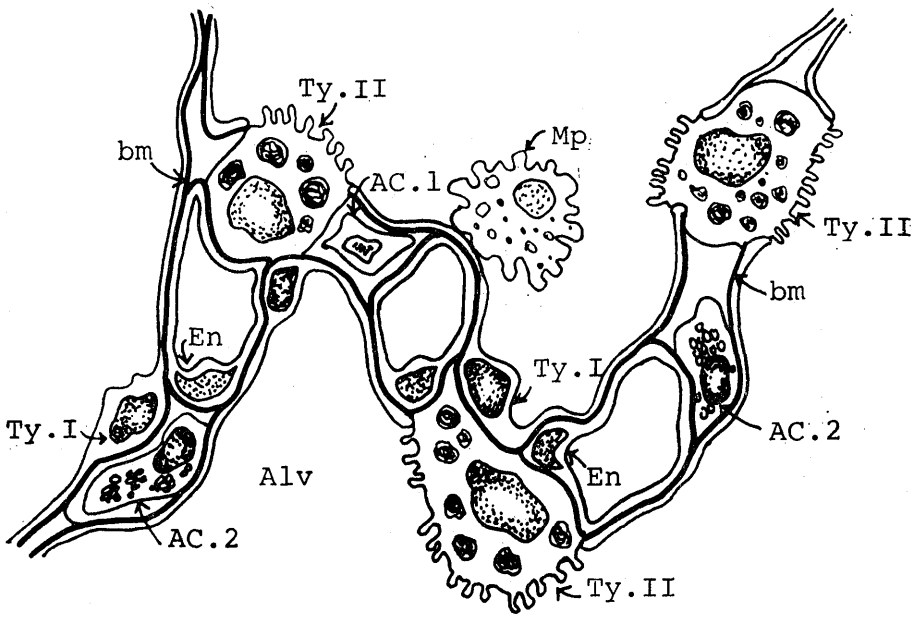



**Fig. D.** Normal alveolar wall. Ty. I., Type I pneumocyte. Ty. II., Type II pneumocyte. AC. 1., Alveolar septal cell. AC. 2., Vacuolated alveolar septal cell. En., Endothelial cell. bm., Basement membrane. Mp., Alveolar macrophage. Alv., Alveolar space.

entodermal origin[19-20], on the alveolar wall, virus particles were detected to adhere to the microvilli of type II pneumocyte and the virus particles were not observed in type I pneumocyte and in the other alveolar cells. This fact suggested that in alveolar wall, influenza virus might multiply in only type II pneumocyte. Virtually, the distribution of type II pneumocyte in alveolar wall was compatible with scattered IF. Moreover, this fact might support the theory that type II pneumocyte and epithelium of bronchial tube were embryologically identical origin.

However, above conception is exceedingly attractive, sufficient electron microscopic study must be renewed.

About virus detection after infection, since 3 hours after inoculation, as the first response to influenza virus infection, heavy secretion from stratified columner epithelium was raised for removaling them, but at this stage, IF could not be detected, because inoculated virus antigen dispersed extensively and which number per cell was too scanty to be fluorescent. Consequently, the virus multiplication could not be detected yet. It seemed that probably this was thie difficulty of IF sensitivity. Many studies were reported on human and murine influenza virus infection and on IF findings at various stage. Hers and Mulder[3,4] reported bright fluorescence in the nucleus of some bronchial epithelium and alveolar cells, using anti-S serum at 4 hours after inoculation, and in the peripheral cytoplasm using anti-V serum at 6 hours after inoculation. Nayak et al[5] described that IF was found in the bronchial epithelium at 8 hours after inoculation using antiserum of the S-15 influenza virus. The difference in time among those experiments seemed to be derived from differences of immune level, types of virus and antiserum and virus quantities. In the present study, anti-S serun and anti-V serum were not used separately but used together as anti-influenza virus A/Tokyo/1/72. In murine lung, brilliant IF was observed in cilia at first, later in cytoplasm of columner stratified cells and finally in the nuclei of desquamated cells derived from them. It seemed that in desquamated cells, the cytoplasm was degenerated and necrotic enough for virus to permeate, so that brilliant IF was detected more strongly in nucleus than in cytoplasm.

Histologically, neutrophile infiltration in tracheal lumen and bronchial tube was recognized since 9 hours after inoculation when positive IF was noticed in epitheium of trachea and bronchus. The Intensity of IF was stronger during 1 to 3 days after inoculation, this fact was nearly consistent with growth curve of influenza virus in CAC (Fig. A). The virus multiplication, HA level and CCA level began to increase at 8 hours after inoculation and reached the highest level and showed plateau during 1 to 2

days after inoculation at the latest. And yet, in fluorecent staining, IF was recognized during 9 hours to 6 days after inoculation, and was most violent at 1 to 3 days. Those findings nearly correponded to growth curve of influenza virus in CAC. However, histological changes were most violent at 2 and 10 days after inoculation. This fact suggested that there was a difference in time between IF intensity and histological changes. Although, the inoculated site was different, Fraser et al[22] also mentioned the difference in time by intracerebral inoculation. He described that in early infection IF was obtained without inflammation and in late infection there were areas of inflammation with no IF. Indeed, in early stage of intracerebral infection in this study, IF was noticed in only ependymal cell without inflammatory change around it.

About the pathogenesis of interstitial pneumonia, Westerberg et al[16] indicated using mouse tracheal organ culture technic that in addition to direct pathologic effect which was confined to the tracheal epithelium, influenza virus led to a reduction of ciliary activity by day 3 and ciliostasis by day 5 and also led to increased morbidity and mortality from secondary bacterial infection, such as staphylococcal pneumonia. Brentjens et al[23] reported experimental interstitial pneumonitis in mouse using multiple injections of anti-bovine albumin rabbit serum and regarded it to be presumably produced by an inflammatory process in immune complexes. Spencer[24] mentioned that proliferation of interstitial cells, interstitial edema, accumulation of polymorphonuclear leukocytes and mononuclear cells, its progressive increase of reticulin fibrils and of interstitial fibrosis were considered the basic element of this disease.

In this study, as response to influenza virus, bronchitis, peribronchitis and perivascular cell infiltration were recognized in almost whole lung. And in severe lesion, alveolar wall was thickened by many inflammatory cell infiltration and formed interstitial pneumonia. And it was speculated that as influenza virus was detected in epithelium of bronchial tube and type II pneumocyte, those inflammatory cells might accumulate around them owing to some toxic substance produced by influenza virus in infected cells. Although, the toxic substance was not examined in this study, some investigators inoculated large dose of unadapted influenza virus intranasally into mice and produced severe lung lesions by toxic effect of the influenza virus[25,26] and Barker and Hoyle[27] described that toxic effect depended on the penetration of virus particles into the lung cells and on the release of the virus nucleic acid, because the virus nucleic acid might itself be toxic but more probably the toxic effects were due to synthesis of some protein under the control of the virus RNA. After all, interstitial pneumonia in the present experiment might be speculated to be secondary

reaction due to toxic substance produced by influenza virus in infected cells. Furthermore, in obtained results, atypism was detected in bronchiolar epithelium, which seemed to be dysplasia due to influenza virus.

## SUMMARY

Influenza virus A/Tokyo/1/72 ($10^{7.5}$ $EID_{50}$) was inoculated into CAC of the hatching egg for examinating of the virus multiplication, and intranasally into murine lung for study of pathogenesis of influenzal pneumonia. These studies were investigated by immunofluorescent, histological and electron microscopical appearances.

In the hatching egg, difference of susceptibility to influenza virus was observed between entoderm and ectoderm. On immunofluorescent study, IF was detected not only in CAM but also in the other membranes and rarely in embryo, especially in respiratory epithelium.

In the murine lung, influenza virus invaded to tracheal and bronchial epithelium and finally to type II pneumocyte of alveolar lining cells. These facts suggested that influenza virus had intense respiratory epitheliotrop ism. In the murine lung, cytoplasmic IF was observed at 9 hours after inoculation in epithelium of trachea and bronchiole at first, and at 12 hours after inoculation, scattered IF in some alveolar wall, and at 1 day after inoculation in nuclei of desquamated epithelial cells in the lumen of the trachea and bronchial tube. IF was most brilliant between 1 to 3 days after inoculation. But in the light microscopic study, most violent histological changes were detected at 2 and 10 days after inoculation. Therefore, there was a difference in time between IF intensity and histolgical changes.

About the pathogenesis of viral pneumonia in this study, many inflammatory cells infiltrated around the trachea, bronchial tube and to alveolar wall, however IF could not be noticed among them. Consequently, it was speculated that interstitial pneumonia might be a reaction caused by some toxic substance resulted from influenza virus in infected cells. So, influenzal pneumonia was seemed to be secondary reaction due to influenza virus infection.

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