STUDIES ON PRIMARY ATYPICAL PNEUMONIA*, ‡

I. Localization, Isolation, and Cultivation of a Virus in Chick Embryos

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Plate 38

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INTRODUCTION

Primary atypical pneumonia (PAP) is a disease entity believed to be of viral origin (3–8). However, there is still no general agreement on its etiology although many workers have reported the isolation of agents from cases of PAP (9–13). Current criteria for the diagnosis of this illness rely largely on clinical and roentgenological findings and to a certain degree on the development of cold agglutinins or streptoccus MG agglutinins in the convalescent sera of patients (14, 15); no specific diagnostic test is yet available.

In 1944, Eaton, Meiklejohn, and van Herick (12) isolated an agent cultivable in chick embryos from the filtered sputum or lung suspensions of PAP patients. Suspensions of lung, trachea, and amniotic membrane from infected chick embryos induced pneumonia after intranasal inoculation into cotton rats and hamsters. This virus-induced pneumonia could be prevented by convalescent sera from patients recovered from PAP (12).

Recently, by the use of fluorescein-labelled antibody, it has been possible in our laboratory to detect the PAP virus in infected chick embryos. This technic provides a new procedure for the isolation and identification of such agents from patients during the acute stage of infection and also a specific serological diagnostic test for this illness is now available. This paper reports observations on the biological and immunological behavior of this agent; these data lead us to believe that this agent is causative of that form of PAP associated with the development of cold agglutinins. PAP unassociated with the development of cold agglutinins may be caused by other agents, including adenovirus (15, 16).

* A portion of this work has been reported to the Society of American Bacteriologists, May 19, 1955 (1) and to the New York Academy of Medicine, November 16, 1955 (2).

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Materials and Methods

Virus Strains.—The Mac strain of PAP virus was isolated from human lung tissue in 1944 in California (17). It has been propagated in chick embryos by amniotic inoculation for the last 10 years and the 55th to 60th passages of pooled tissue suspension of infected lung, trachea, and amniotic membrane were first used in these experiments. Subsequently, eight new strains of PAP virus which were isolated from patients in New England between 1954 and 1956 were included in this study.

Collection of Sputums, Nasal and Throat Washings for Virus Isolation.—These specimens were collected from patients with clinical and roentgenological findings consistent with the diagnosis of primary atypical pneumonia. Most of them also showed a rise of cold and streptococcus MG agglutinins during convalescence. During the acute stage of their illness, the patients were urged to cough. If no sputum could be produced, nasal and throat washings were carried out with 0.85 per cent saline. The materials collected were usually inoculated into chick embryos immediately after their arrival at the laboratory. If inoculations could not be carried out promptly, the specimens were quickly frozen and kept at −20°C. or sealed and stored in a dry ice chest.

The lung specimen was obtained from a patient who died at the Boston City Hospital in 1943. It had been stored in a solid CO_2 cabinet at Dr. Maxwell Finland's laboratory and was kindly made available to us 12 years after the patient's death.

The sputums and the lung were ground in nutrient broth in a glass tissue grinder before being used for inoculation. These specimens as well as the nasal or throat washings were inoculated without filtration. 2,000 units of penicillin were added to each milliliter before inoculation but no streptomycin was added.

Inoculation and Harvest of Chick Embryos.—The Mac strain of PAP virus has always been cultivated in 10-day-old embryos and its activity detected by intranasal inoculation into cotton rats and hamsters (12).

In the present study, cultivation and isolation of PAP virus were carried out in 13-day-old chick embryos by the method of amniotic inoculation previously described (17). Each egg received 0.2 ml. of viral suspension and was incubated at 35°C. for 5 to 6 days. Eggs were harvested without previous chilling. Only the lungs and the lower portion of the trachea were collected from each embryo and ground in 2 ml. of nutrient broth containing 1 per cent of bovine albumin. The pooled ground tissues were estimated to be a 10 per cent suspension; they were divided into 1 to 2 ml. aliquots in sealed glass tubes and were stored in a dry ice cabinet as stock virus. Each passage was tested for bacterial sterility by plating out the suspension on a blood agar plate. Sterile stock suspensions were further passed serially in chick embryos without the addition of antibiotics.

Test for Viral Activity and Titration of Virus.—Although the newly isolated strains of PAP virus also induced pulmonary consolidation by intranasal instillation into cotton rats, the tests for viral activity were carried out on frozen sections of inoculated chick embryo lungs by fluorescent staining with human PAP convalescent sera. Usually 2 to 3 embryos from each passage of virus were checked for the presence of viral antigens and the rest of the eggs were ground up for stock virus. (If the frozen sections were positive, the virus passage was considered to have been successful.)

In the titration of virus, 0.2 ml. of 10-fold serial dilutions of the viral suspensions in broth were inoculated into the amniotic sac of 13-day-old embryos. Five or six eggs were used for each dilution. After incubation, the lungs were harvested and frozen sections were cut. The presence of virus was detected in the bronchial epithelium by fluorescent staining as described below. The EID_{50} was calculated according to the method of Reed and Muench (18).

Preparation of Hyperimmune PAP Rabbit Serum.—Healthy rabbits weighing about 5 pounds each were used for immunization. Each rabbit was inoculated twice weekly with 4

ml. of 10 per cent PAP infected chick embryo lung suspension divided equally between intraperitoneal and intramuscular sites. At intervals, the rabbits were bled from the heart 1 week after the last injection. The antibody titer in the serum was tested by the indirect fluorescent staining method. Some rabbits produced an antibody titer of 1/320 or higher after 6 to 7 injections but some required 10 or more injections to reach this level. The immunized rabbits also developed antibodies against the chick embryo lung tissues which interfered with the interpretation of the specific fluorescent staining of the PAP virus. This difficulty was overcome by absorbing the serum with chicken lung or chick embryo tissue powder.

Preparation of Fluorescein-Labelled Anti-Human or Anti-Rabbit Globulin.—Anti-human globulin sera were prepared by immunizing rabbits with human plasma fraction II obtained from the University Laboratory of Physical Chemistry of Harvard University. The methods of immunization described by Proom (19), using alum-precipitated antigen, or by Gitlin (20), using aluminum hydroxide adsorbed antigen, were used. Sera produced by either method were equally satisfactory. The anti-rabbit glubulin serum[1] was prepared by immunizing a goat with alum-precipitated antigen as described by Proom (19). Both types of sera with a precipitin titer of 1/500 or higher were precipitated by saturated ammonium sulfate solution and labelled with fluorescein isocyanate according to the method of Coons and Kaplan (21). Before use for fluorescein staining, the labelled antibody solutions were absorbed once or twice with mouse liver powder.

Indirect Method of Fluorescent Staining.—The indirect method of fluorescent staining was developed by Watson (22) and has been used successfully in tracing specific antibody of human, rabbit, or chicken origin. Its practical application has been reported in the studies of varicella (23) and poliomyelitis in tissue cultures (24).

In the present study, frozen sections (4 μ) of chick embryo lungs were cut in a cryostat according to the method of Linderstrøm-Lang and Mogensen (25, 26). These sections were first fixed in acetone for 10 minutes and then dried in a 37° oven for 20 minutes. A drop of either human PAP convalescent serum or hyperimmune rabbit serum diluted $\frac{1}{10}$ was put on the section and allowed to react at room temperature for 30 minutes. The excess antibody solution was rinsed off and the slide was dipped into buffered saline (pH=7.0) for 10 minutes. Thereafter, the slide, except the section, was wiped dry and a drop of either fluorescent anti-human globulin rabbit serum or anti-rabbit globulin goat serum was put on the section for 30 minutes. Finally, the slide was washed in buffered saline for 10 minutes and then mounted under a coverslip with buffered glycerol of pH 7.0.

This indirect method is much more sensitive than the direct method of fluorescent staining employing fluorescein-labelled PAP human convalescent serum or hyperimmune rabbit serum. As a matter of fact, the direct staining in this system has not been satisfactory due to the low intensity of specific fluorescence. Furthermore, by means of the indirect method of fluorescent staining, antibody titrations can be carried out. The endpoint of antibody titration by 2-fold dilutions was taken as the highest dilution of serum which succeeds in giving definite specific fluorescence to the infected chick embryo bronchial epithelium.

Virus neutralization test.—The procedure for the neutralization test in cotton rats has been described (27).

EXPERIMENTAL

Localization of PAP Virus in Chick Embryos.—In infected embryos, viral antigens were consistently found in the cytoplasm of the epithelial cells lining the lower trachea, the bifurcation of the bronchi and their larger branches, and the air sacs. No viral antigens were seen in the upper part of the trachea,

[1] Courtesy of Dr. B. K. Watson.

the great vessels, the heart, the esophagus, the parabronchi, the liver, the intestines, or the amniotic membrane. The antigens appeared as homogeneous fluorescence located exclusively in the cytoplasm with its brightest fluorescence at the surface of the epithelium (Figs. 1 and 2). Desquamation of bronchial epithelium was not apparent and pneumonic consolidation was absent. When identical or adjacent lung sections from infected embryos were stained with hematoxylin and eosin, no appreciable histopathological changes were visible. The bronchial epithelium containing viral antigens appeared normal and the cilia were still outlined well (Figs. 3 to 5). The infected embryos did not seem to differ from the uninoculated ones of the same age, though possibly some of the inoculated embryos appeared underdeveloped. Many inoculated embryos survived until the time of hatching.

Several attempts have been made to demonstrate PAP virus by fluorescent staining in cotton rat lungs showing pneumonia. However, although a few

TABLE I

Effect of Age of Chick Embryos and Time of Incubation on the Cultivation of Mac Strain of PAP Virus

Age at inoculation	Time of incubation	Result	Per cent positive
days	*days*		
10	7	4/12*	33
13	5	12/13	92
15	3	1/4	25

* Numerator = number of embryos with viral growth.
Denominator = number inoculated.

scattered macrophages containing the viral antigen were seen, no viral antigen was demonstrable in the bronchial epithelium.

Effect of the Age of Embryos and the Period of Incubation on the Cultivation of Virus.—It has been customary to cultivate the Mac strain of PAP virus by inoculation into the amniotic sac of 10-day-old embryos and to incubate them at 35°C. for 7 days. However, since the lungs of 15 to 16-day old embryos support better growth of the Newcastle Disease virus (28) and also 12-day-old embryos inoculated with influenza virus show more extensive involvement of the cells lining the respiratory tract (29), it was decided to investigate the growth of PAP virus in older embryos.

Embryos of various ages were inoculated with the Mac strain virus and incubated at 35°C. for various periods of time (Table I). As seen from the data, only 4 of 12 embryos 10 days of age showed viral growth in the bronchial epithelium when incubated at 35°C. for 7 days. In contrast, 12 of 13 embryos 13 days of age inoculated in the same manner and incubated for 5 days were infected. When 15-day-old embryos with incubation for 3 days were used, only

1 of 4 became positive. The lung suspensions from the infected 13-day-old embryos inoculated intranasally into cotton rats produced definite and rather extensive pneumonic consolidations.

TABLE II

Relationship of Time of Incubation to Presence of PAP Virus in Inoculated Chick Embryo Lungs

Length of time after inoculation	Virus in lungs	
	Experiment 1	Experiment 2
days		
3	0/3*	0/3
4	2/3	2/3
5	2/3	2/3
6	2/3	nd‡
7	3/4	2/3

* Numerator = number of embryos with viral growth.
Denominator = Number inoculated.
‡ nd = no data.

TABLE III

Isolation of PAP Virus from Human Patients

Strain	Source	Date of isolation	Area	Detected at passage No.	Patients' serum PAP antibody	
					Acute	Convalescent
A.S.	Sputum	Dec., 1954	Boston	1st	<10	320
F.H.	"	" "	"	3rd	<10	160
T.H.	"	Jan., 1955	"	3rd	40	160
K.G.	"	Dec., 1955	"	4th	nd*	
H.B.	"	Aug., 1956	"	3rd	nd	320
D.B.	"	Jan., 1955	Exeter, N. H.	2nd	<10	80
W.S.	Nasal washing	Feb., 1956	" "	2nd	160	160
Sil	Lung‡	June, 1955	Boston	2nd	nd	

* nd = no data.
‡ Patient died at the Boston City Hospital in January, 1943.

Development of Viral Antigen in Inoculated Chick Embryos.—In order to determine the optimum incubation period required for viral growth, a group of 13-day-old embryos were inoculated with the F.H. strain of PAP virus (Table III). Three to 4 eggs were harvested daily starting on the 3rd day of incubation and the lungs were sectioned and examined. Two separate experiments were performed and the results are summarized in Table II.

The data from the two experiments are almost identical. No embryos showed

the presence of PAP virus on the 3rd day after inoculation. Viral antigens appeared in the bronchial epithelium on the 4th day and persisted to the 7th day after inoculation. However, the area of specific fluorescence in the bronchial epithelium incubated for 4 days after inoculation was narrower, less bright, and fewer bronchi were involved. This suggested that less virus was present in the embryos incubated for 4 days when compared to those incubated for 5 to 7 days. Therefore, embryos were incubated for 5 to 6 days for routine passage or isolation of virus.

Isolation of PAP virus.—A total of eight strains of PAP virus were isolated in this laboratory between 1954 and 1956 (Table III). Six strains were isolated from sputums, one from nasal washing, and one from the lung of a patient (Sil.) who had died at the Boston City Hospital in 1943. This case has been reported by Parker *et al.* (30) but no virus was isolated at that time. The lung had been thawed and refrozen several times during the last 12 years in storage at Dr. Maxwell Finland's laboratory. To insure that the isolation of the Sil strain was not a laboratory contamination, two separate attempts 5 weeks apart with precautions to avoid any possible contamination were carried out and in both instances, isolation was successful.

All eight strains of virus were successfully detected by fluorescent antibody staining between the first and the fourth serial passage in embryos. All these specimens except the lung were either inoculated into eggs immediately after receipt or kept frozen in the CO_2 box for less than 2 weeks. From eight additional sputum specimens and three nasal washings, no PAP virus could be isolated. Three of these sputums had been stored for more than 2 years in the CO_2 box and the other five specimens for more than a month at $-20°C$.

When the isolated PAP virus was tested by fluorescent antibody titration against homologous sera, with one exception, there was a definite rise of titer in the convalescent sera. Patient W.S. did not show any rise of antibody titer; however, his acute serum was collected on the 8th day of his illness when he had already developed an antibody titer of 1/160.

Three strains of newly isolated virus, A.S., F.H., and Sil, were inoculated into cotton rats intranasally; all produced definite pneumonic lesions and all were neutralized by convalescent human PAP sera or hyperimmune rabbit PAP sera.

Antigenic Relations between Various Strains of PAP Virus.—By using the fluorescent staining method to test the cross-reactions between five strains of PAP virus (Mac, A.S., T.H., F.H., and Sil) and eight sera (five human sera, A.S., T.H., F.H., W.E., and H.R.; three rabbit sera against A.S., F.H., and Mac), it was found that all the sera at 1/10 dilution cross-reacted to the same degree with different strains of virus. The localization of the virus and the staining character of the bronchial epithelium in infected lungs were so similar in appearance among all viral strains and convalescent sera that it was impos-

sible to distinguish them one from another. The Mac strain isolated in California in 1944 and the Sil strain from a patient who died in Boston in 1943 reacted with sera from recent cases of PAP to the same degree as the recently isolated strains. This suggests that all strains of PAP virus studied were antigenically closely related if not identical.

To elucidate more fully the antigenic relationships among these PAP virus strains, cross-neutralization tests in cotton rats and cross-fluorescent staining of infected chick embryos were carried out. Hyperimmune cotton rat and rabbit sera were prepared against the Mac strain and the F.H. strains of virus. Antibody titrations were carried out on these sera against homologous and heterologous viruses (Table IV). The data show no appreciable differences among

TABLE IV

Antigenic Relationships between the PAP Viruses Isolated in 1955 and 1944

Serum	Virus strains			
	F.H.		Mac	
	C.R.* neut.	F.A.‡ staining	C.R. neut.	F.A. staining
Normal rabbit.....	<8§	<10	nd	<10
Anti-Mac cotton rat....	32	nd‖	32	nd
Anti-Mac rabbit.....	16	40	32	20
Anti-F.H. rabbit.....	>256	160	>256	80

* Neutralization test in cotton rats.

‡ Fluorescent antibody staining on infected chick embryo lungs.

§ Reciprocal of serum dilution.

‖ nd = no data.

them which indicates further the intimate antigenic relationship of these two strains of virus.

Since there is such a close antigenic relationship between all strains of the PAP virus so far studied, it should be possible to use lung sections infected with any of them for serological diagnostic tests. When this was tried, the serum antibody titers were found not to vary appreciably when different strains of virus were used as antigens. Detailed results of the serological diagnosis of human PAP infections will be published in a separate communication (31).

Titration of Virus.—Taking the presence of viral antigen in the bronchial epithelium as the indication for viral multiplication, the results of the titration of several strains of PAP virus in chick embryos are shown in Table V. The EID_{50} of three strains, Sil-7, Mac-62, and Hall-7 were almost equal at about $10^{4.8}$ per 0.2 ml. of inoculum. The F.H. strain in titrations of several passages seemed to have a consistently higher titer than the other strains. Centrifuga-

tion at 1,000 R.P.M. for 10 minutes at 4°C. to settle the coarse particles of the embryo lung tissues did not affect the titer.

Relationship of PAP Virus to Other Agents.—As shown in Table VI, antisera from several viral and rickettsial agents commonly causing pulmonary

TABLE V

Titration of PAP Virus in Chick Embryos

Strain	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	EID_{50} log
F.H.-8*	3/4‡	3/3	2/2	4/5	nd	>5.0
F.H.-8 (centrifuged)	nd	4/4	2/5	2/5	1/4	5.4
F.H.-15	nd	4/4	5/5	3/3	2/4	6.0
Sil-7	nd	6/6	3/6	4/6	0/6	4.8
Mac-62	nd	nd	3/4	2/4	0/4	4.8
T.H.-7	nd	3/4	3/5	2/4	1/4	4.7

* Numerals indicate serial passage numbers.

‡ Numerator = number of embryos with viral growth.
Denominator = number inoculated.

TABLE VI

List of Various Antisera Tested Which Did Not Show Fluorescent Staining against PAP Virus

Sera	No. tested
Human psittacosis* ‡	3
Rabbit psittacosis immune serum‡	1
Human Q fever‡	1
Rabbit Q fever immune serum‡	1
Human APC (Type I to VI pooled)‡	1
Human influenza A′	5
Human influenza B	5
Rabbit PVM immune serum§	1

* Courtesy of Mrs. Joan B. Daniels, Virus Section, State Diagnostic Laboratories, Massachusetts.

‡ Courtesy of Dr. Robert S. M. Chang, School of Public Health, Harvard University.

§ Courtesy of Dr. Frank Horsfall, Jr., The Rockefeller Institute for Medical Research, New York.

infections did not show any cross-reactions with the PAP virus, as tested by the fluorescent antibody staining technic. In addition, two pairs of our human sera with high fluorescent staining and neutralization antibody against PAP virus were sent to Dr. M. R. Hilleman at the Walter Reed Army Medical Center, Washington, D. C. He reported that no complement fixation was demonstrable in these sera when tested against the adenovirus isolated from his PAP patients.

DISCUSSION

The failure of other investigators to confirm the isolation of PAP virus by Eaton *et al.* was probably largely due to the low pathogenicity of the virus. It requires quite a large inoculum to produce pneumonia in cotton rats, and both cotton rats and hamsters vary in their susceptibility to the infection (32). The PAP virus is quite labile (17) and is rapidly inactivated at room temperature. Although the fluorescent antibody-staining technic has shown that the virus multiplies in the cytoplasm of the embryonic bronchial epithelial cells, no injurious effect was detectable by hemotoxylin and eosin staining of such cells. In human autopsied material, Parker *et al.* (30) found that although the bronchioles not infrequently contained polymorphonuclear leukocytes and occasionally some bacteria, the epithelium was intact.

The failure to detect viral antigen in the bronchial epithelium of cotton rats suggests that the virus does not multiply extensively in the cotton rat lungs and this was also borne out by earlier evidence of progressive disappearance of pulmonary lesions during continuous serial passages of lung materials in cotton rats (12). The requirement of a large inoculum for the production of pneumonia in cotton rats might be due to a pneumotropic toxic effect of the PAP virus such as that seen in the intranasal inoculation of Newcastle virus and unadapted influenza virus in mice (33, 34).

The influence of the age of the embryos on the multiplication of PAP virus is interesting. Similarly, it has been shown that Hemophilus pertussis (35), Newcastle disease virus (28), and influenza virus (29) multiply better in more mature lung tissues. In addition, the perforation of the "tracheal plug" in chick embryos, which occurs on the 14th day of the development of chick embryo,[2] apparently allows the virus to gain access to the bronchioles. The

[2] Four sets of serial sections of chick embryos of various ages were examined to determine when the trachea became patent and its lumen continuous with the pharynx. These embryos are part of the Minot Embryological Collection of the Department of Anatomy, Harvard Medical School, and are identified in the table below.

In both the 11- and 13-day-old embryos the larynx was closed by a plug of epithelial cells, the so called "tracheal plug." In both of the 14-day-old embryos, the larynx was open and continuous with pharynx.

The author is grateful to Professor Edward A. Boyden, University of Washington, Seattle, for his expert guidance through the serial sections which he had himself made as a student in 1915. His fortuitous presence on the day the Collection was consulted greatly facilitated the search, and of course substantiated the observations as well.

Embryo No.	Age	Length
1967	11-day old	31 mm. Transverse sections (Set AD)
509	13-day old	43 mm. Sagittal sections (Set BD)
1968	14-day old	55 mm. Transverse sections (Set EX)
515	14-day old	54 mm. Sagittal sections (Set AO)

change from 10-day-old embryos to 13-day-old embryos for the cultivation of the PAP virus, which increased the number of infected embryos to more than 90 per cent, not only gives more consistent results but also provides a more reliable means for the isolation of virus from patients.

The close antigenic relationship of various strains of PAP virus isolated in two different locations, California and Massachusetts, and 12 years apart, is striking. It suggests that the PAP virus is a very homogeneous group. On the other hand, the fact that no cross-reactions were demonstrable between the PAP virus and antisera against psittacosis, Q fever, influenza A and B, adenovirus, and the PVM (pneumonia virus of mice) clearly indicates that the PAP virus is distinct from agents commonly causing pulmonary infections in human beings and animals. It is hoped that immunological means other than the fluorescent antibody-staining method may be developed to study the antigenic relationship among different strains of PAP virus as well as with other agents in greater detail.

Clinicians and laboratory investigators have long considered that PAP is a clinical syndrome which may be caused by several etiological agents. However, since the adenovirus has been isolated from PAP patients without cold agglutinins (15, 16) and the present PAP virus isolated from patients with cold agglutinins, a more specific name should be given to each of these two viral pneumonias of which the etiological agent is now probably known.

SUMMARY

By means of fluorescein-labelled antibody, the primary atypical pneumonia virus was found to multiply exclusively in the cytoplasm of the epithelial cells lining the bronchioles and air sacs of developing chick embryos. When 13-day old embryos were inoculated intra-amniotically and incubated at 35°C. for 5 days or longer, over 90 per cent of the inoculated embryos became infected.

Between 1954 and 1956, seven strains of PAP virus were isolated from sputums or nasopharyngeal washings in patients during the acute stage of the PAP infection. One strain of virus was isolated from the frozen lung of a patient who died at Boston in 1943. All eight recently isolated strains and the Mac strain isolated by Eaton *et al.* in California in 1944 were antigenically closely related if not identical. PAP virus is not related antigenically to agents of psittacosis, Q fever, adenovirus (Types 1 to 6), influenza A or B, or PVM.

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EXPLANATION OF PLATE 38

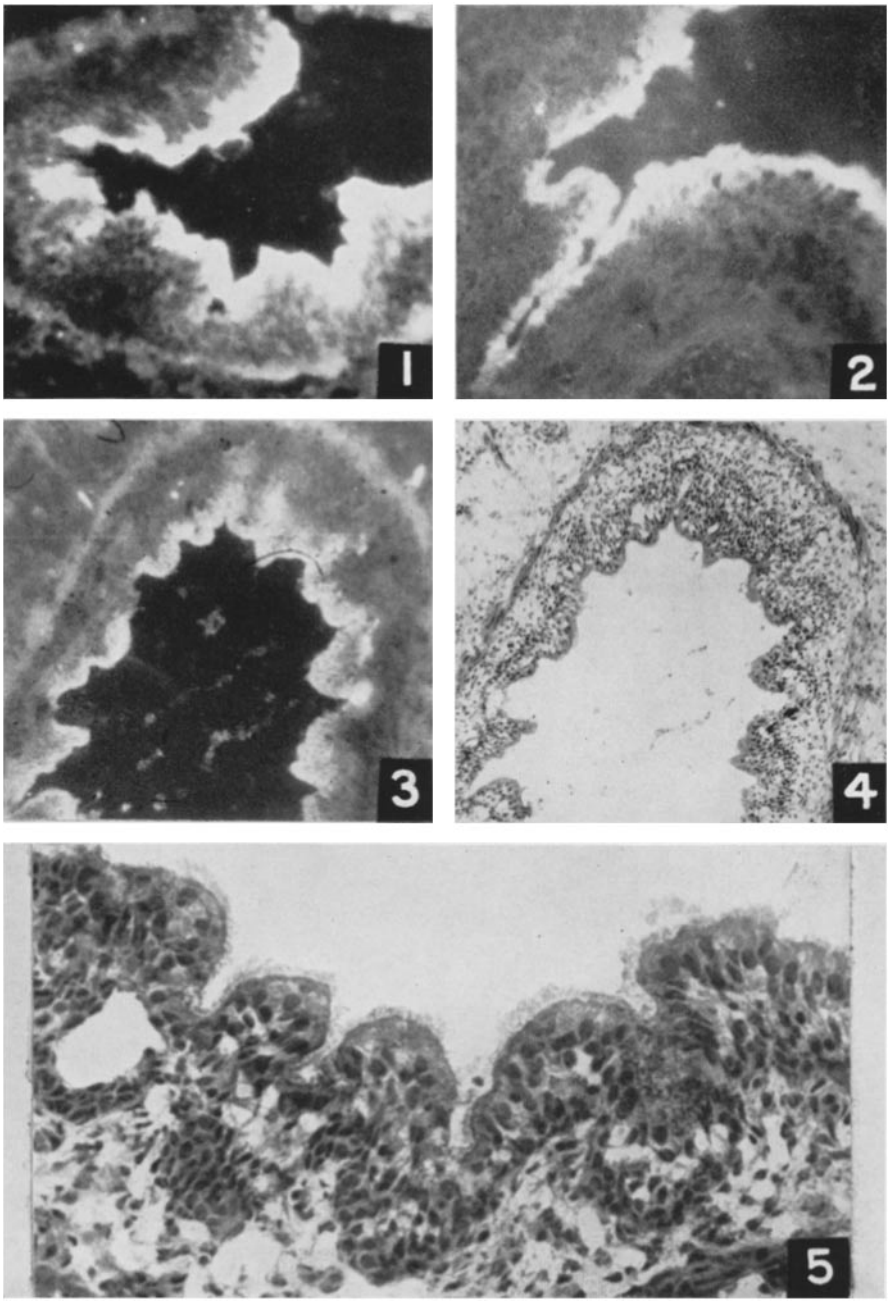
FIG. 1. Fluorescence photomicrograph of a chick embryo lung section infected with T.H. strain of PAP virus. The white area represents PAP viral antigen in the epithelium of a bronchus. × 280.

FIG. 2. Fluorescence photomicrograph of a chick embryo lung section infected with A.S. strain of PAP virus. The viral antigen is localized in the cytoplasm of the bronchial epithelial cells. × 280.

FIG. 3. Fluorescence photomicrograph of a chick embryo lung section infected with F.H. strain of PAP virus. × 140.

FIG. 4. The same section as in Fig. 3. After fluorescent antibody staining, the section was fixed in formalin and stained with hematoxylin and eosin. The infected bronchial epithelial cells appear normal. × 140.

FIG. 5. Adjacent section from the same embryo lung as in Fig. 3. At higher magnification, the infected bronchial epithelial cells appear normal, with intact cilia. Hematoxylin and eosin stain. × 400.



(Liu: Primary atypical pneumonia. I)