STUDIES ON THE ETIOLOGY OF PRIMARY ATYPICAL PNEUMONIA

A Filterable Agent Transmissible to Cotton Rats, Hamsters, and Chick Embryos*

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Plates 15 and 16

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During the past 5 years the cause of primary atypical pneumonia has been ascribed to at least eight separate and distinct infectious agents (1–14). Some of these appear to be new or unidentified viruses (1–8). Others are recently discovered members of the psittacosis–lymphogranuloma venereum group (9–12). In addition, the rickettsiae of Q fever (13) and the virus of lymphocytic choriomeningitis (14) have been shown to be capable of causing pneumonitis in human beings. The available evidence from serological studies and attempted virus isolations indicates that the three last-named agents probably cause less than one-tenth of all cases of atypical pneumonia. Although all three are readily transmissible to mice or guinea pigs, results in these animals generally have been negative.

It was observed during a search for susceptible animals that cotton rats developed non-bacterial lung lesions after inoculation with sputum from patients with primary atypical pneumonia, and evidence that the agent could be passed in these animals was presented (5). Johnson (6) also observed lung lesions in cotton rats inoculated with sputum or throat washings from four of six persons with atypical pneumonia, but passage experiments were not convincing. More recently Horsfall and his collaborators (7) have reported that at least one sputum examined by them consistently produced lung lesions in cotton rats. These workers were unable to reproduce the lesions on passage

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of lung suspensions from one cotton rat to another. They did, however, present evidence that the agent in the sputum could be neutralized by convalescent serums from 11 persons who had had atypical pneumonia.[1] Rose and Molloy (8) were unable to produce evidence of infection by direct inoculation of cotton rats, but obtained pulmonary consolidation in guinea pigs inoculated intranasally with sputum. They subsequently passed the infection from guinea pigs to cotton rats, but their agent was apparently not neutralized by convalescent human serum.

This paper will describe studies on the agent in sputums and lung tissue from certain cases of atypical pneumonia, which produces pulmonary lesions after primary intranasal inoculation of cotton rats. Evidence will be presented that this agent has been adapted to chick embryos and that it is neutralizable by serums taken during convalescence, but not usually by acute-phase serums, from patients with primary atypical pneumonia. The difficulties and confusion introduced into studies of this kind by previously unrecognized latent respiratory viruses of rodents have been noted in some detail.

Materials and Methods

The laboratory studies to be presented were done on material from patients who had primary atypical pneumonia with clinical and roentgenological findings corresponding to those described in numerous published reports (15–17).[2] The clinical details of these cases will be published elsewhere (18). In most cases the disease was mild, in a few severe, and in some the illness terminated fatally. All patients had pulmonary involvement, as determined by x-ray examination, and all had fever of approximately 1 to 3 weeks' duration.

Sputum.—Except in the early phases of the study, all patients were seen by one of us (G. M.), fresh sputum was collected at the bedside, and the specimen was frozen in dry ice within an hour. Specimens were stored at −70°C. until used. An effort was made to obtain the sputum while the patient was febrile and as soon as possible after onset of illness. The sputum was ground with alundum and broth, after which the mucoid portion was centrifuged out at approximately 1500 R.P.M. for 10 minutes. No attempt was made to infect animals or chick embryos with throat washings.

Lung Tissue.—In those cases which came to autopsy a portion of lung tissue was obtained with aseptic precautions whenever possible. In 10 of the 19 fatal cases studied very few bacteria, or none at all, were found in the lung tissue. In 4 the predominant organism was *Staphylococcus aureus*. The tissue was prepared for animal

[1] An indifferent streptococcus has also been isolated from the sputums or lungs of certain cases of atypical pneumonia (34).

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inoculation by grinding with alundum in broth to make a 10 per cent suspension, which was then centrifuged lightly as in the case of sputum.

Serum.—A specimen of blood was collected as early in the disease as possible, and other specimens were usually obtained 10 to 30 days later. After the cold agglutination test was described (19, 20), all blood specimens were allowed to clot at room temperature or allowed to warm to room temperature before the serum was separated. Serum specimens were stored at 4°C. without preservative. Sera for complement fixation and, with a few exceptions, those for virus neutralization were heated to 56°C. for 20 to 30 minutes just before use. The technique employed for the cold agglutination test with 1 per cent group O human cells has been described (21).

Inoculation of Cotton Rats and Hamsters.—The larger portion of the cotton rats used in this study were bred locally from animals of the species *Sigmodon hispidus eremicus*, originally trapped in the vicinity of the lower Colorado River. Eastern cotton rats of the subspecies *hispidus hispidus* and a few *hispidus littoralis* were obtained from two breeders in the eastern United States or were bred locally from stock purchased in the east. The syrian hamsters (*Cricetulus auratus*) were bred locally from descendants of 6 animals out of a group of 100 imported from Syria to New York in 1939 by Dr. W. A. Sawyer. Most of the animals were 6 to 10 weeks of age, although older cotton rats were used in some of the experiments with sputums.

The cotton rats and hamsters were inoculated intranasally under ether anesthesia. The intranasal dose for cotton rats was 0.3 to 0.4 cc. and for hamsters 0.4 to 0.5 cc. During the latter months of the study it was found that more effective aspiration of the inoculum could be produced by lightly reanesthetizing the animal while holding it in a vertical position after inoculation. Animals were sacrificed and autopsied 7 to 14 days after inoculation. Passages were done with 10 or 20 per cent suspensions of lung tissue inoculated intranasally.

Inoculation of Chick Embryos.—Filtered suspensions of sputum or unfiltered suspensions of sterile tissue were inoculated in amounts of 0.1 cc. into the amniotic sac through a 5 mm. opening in the egg shell, according to the method of Burnet (22). The opening was then sealed with Scotch tape.[3] The chick embryos at the time of inoculation were 11 to 12 days old, and they were incubated for 5 days after inoculation. From those embryos which remained alive after the second period of incubation, amniotic membranes, lungs, and trachea were collected for passage. The tissues from each embryo were ground in 2 cc. of broth. The suspension was centrifuged lightly and, after demonstration of bacteriological sterility, was injected by the amniotic route into new embryos.

Examinations for Bacteria.—Sputums and suspensions of human or animal lung tissue were routinely cultured on blood agar plates, and these were incubated for at least 48 hours before being discarded. Direct examinations were made on touch preparations from human or animal tissue stained by the methods of Gram, Giemsa, or Macchiavello. The sterility of chick embryo material and of filtrates was controlled by culture in 1 per cent glucose "hormone" broth.

Storage of Virus.—Tissues, or suspensions of tissues, were stored at −70°C. in

[3] This technique for sealing the opening was described to us by Dr. Carl TenBroeck of The Rockefeller Institute for Medical Research.

rubber-stoppered glass tubes. As an extra precaution against loss of infectivity the suspensions of chick embryo material used in neutralization tests and some samples of sputum and lung tissue were distributed in quantities of 1 or 2 cc. into a number of small pyrex glass tubes which were then sealed in a gas-oxygen flame. The sealed specimens after being opened once were discarded. These refinements of technique were used to keep out carbon dioxide and to avoid repeated freezing and thawing of virus suspensions.

Tests of Cotton Rats and Hamsters for Latent Agents Which Might Be Mobilized by Intranasal Inoculation

In attempts to adapt viruses in human throat washings or sputum to mice by intranasal passages, at least two different latent viruses carried by mice have been encountered (23, 24). The agent of mouse catarrh (25), certain pleuropneumonia-like organisms (26), and pneumotropic strains of the virus of lymphocytic choriomeningitis (27) also produce pulmonary consolidation.

In 1940 a virus related to the pneumonia virus of mice described by Horsfall and Hahn (23) was isolated from hamsters (28). This same virus, or one closely related to it, has been found both in cotton rats and hamsters from time to time. In accordance with the terminology of Horsfall, this agent will be referred to as P.V.M. in this paper. In March, 1942, a passage experiment in apparently normal hamsters, starting with sterile broth, resulted in the isolation of a second unidentified agent, hereafter referred to as agent W1.

Although the presence of latent respiratory viruses in hamsters was fully recognized, it was at first not so evident that cotton rats carried similar agents. Several passage experiments in normal cotton rats were negative. In the period of 2 years since January, 1942, a total of 136 cotton rats and 97 hamsters have been inoculated intranasally with various materials presumed to be free of any infectious agent. The results of these experiments are presented in Table I. It will be noted that the occurrence of lung lesions in the animals tended to be seasonal, the time of highest incidence (about 14 per cent) being in the winter, while practically no lesions were found in the summer months. This is in accordance with our own experience with the pneumonia virus of mice, which has been isolated much more frequently from apparently normal mice in the winter months than in the summer. The results in Table I do not include one group of cotton rats which developed pulmonary consolidation, demonstrated to be due to P.V.M., after inoculation with lung tissue from an apparently normal cotton rat. No lung lesions were found at autopsy in 78 cotton rats inoculated by routes other than intranasal.

The lung lesions observed in the control animals (Table I) could be produced by any one of the several materials and were apparently due to at least four separate and distinct infectious agents which were not bacterial. Two of these, P.V.M. and the unidentified agent W1 isolated from normal hamsters, have already been mentioned. A third agent was isolated from normal cotton rats

and is designated W2. The fourth agent, W3, was not demonstrated in normal cotton rats, but was isolated after multiple intranasal passages in cotton rats starting with human lung tissue or sputum. These four agents exhibited distinctive differences in pathogenic properties and host range. All four were shown to be antigenically unrelated to each other, and none was neutralizable by human convalescent serum. It is impossible because of space limitations to present a detailed description of these agents in this paper.

TABLE I

Results of Intranasal Inoculation of Cotton Rats and Hamsters with Materials Presumably Free of Infectious Agents

Date	Cotton rats		Hamsters	
	No. tested	No. with lung lesions	No. tested	No. with lung lesions
1942				
Jan.–Mar.	14	0	—	—
Apr.–June	9	0	—	—
July–Sept.	7	0	10	1
Oct.–Dec.	10	1	11	0
1943				
Jan.–Mar.	21	3	22	3
Apr.–June	35	2	16	2
July–Sept.	23	0	10	0
Oct.–Dec.	17	1	28	3
Totals	136	7 (4.2 per cent)	97	9 (9.3 per cent)

Cotton rats inoculated as follows: 18, throat washings; 27, heated sputum (60°C. for 1 hour); 44, broth; 30, normal chick embryo suspension; 17, normal cotton rat lung.

Hamsters inoculated as follows: 50, normal chick embryo suspension; 42, broth; 3, normal cotton rat lung; 2, normal hamster lung.

Results of Primary Intranasal Inoculation of Cotton Rats and Hamsters with Human Material

Cotton Rats.—About 370 cotton rats were inoculated intranasally with sputums from 128 persons having atypical pneumonia and with suspensions of lung tissue from 15 patients who had died of the disease. Non-bacterial lung lesions were observed in 28 per cent of the animals, an incidence over six times as great as was seen in control animals. On gross examination these lesions were found most frequently near the hilum and varied in width from 2 to 10 mm. Sometimes several small irregular patches were seen scattered throughout one or more lobes. The lesions, which reached a maximum intensity between the seventh and fourteenth days after inoculation, were gray or grayish-red, and usually level with the surrounding tissue. In lungs containing these lesions the remainder of the tissue was often pink and normal in appearance, or only slightly hyperemic.

Four to eight days after inoculation many cotton rats which subsequently developed lung lesions showed loss of appetite, somewhat labored respiration, drowsiness, and ruffled fur. Fatalities were practically never observed except with one sample of lung tissue, which produced in 21 of 28 animals a non-bacterial pneumonia usually fatal in 3 to 4 days. Although this lung tissue contained *Staphylococcus aureus*, a broth culture of the organism by itself failed to produce lesions. The production of pneumonia in cotton rats was observed for the first time in animals inoculated with this specimen, and the lesions were reproducible over a period of 18 months with material stored at −70°C.

Bacterial pulmonary infections were occasionally seen in cotton rats inoculated with human material. Sputums from seven patients and lung tissue from one patient produced a pneumococcal pneumonia in cotton rats which was

TABLE II

Results of Inoculating Cotton Rats and Hamsters with Sputum or Lung Tissue from Patients with Atypical Pneumonia

Kind of specimen	Negative in cotton rats	Positive results with cotton rats Proportion of animals showing lung lesions with each specimen*	Positive results with hamsters
Sputum.....	90	1/1, 1/1, 1/2(×13), 2/2, 2/2, 2/2, 1/3, 1/3, 2/3, 2/3, 3/3, 3/3, 1/4(×6), 2/4, 2/4, 3/4, 2/5, 5/9, 6/9, 6/10, 7/12	1/2, 2/2, 5/7
Lung.....	12	1/2, 8/27, 21/28	1/1, 6/14

* Each fraction represents specimen from one patient; numerator shows number of animals with lung lesions, denominator shows number tested. 1/2(×13) means specimens from 13 patients each showed result indicated.

fatal for all animals within 48 to 72 hours. In three instances Gram-negative bacilli, probably of the *Hemophilus* group, produced edematous red pulmonary consolidation in some of the animals inoculated. The pulmonary consolidation produced by the bacteria was definitely suppurative, as contrasted with the lesions described in the previous paragraph. Sputums from three other patients and one sample of lung tissue contained psittacosis-like agents, which produced a fatal disease with pneumonia in cotton rats as described in a previous publication (29).

The results of inoculating cotton rats and hamsters with sputum or lung suspensions from patients with atypical pneumonia are recorded in Table II. It will be seen that animals inoculated with sputums from 90 patients and lung tissue from 12 failed to develop lung lesions. In the early phases of the work no great care was used in selection of patients or in obtaining fresh sputum at the optimum period after onset of the disease. Consequently in 55 instances the negative results have little significance because the specimens obtained

were poor and because each sputum was inoculated into only one cotton rat. The development of lung lesions in cotton rats inoculated with material from 41 patients (column 3, Table II) seemed to suggest the presence of an infectious agent in the sputum of these patients. This was particularly true when more than two animals were inoculated with the same specimen and half or more of the number developed lung lesions. The results with two specimens of human lung were particularly interesting. A bacteriologically sterile specimen (case De) produced lesions in 8 of 27 cotton rats. The other, from case B1, was mentioned previously. It is unlikely that these results could be attributed to chance mobilization of a latent virus carried by cotton rats.

TABLE III

Relation of Cold Agglutination Reaction and Time of Collection of Specimen to Results of Primary Inoculation of Cotton Rats

Kind of specimen	Days after onset	Cold agglutination positive		Cold agglutination negative		Cold agglutination not done	
		Cotton rats		Cotton rats		Cotton rats	
		Positive	Negative	Positive	Negative	Positive	Negative
Sputum	3–5	5	1	1*	2	15	16
	6–9	4	3	1*	4	6	22
	10–25	1	3	0	2	1	21
	Unknown	0	0	0	0	4	16
Lung‡	5–9	0	0	0	3	1	3
	10–25	0	1	1	2	1	3
Totals.....		10	8	3	13	28	81

* Lesions in one of four cotton rats inoculated.

‡ Cold agglutinations done with postmortem serum in most of these cases.

Hamsters were inoculated with five specimens of human material which had produced lesions in cotton rats. The lesions in hamsters reached maximum development after 10 to 14 days, and gross pathological evidence of infection was not seen in animals killed at 7 days. With one sample of sputum and one sample of sterile human lung (case De) significant lesions were obtained in hamsters. A number of specimens which were apparently infectious for cotton rats did not produce significant lung lesions in white rats, mice, or microtus. Several specimens which gave negative results in cotton rats also failed to produce lung lesions in hamsters or other animals.

The relation of the production of lesions in cotton rats to the time after onset at which the sputum was collected and to the subsequent development of cold agglutinins in the patient's serum is indicated by the data in Table III. A virus was apparently transmitted to cotton rats from 10 of 18 patients in whom the presence of cold agglutinins was subsequently demonstrated. When the

disease was fatal the patient often died before adequate time had elapsed for the development of cold agglutinins. The results presented in Table III also confirm previous observations (5) that the most frequent production of lung lesions in cotton rats occurs with sputums taken between the 3rd and 5th day and that sputums taken late seldom produce lesions.

Histology of Lung Lesions in Cotton Rats and Hamsters.—The lung lesions in the two species of animals were similar. Microphotographs of sections of lungs with gross lesions are shown in Figs. 1 to 3. Pathological changes resembling those of a virus pneumonia were seen. These changes were sometimes compact and sharply marked off from the surrounding normal tissue, but in other animals the process was diffuse, with scattered small areas throughout the lung. Peribronchial and perivascular infiltration with lymphocytic cells was a prominent feature, and this sometimes occurred in the absence of definite alveolar reaction. In some places there were changes suggestive of thrombosis of small arterioles or venules. The microscopic pathology just described did not differ essentially from that seen with other viruses or rickettsiae, except that some of these agents produced less perivascular or peribronchial infiltration and more alveolar exudate containing a larger proportion of polymorphonuclear leucocytes. There was nothing in the pathology to suggest bacterial infection and no organisms were seen in sections or impression smears.

Passage Experiments in Cotton Rats and Attempts to Demonstrate a Relationship of the Cotton Rat Strains to Human Disease

In a total of 52 cases two or three or more serial intranasal passages were carried out in cotton rats. Sputums from 24 patients failed to produce lung lesions on primary inoculation, and "blind" passages from the lungs of the first passage animals were uniformly negative. In 19 cases in which lesions were obtained in one or more cotton rats on primary inoculation, subsequent passages were either negative or lesions appeared in only the first few passages. Even when the starting material produced extensive pulmonary consolidation or death in animals of the 1st passage, the 2nd passage was frequently negative.

Continued serial passage in nine cases resulted in the eventual establishment of an agent in the cotton rats which consistently produced lung lesions; and in four cases this result was repeated, starting with the original material. In one case two different agents were isolated after starting with the same material. The agents in cotton rats which were responsible for the production of lesions in the animals of the later passages were, however, not identical with the virus in the human material. This was suspected when neutralization by convalescent human serum could not be obtained regularly although homologous immune rabbit or cotton rat serum readily neutralized the agents. Further investigation showed that the agent most frequently obtained in the serial passages was identical with the agent W2 isolated from normal cotton rats.

In two or three experiments an unrelated agent previously designated W3 was found.

Despite the presence of these contaminating agents, some evidence from reinoculation experiments suggested that the original human virus was carried for several passages in cotton rats. The results presented in Table IV show that cotton rats immunized with two "adapted" strains (K1-CR14 and B1-1-CR4-5) in the 4th to 14th cotton rat passage were solidly immune to reinoculation with the sample of human lung (strain B1-HL) which produced marked pulmonary consolidation and frequently death in the control animals. During a 2nd passage series another unrelated agent (strain B1-2) was isolated and

TABLE IV

Reinoculation and Neutralization Experiments with Cotton Rat and Human Material

Immunizing strain*	Test strain*	Lung lesions test animals‡	Lung lesions control animals‡
(Active immunity in cotton rats)			
K1-CR14	B1-H.L.	±, 0, 0, 0, 0	4(D), 4(D), 2, 1
B1-1-CR4-5	"	±, ±, 0	3(D), 3(D)
B1-2-CR9-10	"	4(D), 4(D), 1	
B1-H.L.	B1-1-CR6	±, ±, 0	3, 2, 1, ±
De-H.L.	De-CR6	3, 2, ±, 0	3, 1, 1, ±
BmKs-H.Sp.	K1-CR14	2, 1, ±, 0	4, 1, 1
(Neutralization by immune rabbit serum)			
K1-CR6-14	B1-H.L.	1, 0, 0, 0, 0	4(D), 4(D), 3(D), 3(D), 2, 1, ±, 0
"	De-H.L.	±, ±, ±, 0	2, 1, ±, 0

* In columns 1 and 2 CR = cotton rat lung, H.L. = human lung, H.Sp. = human sputum. Figures after CR indicate number of passage.

‡ In this column figures represent degree of lung consolidation: 4 = ++++, 3 = +++, 2 = ++, 1 = +, ± = questionable lesion, 0 = no lesion, (D) means animal died.

this failed to produce immunity to the human virus. In reciprocal immunity experiments, immunization of cotton rats with human material failed to produce significant immunity to reinoculation with "adapted" strains B1-1, De, and K1. Neutralization of the strains B1 and De in human lung by the serum of a rabbit immunized with the 6th to 14th cotton rat passages of the strain K1 was indicated by the data presented in the lower part of Table IV. Although other interpretations are not excluded, these results suggested that the virus present in the original human material was carried through 6 to 14 cotton rat passages.

The results of experiments to demonstrate neutralization of the cotton rat–adapted strains with convalescent human serum or with the serum of rabbits, hamsters, and cotton rats immunized with infectious human sputum and lung

were irregular and confusing. Some suggestive neutralization of the 2nd to 7th passages was obtained, but neutralization of later passages uniformly failed. All of these observations seemed consistent with the presence of a contaminating "wild" virus in most of the cotton rat material. Passage to hamsters was found to be of little value because the agent latent in cotton rats was transmissible to hamsters.

Experiments with Chick Embryos

Egg Adaptation and Tests for Infectivity in Cotton Rats and Hamsters.—The sample of bacteriologically sterile human lung tissue from a patient with atypical pneumonia (strain De-H.L. in Table IV) and filtered broth suspensions of four sputums were inoculated into the amniotic sac of chick embryos. Passages were carried out as described in the section on materials and methods. Collodion membranes of 600 mμ pore diameter were used for filtration of the sputums. Except for some incidental contaminants which were mostly diphtheroids, no bacteria or other organisms were found by culture or direct examination of smears in the 1st or subsequent chick embryo passages.

Death of the chick embryos occurred very irregularly during the period of 5 days after inoculation; and in some experiments most of the embryos lived for 8 to 9 days, or until the time of hatching. After the 30th passage the infection seemed to be definitely manifested by underdevelopment.

The results of the chick embryo passages are summarized in Table V. Suspensions of the lungs, trachea, and amniotic membrane of chick embryos of the 3rd to 15th passages of the strain De-1 consistently produced lung lesions in hamsters after intranasal inoculation, but only about 30 per cent of the cotton rats inoculated with this material developed lung lesions. In one experiment tenfold or greater dilutions of the chick embryo suspensions failed to produce lesions in hamsters.

The lesions resulting from inoculation of chick embryo suspensions were very similar in gross and microscopic appearance to those found in animals inoculated with human material. Microphotographs of stained sections are presented in Figs. 4 and 5. Intraperitoneal or intracerebral inoculations were without effect. No lesions were obtained by intranasal inoculation of mice or microtus. Attempts to pass the virus in the allantoic fluid or yolk sac of chick embryos have, so far, been unsuccessful.

The chick embryo passage experiments with the human lung were successfully repeated (strain De-2). In two cases (Mu and Bu) the virus was successfully adapted from sputum to the chick embryos and carried in serial passage. Attempts to adapt the agent in two other cases (Yo and Hu) were unsuccessful. With material from the first few passages lung lesions were obtained on intranasal inoculation of cotton rats and hamsters, but subsequent passages have, to the present, been negative.

The results presented in Table V indicate quite definitely that the virus after passages in chick embryos was more virulent for hamsters than for cotton rats. With certain of the later chick embryo passages of the strain De-1 lung lesions were obtained in 50 per cent of the cotton rats at a time when no lesions were found in the controls. The results of inoculating cotton rats with the other strains were, however, of somewhat questionable significance.

TABLE V

Results of Passages in Chick Embryos with Material from Patients with Atypical Pneumonia

Strain	Source	Chick embryo passages	Lung lesions after intranasal inoculation of	
			Cotton rats*	Hamsters*
De-1	Human lung	1–10	1, 1, ±(×4), 0(×6)	3(×3), 2(×9)
		11–20	1(×3), ±, 0(×4)	4, 3, 3, 2(×3), 1, 0(×3)
		21–30	3, 1(×5), 0, 0	2, 1, ±, ±
		31–39	2(×6), 1(×13), ±(×6), 0(×23)	2, 2, 1(×4), 0, 0
De-2	" "	1–10		4, 2(×6), 1(×7), ±, 0
Mu	Sputum	1–3	0, 0, 0, 0	4, 2(×4), 0
		2–5‡	1, 0, 0	3, 3, 2, 2, 1, 1
		6–15	0, 0, 0, 0	2(×3), 1(×8), ±(×3), 0
Bu	"	1–10	1, ±, 0(×5)	2(×4), 1(×7), ±, 0(×4)
		11–22	2, ±, 0(×4)	2(×4), 1, ±(×5), 0(×7)
Yo	"	1–4	3, 1, ±, ±, 0(×4)	2, 1(×3), 0
		5–12	0, 0, 0, 0	±, ±, 0(×5)
Hu	"	1–2	±, 0(×5)	2(×3), 1, ±(×3), 0
		3–5	±, 0(×5)	0, 0, 0, 0

* In this column figures represent degree of pulmonary involvement as follows: 4 = almost complete, 3 = more than half of lung, 2 = less than half of lung, 1 = small lesion, ± = doubtful lesion, 0 = no lesion. Figures in parentheses (×4), (×6), etc. mean the lesion indicated occurred in that many animals.

‡ Passages repeated, starting with second because strain had been lost.

Two strains which had been passed in cotton rats and were contaminated with the latent agent W2 from these animals were tested for adaptability to chick embryos. One of these showed definite evidence of multiplication in the first 3 chick embryo passages, but was subsequently lost. The other strain apparently failed to grow in chick embryos.

In other experiments not included in Table V filtered throat washings from two patients with an influenza-like illness and filtered sputum from one patient with type A influenza were inoculated into the amnion of chick embryos, and serial passages

were carried out. Eight serial blind passages starting with sterile broth were also done. None of the material from the embryos in these control passages produced significant lung lesions in cotton rats or hamsters. These experiments were done concurrently with the passages of the atypical pneumonia virus, using eggs from the same source.

Because the first 10 to 15 passages with strains De-1, De-2, and Yo were done during the winter months, it is likely that intercurrent disease may have accounted for some of the lung lesions observed in the hamsters. It is improbable, however, that mobilization of latent agents would account for the presence of lung lesions in practically all of the hamsters inoculated with the earlier passages of the strains De-1 and De-2. The remaining passages were done during the late spring and summer months when the incidence of pulmonary infections in the control animals was low.

Attempts to Pass the Chick Embryo Virus in Hamsters.—Six separate attempts were made to pass the strain De in hamsters beginning with the 3rd, 5th, 7th, 8th (twice), and 11th chick embryo passages. In the various hamster passages from the 2nd to the 7th, 12 of a total of 37 hamsters developed lung lesions irregularly, the greatest incidence being observed in the 2nd passage. These observations suggested that passage in developing eggs did not make the virus readily adaptable to hamsters.

Filtration Studies.—Suspensions of lung, trachea, and amniotic membrane of the 36th passage of strain De-1 were filtered through collodion membranes of average pore diameter 400 mμ and 483 mμ respectively. In all experiments *Bacillus prodigiosus* was added to the suspension before filtration. The resulting filtrate after demonstration of bacteriological sterility was inoculated and carried for 2 passages in chick embryos by the amniotic route. Tissue suspensions from these chick embryos produced significant lesions in cotton rats. Filters of smaller pore diameter have not yet been tried. Suspensions of the same passage of the strain De-1 after filtration through Berkfeld N filters were inoculated into chick embryos. The 1st and 2nd chick embryo passages from the Berkfeld N filtrate produced significant lesions in cotton rats.

Neutralization by Serum from Patients Convalescent from Atypical Pneumonia

After several preliminary experiments it became evident that the agent in infected chick embryo tissues which produced lung lesions in hamsters and cotton rats was quite labile. Incubation of broth suspensions at room temperature for periods of time even less than an hour or standing overnight in the ice box produced definite diminution or complete loss of activity as judged by animal inoculation. It was found, however, that suspensions containing 25 to 50 per cent normal horse serum or normal human serum retained their activity for at least 30 minutes at 20°C, and thus neutralization tests using this method of incubation were possible.

In the neutralization tests the following procedure was adopted:—

Suspensions of chick embryo tissues which had previously been tested for activity by animal inoculation and stored in sealed glass tubes were thawed, and the precipitate was resuspended thoroughly by drawing it back and forth through a capillary pipette. The suspension was then centrifuged at 1500 R.P.M for 10 minutes and the supernatant immediately mixed with serum. In most experiments 2 parts of suspension were mixed with either 2 parts of normal horse serum, 2 parts of human serum, or 1 part of horse serum plus 1 part of human serum to make mixtures containing 50 per cent serum in all tubes, which were then incubated for 20 minutes at room temperature. Animal inoculations were completed within another 30 minutes. The mixtures containing convalescent serum were always inoculated first and the controls last.

TABLE VI

Neutralization Tests with Chick Embryo Material in Cotton Rats

Case designation	Maximum cold agglutination titer	Sputum: Lung lesions in cotton rats	Chick embryo strain passage No.	Lung lesions after inoculation of virus plus:		
				Acute-phase* serum	Convalescent-phase* serum	Horse serum broth
Sn	320	2/2	De31	2/4	0/4	5/10
Mr	80	—	De32	3/4	1/4	4/12
Kr	160	—	"	2/4	0/4	
Bu	160	5/9	De36	2/4	0/3	5/8
Yo	2560	7/12	"	2/4	0/4	
Mu	1280	6/9	"	3/3	0/4	
Pr	80	1/2	De37	3/3	0/3	
Bj	640	—	De38	2/4	0/3	
Totals				19/30	1/29	14/30

* Final dilutions 1:2 or 1:4 with each pair acute-phase and convalescent serums. Numerator is number of animals showing lung lesions, denominator is number inoculated.

The data in Table VI show the results with acute-phase and convalescent serums from eight cases of atypical pneumonia in neutralization tests with atypical pneumonia virus (strain De-1) from chick embryos. Cotton rats were inoculated intranasally with the serum-virus mixtures. The 2nd column from the left shows the maximum cold agglutinin titer for each case. The cold agglutinin titer of the serum actually used in the neutralization test was usually less than this because the serum was taken later in the disease or had been stored for some time. The 3rd column shows the results of inoculating cotton rats with sputum from the cases indicated. In the last 3 columns it will be seen that 50 per cent or more of the cotton rats inoculated with mixtures of virus and each acute-phase serum developed lung lesions; but animals receiving similar mixtures with convalescent serum failed, with one exception, to develop significant pulmonary changes. Results comparable to those with

acute-phase human serum were obtained by inoculating mixtures of virus and normal horse serum. Acute-phase serum alone gave no lung lesions. The results of two other experiments were not included in Table VI because less than one-fourth of the animals inoculated with virus and normal horse serum showed evidence of infection.

The results of similar neutralization experiments using the hamster as the indicator are presented in Table VII. In four cases (Pk, Ge, Hu, and Sh) there

TABLE VII

Neutralization Tests with Chick Embryo Material in Hamsters

Case designation	Maximum cold agglutination titer	Sputum: Lung lesions in cotton rats	Chick embryo strain passage No.	Lung lesions after inoculation of virus plus:		
				Acute-phase serum	Convalescent-phase serum	Horse serum broth*
So	320	—	Mu 5	3/3	0/3	(0/3)
Mu‡	1280	6/9	Mu 6	3/4	0/4	
Pk	80§	0/2	"	1/4	0/4	
Gi	40§	3/4	"	2/4	0/4	
Bt	80	—	Mu 7	2/4	0/4	5/8
Bu‡	160	5/9	Mu 8	2/4	0/4	(0/4)
Hu	320	6/10	Mu 9	1/4	0/4	7/10
Ir	—§	2/3	Mu 10	3/4	0/4	
Dk	160	—	"	4/4	0/4	4/8
Ge	<10	0/2	"	0/4	0/4	
Hu	(See above)		Hu 1	3/4	2/4	2/2
Mu	(See above)		Hu 1	4/4	2/4	
Sn‡	320	2/2	De 5	2/4	0/4	
Sh	20	1/2	De 7	0/4	0/4	4/8
Bu	(See above)		De 10	2/3	0/3	(1/3)
Pr‡	80	1/2	Bu 22	3/4	0/4	2/2
Ho	20	Psitt.	"	3/4	2/4	

* Figures in parentheses are results of tests in which virus was incubated with plain broth.

‡ Also tested in cotton rats (see Table VI).

§ Cold agglutinin titers of both serum specimens were known to be less than 10 at the time of neutralization test.

was a suggestion of neutralization by acute-phase serum specimens that were collected more than a week after onset (14 days in case Ge). In two of these cases, Ge and Sh, no cold agglutinins were demonstrable. In the remaining tests acute-phase serum collected within a week after onset was used, and more than half of the hamsters receiving virus and these serums developed lesions. Two convalescent serum specimens failed to show definite neutralization of the strain Hu, one of these being from the patient from whose sputum the strain was isolated. As this strain produced rather extensive lesions in the control animals, an excess of virus may have been used in the test. In another test

(not shown in Table VII) with a pool of convalescent serum and a passage of the strain De which showed unusual activity in hamsters, only partial neutralization was obtained.

The case Ho in Table VII was an infection with a psittacosis-like agent. This was demonstrated by isolation of the virus from the sputum and by demonstration of a significant increase in complement-fixing antibody titers with the group-specific antigen. The serum from this case showed no neutralization of the strain Bu and had no significant titer of cold agglutinins. Five other neutralization experiments in hamsters were inconclusive because of insufficient virus activity. In two experiments intercurrent respiratory infections obscured the results.

The strain De was neutralized by the serums of rabbits immune to the strains De, Bu, and Mu grown in chick embryos, but was not neutralized by serums from the same rabbits before immunization.

Several of the serums from the patients listed in Tables VI and VII were tested in mice for neutralization of type A influenza virus and the pneumonia virus of mice. None showed a definite increase in neutralization titer for the strain PR8, and there was no significant neutralization of P.V.M.

Negative Experiments with Complement Fixation.—No complement fixation could be obtained with suspensions of infected chick embryo tissue and serums from patients convalescent from atypical pneumonia. When a method of overnight fixation in the ice box was used, definite fixation of complement was obtained with normal hamster lung. Even stronger reactions were obtained with hamster lung infected with a variety of agents such as W1 from hamsters, W2 from cotton rats, and the virus from cats described by Baker (12). The convalescent serums from cases of atypical pneumonia often showed much higher titers of complement-fixing antibodies against normal or infected hamster lung than did the corresponding acute-phase specimen or normal serums. Similar non-specific or heterogenetic reactions in atypical pneumonia have been reported by other investigators (30, 31). Increases in group-specific complement-fixing antibodies to the viruses of meningopneumonitis or lymphogranuloma venereum propagated in chick embryos were not found in any of the cases.

Cross-Immunity Tests with Egg-Adapted and Human Viruses

The results presented in Table VIII indicate an antigenic relationship between the agent in sputum or lung tissue from patients with atypical pneumonia and the virus propagated in chick embryos, as demonstrated by reinoculation experiments in cotton rats and hamsters. Animals immunized with chick embryo material by repeated intranasal inoculation did not develop significant lung lesions after intranasal inoculation with a sample of sputum (strain Hu) which produced definite signs of pulmonary infection in the con-

trols. Hamsters similarly immunized with human lung (strain De) were immune to the homologous strain adapted to chick embryos. Relationship between homologous and heterologous strains is also indicated in the lower part of Table VIII. Cross-immunity experiments were also done with chick embryo strains and the cotton rat agents W2 and W3. No antigenic relationship between the egg-adapted virus and these cotton rat agents was demonstrated.

TABLE VIII

Cross-Immunity Tests with Human and Egg-Adapted Viruses in Cotton Rats and Hamsters

Animal species	Immunizing strain* passage No.	Test strain passage No.	Lung lesions test animals	Lung lesions control anima s
Cotton rat	De-amn. 33–38	Hu-Sp.	±, 0, 0, 0, 0	3, 1, ±, 0, 0
Hamster	De-amn. 9–10	Hu-Sp.	2‡, 0, 0	2, 1, 1
"	De-H.L.	De-amn. 9–10	1, 0, 0, 0	3, 2, 2, 1, 1
"	De-amn. 7–25	De-amn. 9–10	0, 0, 0, 0	
"	De-amn. 9–10	Mu-amn. 7	0, 0, 0, 0	2, 2, 1, 1

* Amn. = strain propagated in the amnion of chick embryos. Other symbols as in Table IV.

‡ Lesion not typical, possibly non-specific.

Relation to Other Viruses

Failure to infect mice by the intranasal route, the consistent absence of characteristic elementary bodies in impression smears of infected lung or chick embryo tissues stained by the method of Macchiavello, differences in gross and microscopic pathology, and lack of antigenic relationships all indicate that the virus isolated from cases of atypical pneumonia as described in this paper is apparently not a member of the psittacosis–lymphogranuloma venereum group. This includes the agent isolated by Baker from cats (12, 32).

The pathogenicity of the atypical pneumonia virus for guinea pigs by the intranasal route has not been studied in detail because of the fact that these animals frequently developed lung lesions after intranasal inoculation of broth. One agent isolated by intranasal passage in guinea pigs produced pulmonary consolidation in cotton rats and hamsters and was very similar to the agent W1 isolated from normal hamsters. This latter agent was apparently not neutralizable by homologous immune hamster or rabbit serums. Its relation to the virus described by Rose and Molloy (8) has not yet been investigated.

The identity of the virus transmitted to mongooses by Weir and Horsfall

(2) with the virus which we have isolated from cases of atypical pneumonia has not been determined because of lack of suitable experimental material. It is hoped that this question may be cleared up eventually. Recently Horsfall and his associates (7) have stated that inoculation into cotton rats and rabbits of sputum or plasma from certain patients with atypical pneumonia, of chick embryo passage material from these patients, or of passage material from mongooses all produced antibodies to the pneumonia virus of mice (P.V.M.). During the present studies a virus closely related to P.V.M. has been isolated from cotton rats, and it has also been observed that apparently normal cotton rats have antibodies to this virus.

A number of rabbits had been immunized with lung tissue or sputum from patients with atypical pneumonia and with material from chick embryo, cotton rat, and hamster passages. When the work just referred to was published, neutralization studies with our strain of P.V.M. from mice, which is antigenically related to Dr. Horsfall's strain, were done on these rabbit serums. Two rabbits immunized with human lung (case De) and one rabbit immunized with chick embryo passages of the strain De developed antibodies to P.V.M. Serums from three other rabbits immunized with infectious human lung or sputum and from two immunized with the atypical pneumonia virus adapted to chick embryos failed to neutralize P.V.M., but the latter two serums did neutralize the chick embryo strain De. These results assumed a somewhat different significance when development of antibodies to P.V.M. was observed in two rabbits not inoculated with atypical pneumonia virus. One of these had been inoculated with human lung heated to 60°C. for 2 hours, and the other with rabbit serum. Nine normal rabbits were also tested for antibodies to P.V.M., and one showed definite neutralization. Further details of these investigations will be published when they have been completed.

DISCUSSION

The evidence presented indicates that the causative virus in certain cases of atypical pneumonia is of low virulence for cotton rats, hamsters, and other experimental animals, is relatively labile as compared with other filterable viruses, and has a longer incubation period than several other agents which also cause pulmonary infections in cotton rats and hamsters. These properties of the virus introduce difficulties in obtaining definite evidence of its multiplication in experimental animals and facilitate contamination or replacement by latent respiratory viruses of animals. The virus of atypical pneumonia seems to be propagated more readily by amniotic inoculation into chick embryos than by intranasal passage in animals. Evidence of infection of the chick embryo was based on the demonstration of small to moderate sized lung lesions in cotton rats and hamsters inoculated intranasally, and neutralization by convalescent serum was indicated by inhibition of these lesions. Although a

similar kind of evidence has frequently been used in other virus diseases, for example in the early work on ferret influenza (33), it is necessarily less convincing than is death of the experimental animal with characteristic lesions and prevention of death by immune serum.

The virus in human material and that propagated in chick embryos apparently failed to produce significant lung lesions on passage in cotton rats and hamsters even when definite pulmonary consolidation was evident after the primary inoculation of these animals. This observation suggests that the effect of heterologous tissue proteins or some other tóxic factor may be operative in conjunction with the virus when human or chick embryo material is inoculated intranasally into these animals. This factor, which may participate in the production of lung lesions, seems not to be effective in passages with homologous tissue subsequent to the primary inoculation.

The question as to what proportion of all cases of primary atypical pneumonia is caused by the virus just described has not been answered. This can probably be done most effectively by neutralization tests on a large series of cases. The results of inoculating cotton rats with sputum suggested that when fresh material is taken soon after onset of the disease almost half of the specimens contain the infectious agent. The available evidence also indicates an association of the virus transmissible to cotton rats with cases in which cold agglutinins are found in the serum. It is considered quite probable that certain forms of respiratory disease without pneumonia may be caused by the same virus.

SUMMARY

1. A filterable virus from certain cases of primary atypical pneumonia was transmitted to chick embryos by inoculation into the amnion of suspensions of bacteriologically sterile lung tissue or filtered sputum, and three strains were adapted by passage.

2. After intranasal inoculation into cotton rats or hamsters, suspensions of the infected chick embryo tissues produced pulmonary lesions which were similar to those seen after instillation of infective human material.

3. The agent propagated in chick embryos was specifically neutralizable by serum from patients recovered from primary atypical pneumonia and was not neutralized by the acute-phase specimens.

4. Passages of the virus in cotton rats and hamsters gave confusing results because of contamination with latent respiratory agents already present in the animals.

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EXPLANATION OF PLATES

PLATE 15

All sections stained with hematoxylin and phloxine.

Photographs by W. C. Matthews.

FIG. 1. Section of lung from a cotton rat inoculated intranasally with sputum from a patient with primary atypical pneumonia and sacrificed after 10 days. At the lower part the tissue appears to be partially collapsed. In several places the alveolar walls are thickened and merge with patchy areas of consolidation. Round dark spots represent perivascular and peribronchial infiltration. Normal lung tissue at upper left. ×15.

FIG. 2. Same, showing infiltration with lymphocytic cells around blood vessels and bronchioles. Exudate in bronchial lumen consists of polymorphonuclear leucocytes and lymphocytes. Alveolar thickening of various degrees is seen in surrounding areas. ×185.

FIG. 3. Same, showing cellular detail in alveoli. At the center is alveolar exudate consisting of mononuclear cells and a very few polymorphonuclear leucocytes. Alveolar walls contain pathological cells of polyhedral form with large pale-staining nuclei. ×500.

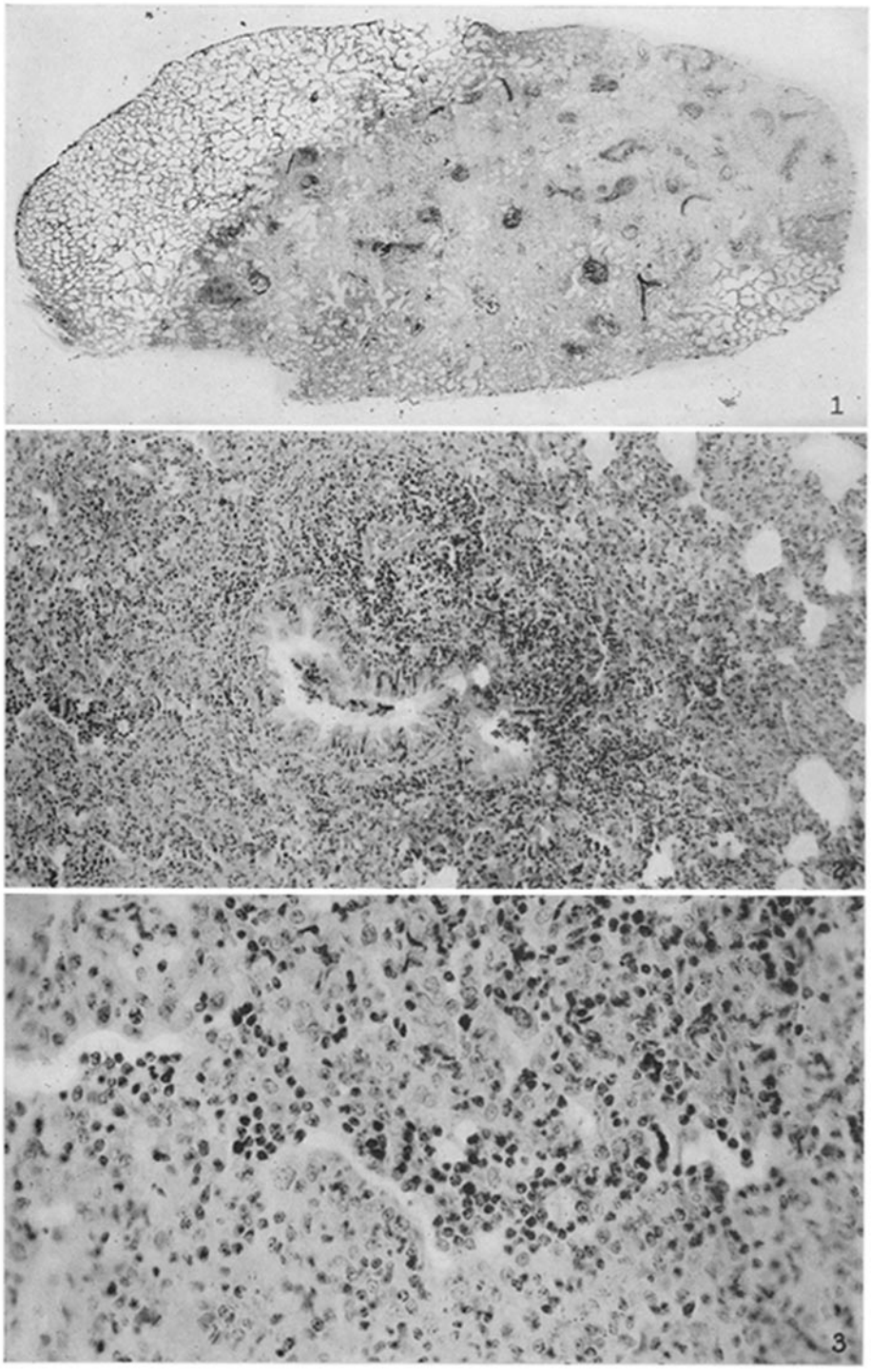

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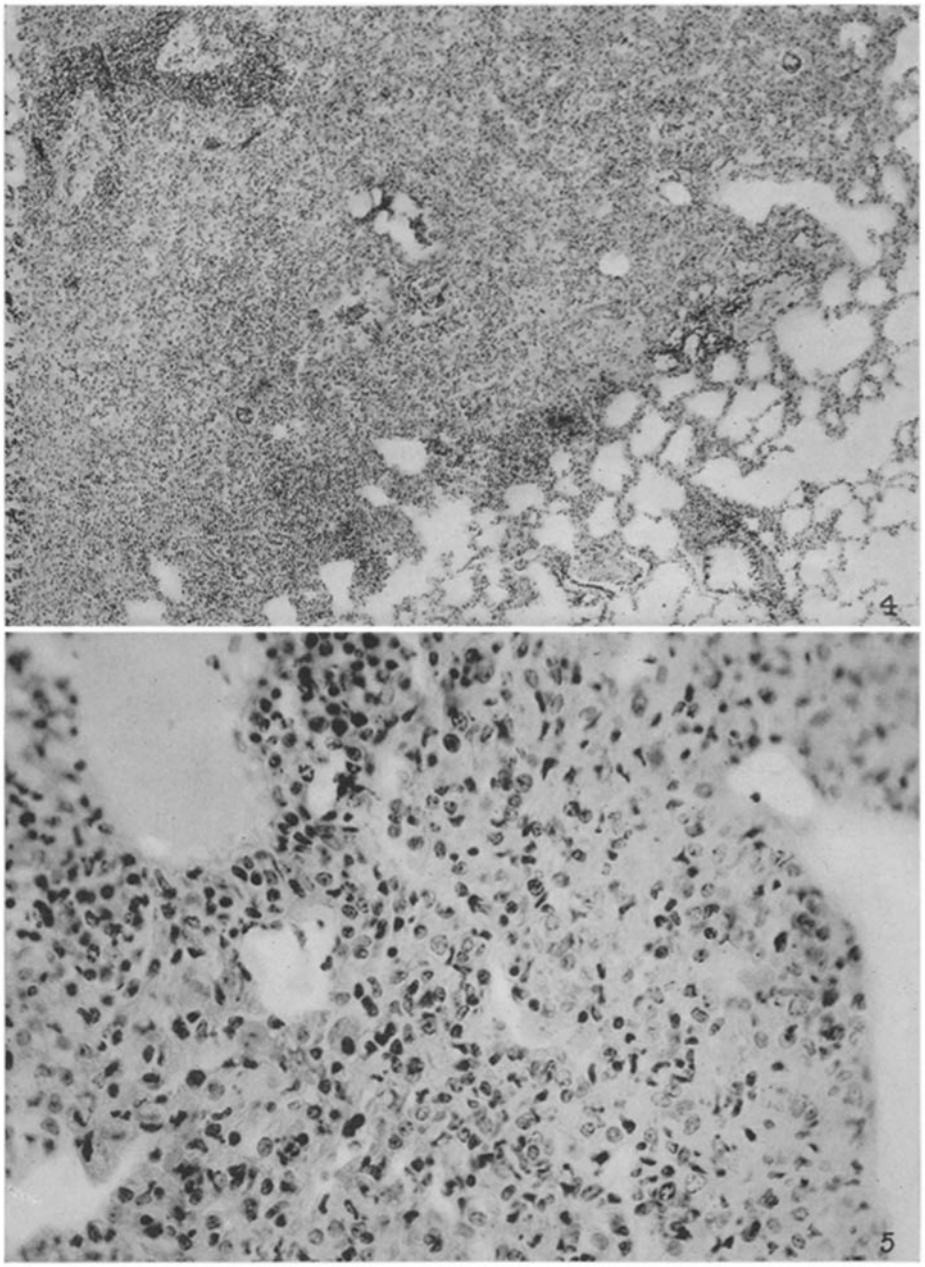
PLATE 16

FIG. 4. Section of lung from a cotton rat inoculated with the 31st chick embryo passage of the virus of atypical pneumonia strain De, and killed at 7 days. Two arterioles with marked lymphocytic cuffing can be seen at upper left. Bronchiole in center contains leucocytes. Near the edge of the lesion at lower right corner is another bronchiole with surrounding mononuclear infiltration. ×92.

FIG. 5. Section of hamster lung from an animal inoculated with a chick embryo passage of the strain Bu and killed at 7 days. Small blood vessel with surrounding lymphocytic reaction is seen at upper left. Cellular detail in alveoli is similar to that of Fig. 3. A few large mononuclear cells with frothy cytoplasm and eccentrically placed nuclei are to be found in the few remaining air spaces. ×500.



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