STUDIES ON THE ETIOLOGY OF PRIMARY ATYPICAL PNEUMONIA

II. Properties of the Virus Isolated and Propagated in Chick Embryos*

By MONROE D. EATON, M.D., GORDON MEIKLEJOHN, M.D., WILLIAM VAN HERICK, AND MARILLA COREY

(From the Research Laboratory of the California State Department of Public Health, Berkeley)

(Received for publication, July 13, 1945)

The isolation of a new virus, unrelated to the agents of the psittacosis group or of influenza, from patients with atypical pneumonia, by inoculation of filtered suspension of sputum or unfiltered suspensions of bacteriologically sterile human lung tissue into the amnion of chick embryos, has been described in the first paper of this series (1). Passage of the virus in chick embryos was successful; but no marked increase in virulence was observed, and the pathological changes produced in the embryos were not well defined or constant. Consequently the development of small pulmonary lesions in hamsters or cotton rats after intranasal inoculation of suspensions of the lungs, tracheas, and amniotic membranes of experimentally infected chick embryos was used as a means of detecting the virus. Passage of the agent in cotton rats or hamsters was found to be impracticable because of the contamination with respiratory viruses from these animals and the failure of the human virus to become adapted to them.

This paper will present further evidence that a filterable infectious agent was carried by amniotic passage in chick embryos primarily inoculated with material from patients with atypical pneumonia and a description of the properties of this virus will be given. In this paper and the one following the agent propagated in chick embryos will be provisionally designated as the virus of atypical pneumonia. Experiments showing the lack of cross-immunity between the atypical pneumonia virus passed in chick embryos and the respiratory viruses of hamsters and cotton rats will also be presented.

Materials and Methods

The methods for the collection of sputum or lung tissue for virus isolation have been described (1).

*The studies and observations reported in this paper were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in cooperation with the California State Department of Public Health and the Commission on Influenza under the Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, United States Army.

Inoculation of Chick Embryos.—All of the fertile eggs were obtained from one hatchery in Hayward, California. Most of these were from white Leghorn chickens, but an experiment with chick embryos of one other variety indicated that these were equally susceptible. The embryos were allowed to develop for 11 days before inoculation. Amniotic inoculation was done by a modification of the stab method described by Hirst (2). The egg was transilluminated from below and the air sac elevated with the long axis at an angle of about 45° to the horizontal, then rotated so that the embryo could be located in the angle formed by the inner air sac membrane and the shell. A hole was made with a sharp needle over the middle of the air sac and a 1½ inch, 24 gauge, long bevel needle attached to a 1 cc. tuberculin syringe was inserted in the direction of the embryo. By a short quick motion the embryo was gently impaled on the point of the needle, and 0.1 cc. of the inoculum was injected. If the latter operation was performed so that only the point of the needle entered the embryo, most of the inoculum passed through the remaining open part of the bevel into the amniotic cavity. After a period of incubation at 36.5°C., usually for 5 to 7 days (see next section), the amniotic membranes, lungs, and trachea of the embryos were collected, with precautions as to sterility, and were ground in broth to make a 20 per cent suspension. The tissues from 3 or 4 embryos were pooled before grinding. The resulting suspension was cultured in glucose broth and on blood agar, and distributed in 1.5 to 2.0 cc. amounts into several small glass tubes, which were sealed and immediately stored at −70°C., as previously described (1). For intranasal tests in hamsters or cotton rats aliquot samples from 2 to 5 pools were usually combined in a lot representing 6 to 20 embryos.

Inoculation and Autopsy of Cotton Rats and Hamsters.—Before inoculation, animals were examined for signs of respiratory illness such as labored respiration, audible rhonchi, or evidence of nasal involvement. The method of intranasal instillation with repetition of the anesthesia after inoculation has been described (1). Cotton rats inoculated with chick embryo tissue were autopsied 10 to 11 days after inoculation while hamsters were sacrificed at 8 days. Animals were killed with chloroform and autopsied as soon as respiration had ceased. The pulmonary lesions were graded immediately after removing the lungs and each lobe was examined carefully because the lesions were often small. The lesions occurred as irregular, but well demarcated, grayish-red patches of consolidation 2 mm. to 10 mm. in width often near the hilum or sometimes scattered throughout one or more lobes. A more detailed description and microphotographs will be found in the first paper of this series (1). Lungs of cotton rats and hamsters which were normal on gross examination immediately after autopsy frequently developed areas of collapse or local accumulations of blood after standing 15 minutes on more, which might be mistaken for lesions.

Observations on the Growth of the Virus in Chick Embryos

Strains Isolated.—A total of 5 strains have been isolated and adapted directly to the chick embryo from human material without previous passage in cotton rats or hamsters. Of these, 2 were obtained from lung tissue and 3 from sputums. Evidence that these 5 strains were antigenically related will be presented in a later section of this paper. Three other sputums and one throat washing yielded negative results. In the present work 2 strains, De and Mac, were studied in detail. The strain De was isolated in December, 1942 (1) and the strain Mac was obtained from human lung tissue in April, 1944. Some of the properties of 3 other strains, Bu, Da, and Mu, have been studied.

Effect of the Time of Incubation.—Chick embryos of ages varying from 7 to 11 days were inoculated by the amniotic route, and the pooled lungs, tracheas,

and amniotic membranes of 3 or 4 of them were tested by intranasal inoculation of hamsters or cotton rats after intervals of incubation from 2 to 12 days. The results presented in Table I indicate that the best growth of virus was obtained with long incubation periods. Of the animals inoculated with material collected at 2 or 4 days only about 30 per cent developed lung lesions while of those receiving tissues from embryos incubated 7 days or more about 60 per cent developed infiltration. There appeared to be no definite advantage to using embryos younger than 11 days.

Tissues of the Embryo Infected.—The best yields of virus were obtained by the amniotic route of inoculation. Chorionic and allantoic passages were apparently unsuccessful.

TABLE I

Effect of Age of Embryo and Period of Incubation in Chick Embryo Passages

Strain* and passage	Age of chick embryos	Pulmonary lesions produced by material collected after days indicated‡					
		2	4	5–6	7–8	9–10	11–12
	days						
Mu 14.....	11	1,0,0,0	—	2,2,1,1,1,1,0,0	2,2,1,1	—	—
De 40.....	11	—	1,1,1,0,0,0,0	—	2,1,1,1,1,1,0,0	—	—
De 11–12..	10	—	—	1,0,0,0,0,0	1,1,1,0,0,0	1,1,0	—
De 11–12..	9	—	—	—	1,0,0,0,0,0	1,1,1,0,0,0	—
De 11–12..	7	0,0,0	1,0,0	1,0,0	1,1,1,0,0,0	1,1,0	2, 1, 1

* All tests except that with De 40 were performed in hamsters; De 40 was tested in cotton rats.

‡ 0 = no lesion; 1 = one plus pulmonary lesion involving 1/8 to 1/4 of lungs; 2 = two plus pulmonary lesion involving about half of lung.

Yolk sac inoculations from the 38th and 45th amniotic passages of the strain De and the 18th and 24th amniotic passages of the strain Bu were done in 6 day old chick embryos and, at intervals of 7 to 10 days, 3 further passages from the yolk sacs were carried out. Significant pulmonary lesions occurred in only 2 of the 16 hamsters inoculated with this yolk passage material. The yolk sacs were then inoculated into the amnion of chick embryos and the lungs, trachea, and membranes of this first passage tested for the presence of virus by intranasal inoculation of hamsters. Ten of the 14 animals receiving this material developed pulmonary lesions. These results indicate that the virus survived the yolk sac passages, but did not increase to the degree necessary for the production of pulmonary lesions in hamsters.

When the virus was inoculated into the amnion, suspensions of the remainder of the embryo after removal of the lungs, trachea, feet, and head produced lesions in cotton rats and hamsters as shown in Table II, but this finding was

rather irregular. In one experiment suspensions of liver and spleen produced lesions, but brain did not.

Variations in the Incidence of Pulmonary Lesions Produced by Different Lots of Chick Embryo Material.—Table III summarizes the results of inoculating hamsters or cotton rats with 62 different lots of chick embryo material. Only those lots which represented pools of material from 8 or more embryos and which were tested in 8 or more animals have been included. The results were similar with 25 other lots representing smaller numbers of embryos or tested in fewer animals. In general, each lot of material was derived from pools of embryos inoculated with the same seed virus within the same week. In many instances the results represent the sum of repeated tests on a given lot of virus. Three lines of one strain, De (R') in the 10th to 13th passages, De (R) in the 11th to 15th passages, and De (O) in the 37th to 45th passages; and one line of a different strain, Mac, in the 2nd to 9th passage, are included. These passages extended over a period of about 1 year.

TABLE II

Comparison of Lesions Produced by Embryo Tissues

Strain passage	Test animal	Pulmonary lesions produced by	
		Amnion, lungs, and tracheas	Remainder of embryo
De 41	Cotton rat	1, 1, 1, 0	2, 1, 0, 0
De 39	Hamster	1, 1, 0	1, 1, 0*
De 13	Hamster	1, 1, 0, 0	1, 1, 0, 0
Mac 9	Cotton rat	1, 1, 1, 1	1, 1, 0, 0, 0, 0
Mac 9	Cotton rat	1, 1, 1, 1	0, 0, 0, 0

* Liver and spleen only, no pulmonary lesions obtained with brains.

The data presented in Table III indicate that 10 (16 per cent) of the lots of chick embryo material produced pulmonary lesions in less than one-fourth of the number of the animals inoculated and 19 (30.5 per cent) produced lesions in over three-fourths of the number inoculated. The remainder were intermediate in pathogenicity.

Of the total of 970 animals inoculated with the infected tissues, 572, or 59.0 per cent, developed pulmonary lesions. Only 8, or 2.24 per cent, of the 357 control animals inoculated concurrently with suspensions of the lungs, tracheas, and amniotic membranes of normal 19 day old chick embryos developed what appeared to be lesions.

The two most obvious reasons for the differences in production of pulmonary lesions by various lots of infected chick embryo tissue seemed to be variations in the susceptibility of the hamsters and rats or inconsistencies in the multiplication of virus in the chick embryos. The animals were between 6 and 10 weeks of age when used and were obtained from several breeders. In the series of intranasal tests from which the data in Table III were derived, records were kept of the age and source of the animals. Analysis of these data revealed

TABLE III

Results of Intranasal Inoculation of Hamsters and Cotton Rats with Individual Lots of Infected Chick Embryo Tissue

Strain	Test animal	Percentage of animals with lesions after intranasal inoculation of individual lots*				Total
		Less than 25 per cent	25–50 per cent positive	55–75 per cent positive	Over 75 per cent positive	
De (R′).....	H	9/67 (6)‡	3/12, 3/12, 4/15, 4/11	9/15, 9/12, 20/30, 19/26	19/25, 11/13, 12/14, 13/14	135/266
Controls§....	H	0/7	— — 0/3 , 0/4	0/4 , — 0/16, 0/20	0/19, 0/10, 1/8 , 0/8	1/99
De (R).....	H	2/12, 2/16	2/8 , 4/16, 13/26	10/18, 10/18, 15/22, 8/12, 21/30, 24/35	20/25, 11/12, 22/24, 16/16, 10/10	191/300
Controls.....	H	— —	0/3 , — 1/11	0/4 , 0/11, 0/4 , 0/4 , 0/16, 0/14	0/8 , 0/8 , 0/10, 0/8 , 0/6	1/107
De (O).....	CR, H	1/10	5/11, 4/8 , 4/8	8/14, 8/13, 9/14, 13/20, 6/8	13/17, 13/16, 14/18	98/157
Controls.....	CR, H	—	1/4 , — —	0/8 , 0/4 , 0/8 , 1/11, 0/4	0/7 , 1/13, 0/7	3/66
Mac.....	CR	0/8	7/21, 6/16, 9/23, 13/28	20/34, 9/13, 8/13, 16/22	14/17, 7/8 , 13/15, 16/18, 10/11	148/247
Controls.....	CR	—	— 0/7 , 0/8 , 0/10	1/16, 0/3 , — 0/11	0/9 , 1/2 , 0/4 , 1/7 , 0/8	3/85
Total No. lots		10	14	19	19	62
Total, *per cent*.....		16	23	30.5	30.5	100

* Numerator of fraction = number of animals with pulmonary lesions; denominator = number tested.

‡ Sum of 6 lots.

§ Animals inoculated with normal chick embryo tissues concurrently with test group just above.

no important influence of such factors on the incidence of lung lesions. The possibility of fluctuations in the susceptibility of the animals from one week to another was also considered. Many of the more active lots of material were used in neutralization tests and, therefore, were tested repeatedly over periods up to 12 weeks. Since these lots of material consistently produced lesions when tested at different times, while less active or poor lots when repeatedly tested produced a low incidence of lesions, fluctuations in the susceptibility of the animals seemed improbable. There were also marked differences in the incidence of lesions between various lots tested simultaneously in one group of animals.

It was found that certain lots of seed virus rather consistently failed to produce active material on passage in chick embryos. Whether this was due to some variation in the virus with serial passage or merely to loss of activity

TABLE IV

Titrations of the Virus of Atypical Pneumonia

Strain passage	Titration in	Pulmonary lesions with dilutions of infected chick embryo							Normal tissue
		10^{-1}	2×10^{-2}	4×10^{-3}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
De 13.....	Hamsters	7/7	6/6	1/6	—	—	—	—	0/6
De 13.....	Hamsters	4/4	—	3/3	0/3	—	—	—	0/6
De 15.....	Hamsters	3/3	1/3	3/4	0/4	—	—	—	0/4
De 46.....	Chick embryos	—	—	—	4/4	2/4	0/4	0/4	0/4
De 40.....	Chick embryos	2/4	3/4*	—	3/4	2/4	—	—	0/4

* Tested at 1×10^{-2}.

during storage has not been determined. The possibility of large variations in the susceptibility of individual embryos, such as have been observed with influenza virus (3), was not excluded.

Titrations in Hamsters and Chick Embryos

The results of titrations are presented in Table IV.

In 3 experiments the tissue suspensions were diluted in 50 per cent normal horse serum and the dilutions were inoculated directly into hamsters. In 2 other experiments the dilutions were inoculated into the amnion of chick embryos and after an incubation period of 7 days the lungs, tracheas, and amniotic membranes of the embryos surviving from each virus dilution were pooled, suspended in broth, and tested for virus activity by inoculation of hamsters.

The findings suggested that about 25 to 100 times as much virus was required to produce pulmonary lesions in hamsters as to infect chick embryos.

TABLE V

Stability Tests by Direct Inoculation of Hamsters or Cotton Rats

Strain	Test animal	Control test	Conditions of treatment	Result
De (O).....	CR	6/8	Storage sealed at −70°C. 7 mos.	7/12
De (R).....	H	3/4	Storage sealed at −70°C. 14 mos.	3/8
Mac.....	H	3/4	Storage with rubber stopper at −70°C. 10 days	4/4
De (R).....	H	4/7	Storage with rubber stopper at −70°C. 1 mo.	0/8
De (O).....	CR	9/11	Storage with rubber stopper at −70°C. 7 mos.	1/11
Mac.....	CR	8/8	Serum broth 5°C. 18 hrs.	6/6
			Serum broth 37°C. 3 hrs.	1/6
De (R).....	CR	12/13	Plain broth 20°C. 2 hrs.	4/11
De (R).....	H	16/17	Serum broth 20°C. 2 hrs.	8/16
De (R).....	H	3/4	Serum broth 20°C. 4 hrs.	1/4
De (R).....	H	2/4	Serum broth 56°C. 15 min.	0/4
De (R).....	H	2/3	*F.D., stored at 4°C. 4 mos.	0/4

* F.D. = frozen and dried *in vacuo*.

TABLE VI

Stability Tests by Amniotic Passage and Inoculation of Cotton Rats or Hamsters

Strain	Test animal	Control test (direct)	Conditions of treatment	Test after amniotic passage
De (O).....	CR	3/4	Storage sealed at −70°C. 8 mos.	11/16
			Storage sealed at −70°C. 11 mos.	0/6*
De (R).....	H	1/4	Storage sealed at −70°C. 14 mos.	9/12*
De (R).....	H	1/8	Serum broth 20°C. 30 min.	4/4
			Serum broth 20°C. 2 hrs.	3/4
			Serum broth 20°C. 6 hrs.	0/4
			Serum broth 37°C. 1 hr.	4/4
De (O).....	H	3/4	‡F.D. and stored at 4°C. 10 days	1/4
Mu.....	H	2/3	F.D. and stored at 4°C. 10 days	2/3
			F.D. and stored at 4°C. 4 mos.	2/4

* After 2 amniotic passages.

‡ F.D. = frozen and dried *in vacuo*.

Stability of the Virus

The effects of temperature and conditions of storage on the virus of atypical pneumonia in plain broth or serum broth suspensions of chick embryo tissues (10 to 20 per cent by wet weight) are shown in Tables V and VI.

In the experiments listed in Table V the material was first tested as a control immediately after removal from the embryo or after a short period of storage at −70°C., then again after the treatment indicated, with the result presented in the last column. Table VI contains data from experiments that were similar, except that after the treatment one or 2 passages through the amnion of chick embryos were carried out and the resulting material was tested by intranasal inoculation of animals. The control tests were done in the same manner as in the experiments shown in Table V; that is, without amniotic passage.

Material could be stored in sealed glass tubes at −70°C. for as long as 14 months without complete loss of activity, but storage at this temperature in rubber-stoppered tubes was less successful, many preparations losing the ability to produce pulmonary lesions after 1 to 7 months. The virus remained active for at lest 18 hours at 5°C.

TABLE VII

Results of Filtration of Infected Chick Embryo Suspensions through Collodion Membranes

Experiment No.	Strain and passage	Filter A.P.D. mμ	Lesions after passage through amnion and inoculation of cotton rats*
1	De (O) -35	Not filtered	2,1,1,1
1	De (O) -35	483	2,2,1,0,0,0
1	De (O) -35	400	1,1,0,0,0,0
1	Normal tissue	—	0,0,0,0
2	De (O) -46	483	1,1,0
2	De (O) -46	300	0,0,0
2	Normal tissue	—	0,0,0
3	De (R) 14	366	1,1,0,0
3	De (R) 14	336	0,0,0,0

* Lesions graded as in Table I.

Preparations kept at 20°C. for 2 hours in plain broth or for 4 to 6 hours in 50 per cent serum broth showed diminution or complete loss of activity. One lot of frozen and dried material failed to produce significant lung lesions when inoculated directly into animals, but after passage of this lot and one other through the amnion of chick embryos evidence of virus activity was obtained.

Filtration Experiments

Suspensions of chick embryo lung, trachea, and amniotic membrane in 10 per cent horse serum broth to which *Bacillus prodigiosus* had been added were filtered through collodion membranes of average pore diameters between 300 and 483 millimicrons. After demonstration of bacteriological sterility the filtrates were inoculated into the amnion of chick embryos, and the passage material was tested for activity in cotton rats. In 2 experiments amniotic passages were also done with normal tissue.

As shown in Table VII, the virus was apparently retained by filters of 300 and 336 mμ average pore diameter, but passed filters of 366 and 400 mμ

(A.P.D.) indicating a particle size of about 180 to 250 mμ. Because of the irregularities of propagation of the virus in chick embryos previously noted, and other uncertainties of the filtration technique, these results can by no means be considered as finally establishing the size of the virus particle. They do, however, give some idea of the probable maximum size.

Antigenic Relationship of Five Chick Embryo Strains of the Virus of Atypical Pneumonia

Active Immunity.—Hamsters were immunized by inoculating them intranasally 3 times at intervals of 2 weeks with 10 per cent suspensions of the chick embryo strain De. Control hamsters were inoculated intranasally at the same time with comparable suspensions of normal chick embryo tissue. Two to 4 weeks after the last immunizing inoculation the animals were tested by the intranasal route for immunity to each of the 5 strains of atypical pneumonia virus which have been isolated in this laboratory.

TABLE VIII

Cross-Immunity with 5 Strains of Atypical Pneumonia Virus

Immunizing strain (intranasally)	Test strain inoculated intranasally				
	Dea	Mac	Mu	Da	Bu
De amniotic	0,0,0,0	1,0,0,0	0,0,0,0	0,0,0	0,0
Normal amniotic (control)	3,2,2,1,1	3,2,2,1	2,2,1,1	3,2,1	1,1

The results presented in Table VIII indicate antigenic relationship to strain De of the 4 other strains, but they do not necessarily mean antigenic identity of these strains.

Neutralization with Immune Rabbit Serum.—Further evidence of the antigenic similarity was obtained with cross-neutralization tests. Rabbits were immunized by repeated intraperitoneal inoculation with active virus, 2 animals receiving the strain Bu and one each the strains De and Mac respectively. Neutralization tests in hamsters and cotton rats were performed according to the method previously described (1). None of the rabbits had neutralizing antibodies before inoculation. After immunization the serum of each of the 4 animals neutralized the strain De and the serum of the rabbit immune to De also neutralized the strain Da.

Cross-Immunity Tests with the Chick Embryo Strains of Atypical Pneumonia Virus and Non-Bacterial Agents Isolated from Hamsters and Cotton Rats

In a previous publication (1) 4 unidentified non-bacterial agents of which one (W1) was isolated from hamsters and 2 (W2 and W3) from cotton rats were described briefly. It seemed doubtful that the agents W1 and W3 were important in the production of lung lesions in hamsters or cotton rats after

primary inoculation because of the fact that they were isolated infrequently and only after a number of serial intranasal passages of lung material in these animals. The agent W2 was isolated about 15 times during intranasal passages in cotton rats and also produced pulmonary lesions in hamsters and certain other species of rodents without adaptation. The fourth agent, which was by far the most troublesome in producing lesions on primary inoculation, was related or identical with the pneumonia virus of hamsters (PVH) and the pneumonia virus of mice (PVM) as discussed in previous publications from this laboratory (4, 5).

To investigate the possible relation of these agents to the virus propagated in chick embryos cross-immunity tests were performed.

TABLE IX

Hamsters Immunized with "Wild" Agents and Tested with the Virus of Atypical Pneumonia from Chick Embryos

	Immunizing strain				
	Atypical pneumonia I.n.	W2 I.n.	W3 I.n.	PVH I.p.	Nil
Experiment 1 *a*.....	0,0,0,0	3,3,3,2	—	—	3,2,2,1,1
b.....	—	2,2,2,2,0	—	—	2,2,1,1,0
Experiment 2.....	0,0,0,0	—	2,2,1,1	—	2,2,1,1,0
Experiment 3 *a*.....	—	—	—	1,1,1,1,0,0	1,1,1,1,1,0
b.....	—	—	—	1,1,1,1	1,1,1,1
				1,1,0,0	1,0,0,0

I.n. = intranasal.
I.p. = intraperitoneal.

Hamsters were immunized by 2 or more intranasal inoculations at intervals of 2 to 4 weeks with 10 per cent tissue suspensions of the virus of atypical pneumonia, the agents W2 and W3, and by 2 intraperitoneal inoculations with 10 per cent hamster lung suspension containing active pneumonia virus of hamsters (PVH). These procedures were demonstrated to produce solid immunity to the homologous strains inoculated intranasally. The agent W1 produced chronic and persistent lung lesions and did not immunize by the intraperitoneal route so that satisfactory cross-immunity tests could not be done with it.

Two to 4 weeks after immunization the animals were tested for immunity by intranasal inoculation of chick embryo material containing the virus of atypical pneumonia.

The results of representative experiments are presented in Table IX, where it can be seen that PVH, W2, and W3 produced no immunity in hamsters to the virus propagated in chick embryos. In reciprocal tests cotton rats were immunized by the intranasal route with atypical pneumonia virus and tested with the agent W3. The results of these tests indicated no cross-immunity.

Control of Epizootics Caused by the Pneumonia Virus of Hamsters

In hamsters naturally occurring infections with PVH appeared to be confined to the upper respiratory tract in most cases. During epizootics of this disease a large proportion of animals inoculated intranasally with saline, broth, or normal chick embryo material developed pulmonary consolidation and at such times it became impossible to use these animals for demonstration of the atypical pneumonia virus carried in chick embryos. Consequently, the detection and control of infections with PVH assumed importance.

When the epizootic in a colony of hamsters subsided after a period of 3 to 4 weeks, pulmonary lesions were no longer produced by intranasal inoculation of non-infectious material and solid immunity to intranasal inoculation of PVH was demonstrable, while control hamsters obtained from an outside source were fully susceptible. New animals brought into the infected colony invariably contracted the disease with consequent prolongation of the epizootic. Evidence of active infection or immunity resulting from previous natural infection was sometimes detected in the hamsters from certain breeders, on arrival at the laboratory.

Attempted control by isolation was only partially successful. Each lot of hamsters on arrival at the laboratory was tested by inoculating a sample of 4 to 6 with broth or normal chick embryo material and immediately placing the remainder in a ventilated isolation cubicle (6). On 2 or 3 occasions small epizootics appeared in groups of 20 to 30 animals kept in isolation.

A method more reliable than isolation was the immunization of all hamsters before use in the work on atypical pneumonia.

Hamsters from breeders whose stock was known to be susceptible were inoculated intranasally with PVH, and the consolidated lungs were collected after 6 to 7 days. Hamsters were immunized with 2 intraperitoneal injections of 0.5 cc. each of a 10 per cent suspension of infected hamster lungs given 6 days apart. After an additional 16 to 18 days these animals were found to be solidly immune to intranasal inoculation with PVH.

This procedure was found to be useful, especially during the winter months in controlling intercurrent respiratory infection with PVH among hamsters used in neutralization tests and other experiments, and it did not affect the susceptibility of animals inoculated with the atypical pneumonia virus. Groups of hamsters which had become immune to PVH as a result of exposure to the agent in an infected animal room also remained susceptible to the atypical pneumonia virus.

DISCUSSION

The virus obtained from human cases of atypical pneumonia apparently multiplies slowly and somewhat irregularly in the amnion of chick embryos. The agent may grow in the respiratory tract and other tissues of the embryo,

but it has not been propagated satisfactorily in the yolk sac or extraembryonic membranes. The low susceptibility of hamsters and cotton rats is emphasized by the observation that infected chick embryo tissues will not produce pulmonary lesions at dilutions over 4×10^{-3}, although higher dilutions (up to 10^{-4}) infect chick embryos and may produce inapparent infections in animals.

The lability of the virus probably causes some of the difficulties in isolation. For example, if sputum were allowed to stand several hours at room temperature it is possible that the virus which it contains would lose infectivity for chick embryos. The data presented in this paper indicate that the virus may be slowly inactivated, presumably by carbon dioxide, when stored in a dry-ice refrigerator in rubber-stoppered tubes or in any other manner which would allow access of the carbon dioxide gas to the virus suspensions.

The observations establish quite definitely that the pulmonary lesions produced in hamsters or cotton rats by inoculation with chick embryo tissue are due to an agent propagated in the embryos and not to mobilization of a latent virus in the animals by some unknown factor in the inoculum. The virus carried in the chick embryos can readily be distinguished by cross-immunity tests from the respiratory agents of hamsters or cotton rats most likely to produce pulmonary lesions in these animals.

SUMMARY

Experiments to determine the optimum conditions for propagation of the virus of atypical pneumonia in chick embryos are described. Variations in the activity of infected chick embryo material were investigated.

The highest dilution of chick embryo suspension producing pulmonary lesions in hamsters and cotton rats is not over 10^{-3}. Dilutions of 10^{-4} infect chick embryos.

The virus is unstable at room temperature and also loses activity when stored in a dry-ice refrigerator unless the suspensions are kept in sealed glass tubes.

Filtration experiments indicate a maximum particle size of 180 to 250 mμ.

The virus propagated in chick embryos produces pulmonary lesions in hamsters and cotton rats which have been immunized to their own non-bacterial agents inducing pulmonary lesions. Of these, the pneumonia virus of hamsters most frequently causes intercurrent respiratory infections, and methods of controlling epizootics due to this agent are described.

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