# STUDIES ON THE ETIOLOGY OF PRIMARY ATYPICAL PNEUMONIA

## III. Specific Neutralization of the Virus by Human Serum*

By MONROE D. EATON, M.D., WILLIAM van HERICK, and GORDON MEIKLEJOHN, M.D.

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

(Received for publication, July 13, 1945)

In a previous article (1) preliminary observations were reported which indicated that a new virus isolated from patients with atypical pneumonia, by chick embryo passage, could be neutralized by serum from persons recovered from this disease. This paper will present more detailed data on the neutralization of the virus, including titrations of the human antibodies and further evidence for the specificity of the neutralization in hamsters and cotton rats. The relationship of cold hemagglutinins (3) and agglutinins for an indifferent streptococcus (No. 344) described by Thomas and others (4) to the virus-neutralizing antibodies will be considered.

### *Materials and Methods*

*Virus.*—The inoculation of chick embryos by the amniotic route and the preparation of virus suspensions have already been described (2). Material used in the neutralization tests consisted of broth suspensions of lungs, tracheas, and amniotic membranes, containing the strains De or Mac, from 10 to 20 chick embryos. Each lot was divided into small portions and stored at about −70°C. in sealed glass tubes so that 5 or 6 neutralization tests could be performed with the same lot without opening or thawing the material more than once. All pools of chick embryo material were tested in at least 4 hamsters or cotton rats, and only those which in the preliminary test produced pulmonary lesions in over half of the number of animals inoculated were used in neutralization tests. About 40 per cent of the total number of virus lots prepared could not be used for neutralization because the incidence of lesions in the control animals was too low to assure clear-cut results. The virus suspensions were 5 to 20 per cent by wet weight of tissue and contained about 10 to 50 times the minimal dose producing pulmonary lesions in hamsters.

*Serum.*—Whenever possible, enough blood was collected to furnish at least 5 cc. of serum. The serum was stored at 5°C. without preservative. Sera showing evidence of gross contamination after storage were not used in neutralization tests. For negative controls normal horse serum was used. All sera were heated to 56°C. for 20 minutes and cooled to room temperature before being diluted and mixed with the virus.

* 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.

*Method of Neutralization.*—The human sera were diluted in normal horse serum and mixed with an equal part of virus suspension. One control containing virus with 50 per cent normal horse serum and another with suspensions of lungs, tracheas, and amniotic membranes of normal 19 day old chick embryos in 50 per cent normal horse serum were included. All were incubated for 20 minutes at room temperature according to the technique of neutralization previously described (1). Three or 4 animals were inoculated intranasally with amounts of 0.4 cc. from each tube of serum dilution and virus. The technique of inoculating the hamsters and cotton rats was the same as before (1). Hamsters were sacrificed and autopsied at 8 days after inoculation and cotton rats at 10 or 11 days.

Acute-phase and convalescent serum specimens from the same individual were, with a very few exceptions, tested at the same time. Preliminary tests with single serum dilutions of 1:4 or 1:8 were usually done. After the presence or absence of neutralization at these dilutions had been determined lower or higher dilutions were tested against additional samples of the same stored lot of virus suspension for the purpose of estimating the antibody titer. The highest final dilution of serum which completely prevented the appearance of macroscopic pulmonary lesions in the animals was taken as the end point. Although partial neutralization, as evidenced by a smaller incidence of lesions in the animals receiving virus and human serum than in those receiving virus and normal horse serum, was frequently observed; end points based on a percentage of lesions were not calculated. In a number of tests the titrations were incomplete because of an insufficient quantity of serum.

### *Titration of Neutralizing Antibodies in Cases of Atypical Pneumonia*

To the present time acute-phase and convalescent serum specimens from 69 patients with a clinical diagnosis of primary atypical pneumonia have been tested for neutralization. In 42 of these, or 61 per cent, a fourfold or greater increase in neutralizing antibodies during or after illness was demonstrated. In 7 cases the result was in doubt either because the acute-phase serum was obtained more than 10 days after onset and the antibody titer of both specimens was high or because the observed increase was less than fourfold. In 20 cases, or 29 per cent, no increase was found and none showed a decrease in antibodies.

Detailed results of neutralization tests in hamsters and cotton rats with sera of 28 persons representative of those in whom a significant antibody increase was observed, are presented in Tables I, I*a*, and II. The serum specimens were obtained at times varying from 1 day to 2 years after onset of illness as indicated in the second column of each table. Cold agglutination titers on fresh serum specimens and the results of agglutination with the indifferent streptococcus No. 344[1] (4) are shown in the third and fourth columns.

The results of the neutralization tests are indicated by fractions in which the numerator represents the number of animals showing gross pulmonary lesions and the denominator the number tested. Minute lesions or small areas of hyperemia were not counted. The data for virus controls and normal tissue controls presented in the last two columns of the tables represent the sum of the results with the several sets of control animals used in successive tests on the sera. In those persons having little or no neutralizing antibodies at the onset of illness the results with the acute-phase serum also served as a control on virus activity, as the tests with acute-phase and convalescent serum specimens were done at the same time.

[1] A culture of this organism was given to us by Dr. F. L. Horsfall, Jr. The method of performing the agglutination test was identical with that used by Thomas *et al.* (4).

Of the 23 acute-phase serum specimens for which approximate end points were available, 19 (83 per cent) had titers of 4 or less. The acute-phase speci-

TABLE I

*Virus Neutralization in Hamsters by Sera from Persons with Atypical Pneumonia*

| Case No. | Days after onset | Cold agglutination | Streptococcus agglutination | Virus lot (strain De) | Pulmonary lesions after inoculation of* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Virus and serum dilutions of | | | | | | Virus and horse serum | Normal chick embryo |
| | | | | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | | |
| 1 | 5 | 10 | 0 | 7 | 4/4 | 3/4 | — | — | — | — | 8/15 | 0/12 |
| 1 | 11 | 40 | 10 | 7 | — | — | 0/4 | 2/5 | — | — | | |
| 1 | 16 | 160 | 20 | 7 | — | — | 0/4 | 3/5 | 2/4 | — | | |
| 1 | 25 | — | — | 7 | 0/4 | — | — | 0/4 | 2/4 | — | | |
| 2 | 5 | 20 | — | 7 | 3/3 | — | — | — | — | — | 9/15 | 0/8 |
| 2 | 10 | 320 | 0 | 7 | — | — | 0/4 | — | 3/5 | — | | |
| 2 | 17 | 160 | 0 | 7 | — | — | — | — | 0/3 | — | | |
| 2 | 25 | 80 | 0 | 7 | 0/3 | — | — | 0/4 | — | 1/5 | | |
| 3 | 6 | 80 | 0 | 7 | — | — | — | 5/8 | — | — | 10/12 | 0/12 |
| 3 | 26 | 1280 | 10 | 7 | — | — | — | 0/4 | — | 1/4 | | |
| 3 | 33 | 320 | 10 | 7 | — | — | — | 0/3 | 0/4 | 0/4 | | |
| 4 | 4 | 0 | — | 8 | 2/4 | — | 2/3 | — | — | — | 4/7 | 1/4 |
| 4 | 25 | 640 | 40 | 8 | — | — | — | 0/3 | — | 0/4 | | |
| 4 | 75 | 320 | 20 | 8 | 0/4 | — | — | 0/3 | — | 0/4 | | |
| 5 | 8 | 0 | 0 | 8 | — | 0/4 | — | 0/3 | 2/4 | 2/4 | 3/11 | 0/7 |
| 5 | 28 | 20 | 0 | 8 | — | 0/4 | — | 0/3 | 0/4 | 0/4 | | |
| 6 | 3 | 0 | 0 | 5 | — | — | — | 2/4 | 2/4 | — | 8/12 | — |
| 6 | 63 | 0 | 0 | 5 | — | — | 0/4 | — | 0/4 | 0/4 | | |
| 7 | 5 | 0 | 0 | 5 | — | — | 0/4 | 1/4 | 3/4 | — | | |
| 7 | 35 | 0 | 0 | 5 | — | — | 0/4 | — | 0/4 | 0/4 | | |
| 8 | 2 | — | 0 | 3a | 1/3 | 2/3 | 2/3 | — | — | — | 6/6 | 0/3 |
| 8 | 50 | — | 0 | 3a | — | — | 0/3 | 0/3 | 0/3 | — | | |

* In this and following tables results are shown as fractions with numerator representing number of animals with definite pulmonary lesions, denominator representing number tested. Serum dilutions after adding virus suspension are given.

men of case 7 had a titer of 8, and the specimens of cases 5, 18, and 21 had titers of 16. In 5 cases the minimum titer was not determined. The titers of the convalescent serum specimens varied from 4 in one instance (case 13) to levels as high as 128 (cases 9, 10, 18, 21). In 24 (86 per cent) of the 28 convalescent

serum specimens, titers of 16 or greater were observed. The demonstrated antibody increases associated with the illness were at least fourfold in 5 cases

TABLE I*a*

*Virus Neutralization in Hamsters by Sera from Persons with Atypical Pneumonia (Continued)*

| Case No. | Days after onset | Cold agglutination | Streptococcal agglutination | Virus lot (strain De) | Pulmonary lesions after inoculation of | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Virus and serum dilutions of | | | | | | | Virus and horse serum | Normal chick embryo |
| | | | | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | | |
| 9 | 3 | 0 | 0 | 2 | — | 0/4 | — | 3/3 | — | 2/4 | — | | |
| 9 | 10 | 0 | 0 | 2 | — | — | — | 0/3 | — | 0/4 | — | 7/7 | 0/7 |
| 9 | 17 | 0 | 0 | 2 | — | 0/4 | — | 0/3 | — | 0/4 | 0/4* | | |
| 10 | 1 | — | 0 | 2 | — | 0/4 | — | 3/7 | — | 2/4 | — | | |
| 10 | 39 | — | 0 | 2 | — | — | — | 0/7 | 0/3 | 0/4 | 0/3‡ | 9/11 | 0/11 |
| 10 | (2 yrs.) | — | 0 | 2 | — | — | — | — | 0/4 | — | 1/3 | | |
| 11 | 4 | — | 0 | 2 | 3/4 | — | 2/4 | — | | | | | |
| 11 | 32 | — | 10 | 2 | 0/4 | — | 0/4 | — | 3/4 | — | 3/4 | 7/8 | 0/8 |
| 11 | 92 | — | 0 | 2 | 1/4 | 1/4 | 1/4 | — | | | | | |
| 11 | (2 yrs.) | — | 0 | 2 | 1/4 | — | 2/4 | — | | | | | |
| 12 | 4 | 10 | 0 | 6 | — | 4/8 | 2/4 | — | | | | 7/11 | 0/8 |
| 12 | 14 | 320 | 0 | 6 | — | 0/8 | 0/4 | 1/6 | | | | | |
| 13 | 1 | — | 0 | 6 | 4/4 | 3/3 | — | | | | | | |
| 13 | 10 | — | 0 | 6 | 2/3 | 2/3 | — | | | | | 10/11 | 0/3 |
| 13 | 63 | — | 0 | 6 | — | 0/3 | 2/3 | | | | | | |
| 13 | 210 | — | 0 | 6 | 0/3 | 1/7 | — | | | | | | |
| 14 | 5 | 10 | 0 | 4 | 2/4 | 3/4 | — | | | | | | |
| 14 | 14 | 160 | 0 | 4 | 0/4 | — | — | 0/4 | — | 1/4 | — | 7/8 | 0/8 |
| 14 | 137 | 0 | — | 4 | — | 0/4 | — | 2/4 | — | — | — | | |
| 15 | 7 | 0 | 0 | 9 | — | 0/4 | — | 2/4 | | | | 5/8 | 0/8 |
| 15 | 20 | 0 | 0 | 9 | — | 0/4 | — | 0/4 | — | 0/4 | | | |
| 16 | 6 | 0 | 0 | 10 | — | — | 3/4 | — | 3/4 | — | — | 3/4 | — |
| 16 | 13 | 0 | 0 | 10 | — | — | 0/4 | — | 0/4 | — | — | | |

* No neutralization at 1:256.

‡ No neutralization at 1:512.

(Nos. 5, 12, 13, 20, 23) and were larger in the remaining cases, 6 of these having increases of 16- to 32-fold (cases 2, 8, 9, 10, 17, 25) and one an increase of

TABLE II

*Virus Neutralization in Cotton Rats by Sera from Persons with Atypical Pneumonia*

| Case No. | Days after onset | Cold agglutination | Streptococcal agglutination | Virus* lot | Pulmonary lesions after inoculation of | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Virus and serum dilutions of | | | | | | Virus and horse serum | Normal chick embryo |
| | | | | | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | | |
| 17 | 6 | — | 0 | 11 | 2/3 | — | — | — | — | | 6/6 | 0/3 |
| 17 | 41 | — | 10 | 11 | 0/3 | 1/3 | — | 0/3 | 0/3 | | | |
| 17 | 2 yrs. | — | 0 | 11 | 1/4 | — | 2/3 | — | 3/3 | | | |
| 18 | 3 | 10 | 0 | 11 | — | — | 0/4 | 3/4 | — | — | 6/8 | 1/4 |
| 18 | 16 | 10 | 0 | 11 | — | — | 0/4 | — | 0/4 | 0/4 | | |
| 19 | 4 | 0 | 0 | 12 | 3/4 | — | — | — | | | 5/8 | 0/4 |
| 19 | 31 | 80 | 0 | 12 | 0/4 | — | 0/4 | 2/4 | | | | |
| 20 | 3 | — | 0 | 12*a* | 3/7 | — | — | — | | | 4/7 | — |
| 20 | 29 | — | 40 | 12*a* | 0/7 | — | 1/7 | — | | | | |
| 21 | 2 | — | 0 | 13 | 0/4 | 1/4 | 0/4 | 1/4 | 2/4 | — | 9/12 | 0/12 |
| 21 | 22 | — | 0 | 13 | — | 0/4 | — | 0/4 | 0/8 | 0/4 | | |
| 22 | 6 | 80 | 0 | 14 | 1/3 | 2/3 | 2/4 | — | — | — | 4/11 | 0/5 |
| 22 | 19 | 160 | 0 | 14 | — | 0/6 | 0/4 | 0/3 | 1/4 | — | | |
| 23 | 8 | 80 | 0 | 16 | — | 1/3 | 2/3 | — | — | — | 7/7 | 0/3 |
| 23 | 21 | 10 | 0 | 16 | — | 0/3 | 0/4 | 1/3 | 1/4 | — | | |
| 24 | 7 | 20 | 0 | 8 | 2/4 | 2/4 | — | — | | | 2/8 | 0/4 |
| 24 | 18 | 320 | 40 | 8 | 0/4 | 0/4 | — | 0/4 | | | | |
| 25 | 8 | — | 0 | 17 | 2/3 | 2/3 | — | — | — | — | 3/6 | 0/3 |
| 25 | 26 | — | 40 | 17 | 0/3 | — | 0/6 | — | 0/3 | — | | |
| 26 | 2 | — | 10 | 18 | 3/4 | 2/3 | — | | | | 5/7 | 0/7 |
| 26 | 13 | — | 10 | 18 | — | 0/3 | 0/3 | | | | | |
| 27 | 3 | — | 0 | 19 | 3/4 | — | — | | | | 3/8 | 0/8 |
| 27 | 16 | — | 10 | 19 | 0/4 | — | 0/4 | | | | | |
| 28 | 1 | — | 0 | 19 | — | 2/4 | — | 2/3 | — | — | | |
| 28 | 19 | — | 10 | 19 | — | 0/4 | — | 0/4 | — | — | | |

* Lots 11, 12, 12a, 13, 14, and 16 were strain Mac. Remainder strain De (0).

64-fold (case 4). It should be noted that in some cases the end point of the convalescent serum was not reached.

Tables I, I*a*, and II contain some preliminary data on the time of appearance

and duration of neutralizing antibodies. An early increase in antibodies between the 3rd and 11th day of illness was noted in cases 1, 2, and 9, while no increase had occurred by the 10th day in case 13. Further increases between the 10th and 33rd day were indicated in cases 1, 2, and 3. Late serum specimens from 6 cases were tested. No decrease was found at $2\frac{1}{2}$ months in case 4. A decrease in antibody titer, but apparently to a level higher than that of the acute-phase serum, was observed at $4\frac{1}{2}$ months in case 14, at 7 months in case 13, and at 2 years in case 10. Return of the antibody titer to the original level was observed at 3 months in case 11 and at 2 years in cases 11 and 17.

*Cold Agglutinins and Streptococcal Agglutinins.*—Of the 28 patients listed in Tables I, I*a*, and II, 11 were shown to develop cold agglutinins during the course of illness, 6 did not develop cold agglutinins, and 11 were not tested because the sera had been collected some time before the cold agglutination test in atypical pneumonia was described (3). All sera were tested for cold agglutinins within 1 week of collection, but usually the neutralization tests were done much later and at a time when the cold agglutinins had decreased or disappeared as a result of storage of the sera. The maximum cold agglutination titers varied from 20 to 1,280. In patients 3, 22, and 23 appreciable amounts of cold agglutinins were present on the 6th to 8th days after onset or before any marked increase in neutralizing antibodies had occurred.

Agglutination with the indifferent streptococcus No. 344 was observed in 11 of the 28 cases. The titers varied from 10 to 40, a titer of less than 10 being considered negative. Previously published data (5) have indicated that in nearly all cases of primary atypical pneumonia in which streptococcal agglutinins are present cold agglutinins are present also. On the basis of this observation the presence of streptococcal agglutinins may be taken as an indication of the probable occurrence of cold agglutination in those patients whose sera could not be tested. Among 20 patients with no significant increase in neutralizing antibodies, 2 had streptococcal agglutinins and 4 others had cold agglutinins.

*Geographical Distribution.*—Most of the persons with clinical diagnosis of atypical pneumonia included in this study were from the Pacific Coast, but antibody increases were also found in 17 patients from the East. Among the latter, 3 from North Carolina (Nos. 6, 7, and 8) and 4 from Minnesota (Nos 25 to 28 inclusive) are presented in the tables.

*Effect of the Age of the Serum.*—Usually the sera were tested between 4 and 18 months after collection. The specimens from patients 10, 11, 13, 17, 25, 26, 27, and 28 were stored for 18 to 24 months, and those from patients 20 and 21 for 30 months, before the neutralization tests were done. The titers of the older convalescent serum specimens were not appreciably lower than those of specimens recently collected. Neutralizing antibodies were found in a few samples of serum which had been stored for 3 to 4 years.

*Effect of Immunization against Pneumonia Virus of Hamsters.*—During the latter part of the neutralization study hamsters were used almost exclusively, and most of these animals were immunized against the pneumonia virus of hamsters to prevent epizootics of this disease, as discussed in the previous paper (2). Immunization against this virus did not affect the results of neutralization with the virus of atypical pneumonia and human serum.

*Neutralization in Cases of Pneumonia Attributed to Other Viruses or Bacteria*

Sera from 9 persons who had type A influenza with pneumonia as a primary or secondary complication were titrated for neutralizing antibodies to the virus of atypical pneumonia. In all of these cases increases in antibodies to type A influenza virus were demonstrated by complement fixation and inhibition of the agglutination of chicken erythrocytes, and in 7 cases influenza virus was demonstrated in the throat washings by inoculating ferrets or hamsters intranasally and testing the animals for antibody response. Five cases which clinically appeared to be pneumococcal pneumonia with elevated leucocyte counts and significant numbers of typable pneumococci in the sputum, and one case of streptococcal pneumonia with bacteremia were also included in this series of control cases.

The results given in Table III show that none of these 15 controls developed significant increases in neutralizing antibodies for the virus of atypical pneumonia. Several of the lots of virus were the same as those used for the cases in Tables I, I*a*, and II. Only 3 (20 per cent) of the sera had titers of 16 or over, as compared with the 86 per cent of the sera of atypical pneumonia patients with titers at this level or higher. In one case (No. 32) a type VI pneumococcus was isolated from the sputum, but a slight increase in virus-neutralizing antibodies was indicated. The tests for this case could not be completed because of insufficient quantity of serum.

A definite increase in cold agglutinins was found in 3 of the patients with type A influenzal pneumonia (Nos. 38, 39, and 40) and a twofold increase was observed in patients 32 and 35. A number of persons having type A influenza without pneumonia, or undifferentiated respiratory disease, have been tested for cold agglutinins with negative results (9). None of the patients listed in Table III had agglutinins for streptococcus No. 344.

In addition to tests of the sera of the 15 patients just discussed, neutralization tests were done with sera of 8 persons having virus pneumonia in which the presumptive cause was a virus of the psittacosis group as judged by complement-fixation tests and isolation of the virus. In 4 cases acute-phase and convalescent serum specimens were tested and no increase in neutralizing antibodies was found. In the remaining 4 cases titers under 4 were obtained in the convalescent specimen. Details will be presented in a separate paper.

TABLE III

*Virus Neutralization in Hamsters with Sera from Persons with Influenzal or Bacterial Pneumonia*

| Case No. | Clinical diagnosis | Days after onset | Cold agglutination | Streptococcal agglutination | Virus lot (strain De) | Pulmonary lesions after inoculation of | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Virus and serum dilutions of | | | | | Virus and horse serum | Normal chick embryo |
| | | | | | | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | | |
| 29 | Pneumococcal | 4 | 0 | 0 | 7 | 0/4 | — | 0/4 | — | 2/4 | 7/7 | 0/4 |
| 29 | pneumonia | 15 | 0 | 0 | 7 | 0/4 | — | 0/4 | — | 2/4 | | |
| 30 | Pneumococcal | 8 | 0 | — | 7 | 0/4 | — | 3/4 | — | — | 6/8 | 0/8 |
| 30 | pneumonia | 24 | 0 | — | 7 | 0/4 | — | 1/4 | — | — | | |
| 31 | Pneumococcal | 6 | 0 | — | 10 | 2/4 | — | 3/4 | — | — | 3/4 | 0/4 |
| 31 | pneumonia | 17 | 0 | — | 10 | 2/4 | — | 2/4 | — | — | | |
| 32 | Pneumococcal | 5 | 10 | 0 | 4 | 1/3 | — | 1/3 | — | — | 9/10 | 0/7 |
| 32 | pneumonia | 15 | 20 | 0 | 4 | 0/6 | 1/4 | 2/3 | — | — | | |
| 33 | Streptococcal | 3 | — | 0 | 4 | 3/3 | — | 2/3 | — | — | 2/3 | 0/3 |
| 33 | pneumonia | 32 | 0 | 0 | 4 | 3/3 | — | 2/3 | — | — | | |
| 34 | Pneumococcal | 1 | 10 | — | 3 | 1/8 | — | 2/4 | — | — | 7/8 | — |
| 34 | pneumonia | 17 | 10 | 0 | 3 | — | — | 3/4 | — | — | | |
| 35 | Influenza A* | 2 | 10 | 0 | 3 | 0/4 | — | 3/4 | — | — | 5/8 | 0/4 |
| 35 | pneumonia | 14 | 20 | 0 | 3 | — | — | 3/4 | — | — | | |
| 36 | Influenza A* | 1 | 10 | — | 3 | 0/4 | — | 0/4 | — | 2/4 | | |
| 36 | pneumonia | 26 | 10 | 0 | 3 | — | — | 0/4 | — | 2/4 | | |
| 37 | Influenza A* | 2 | 0 | 0 | 3*a* | 1/3 | — | 1/4 | — | — | 9/10 | 0/6 |
| 37 | pneumonia | 15 | 0 | 0 | 3*a* | 1/3 | — | 3/4 | — | — | | |
| 38 | Influenza A | 4 | 0 | 0 | 3*a* | 2/3 | 3/4 | — | — | — | | |
| 38 | pneumonia | 42 | 40 | 0 | 3*a* | 2/6 | 2/4 | — | — | — | | |
| 39 | Influenza A* | 2 | 10 | — | 2 | 0/4 | — | 4/8 | — | 1/4 | 7/8 | 0/4 |
| 39 | pneumonia | 14 | 320 | 0 | 2 | 1/4 | — | 2/8 | — | 3/4 | | |
| 40 | Influenza A* | 1 | 0 | 0 | 2 | 0/4 | — | 4/7 | — | — | 10/11 | 0/7 |
| 40 | pneumonia | 13 | 40 | 0 | 2 | 0/4 | — | 3/7 | — | — | | |
| 41 | Influenza A* | 1 | 0 | 0 | 2 | 1/8 | — | 0/4 | — | 0/4‡ | 6/12 | — |
| 41 | pneumonia | 18 | 0 | 0 | 2 | — | — | 0/4 | — | 0/4‡ | | |
| 42 | Influenza A* | 2 | — | 0 | 10*a* | 3/4 | — | — | — | — | 4/4 | 0/4 |
| 42 | pneumonia | 30 | — | 0 | 10*a* | 2/4 | — | — | — | — | | |
| 43 | Influenza A | 3 | — | 0 | 10*a* | 1/4 | — | — | — | — | | |
| 43 | pneumonia | 31 | — | 0 | 10*a* | 2/4 | — | — | — | — | | |

* Influenza virus demonstrated in throat washings.

‡ No neutralization at 1:256.

*Experiments on Neutralization in Chick Embryos*

It has been suggested (1) that a toxic factor may participate in the production of pulmonary lesions in hamsters and cotton rats by human sputum or chick embryo passage material from cases of atypical pneumonia. If this were the case the observed neutralization by human serum might be antitoxic rather than antiviral. To learn whether a transmissible agent in the chick

TABLE IV

*Virus Neutralization in Chick Embryos with Sera from Persons with Atypical Pneumonia*

| Case No. | Days after onset | Neutralization* titer direct | Virus De | | Chick embryos | | Intranasal passage to animals | |
|---|---|---|---|---|---|---|---|---|
| | | | Passage | Dilution | No. inoculated | No. survived | Test animal | Pulmonary lesions‡ |
| 11 | 4 | <2 | 11 | $10^{-2}$ | 6 | 4 | H | 2,2,2,2 |
| 11 | 32 | 8 | 11 | $10^{-2}$ | 6 | 4 | H | 1,0,0,0 |
| 12 | 4 | <4 | 45 | $10^{-3}$ | 16 | 7 | CR | 2,2,2,2,1,1,0,0 |
| 12 | 14 | 8 | 45 | $10^{-3}$ | 16 | 10 | CR | 2,2,1,0,0,0,0,0 |
| 12 | 4 | <4 | 12 | $10^{-3}$ | 6 | 3 | H | 1,1,1,0 |
| 12 | 14 | 8 | 12 | $10^{-3}$ | 6 | 4 | H | 1,1,1,0 |
| 14 | 5 | <2 | 34 | $10^{-2}$ | 17 | 7 | CR | 2,2,2,2,1,1 |
| 14 | 14 | 16 | 34 | $10^{-2}$ | 17 | 10 | CR | 1,0,0,0,0,0 |
| 14 | 5 | <2 | 11 | $10^{-3}$ | 8 | 2 | H | 2,2,2,1 |
| 14 | 14 | 16 | 11 | $10^{-3}$ | 7 | 5 | H | 1,1,0,0 |
| 19 | 4 | <4 | 11 | $10^{-2}$ | 6 | 4 | H | 2,1,1,1 |
| 19 | 31 | 16 | 11 | $10^{-2}$ | 6 | 4 | H | 0,0,0,0 |
| 19 | 4 | <4 | 12 | $10^{-3}$ | 6 | 3 | H | 1,1,1,0 |
| 19 | 31 | 16 | 12 | $10^{-3}$ | 7 | 4 | H | 1,1,1,0 |

* See Tables I*a* and II.

‡ 2 = lesion involving about 1/2 of lung; 1 = lesion involving about 1/8 or 1/4 of lung.
0 = no lesion.
H, hamster; CR, cotton rat.

embryo passages was neutralizable, experiments were performed in which the serum and virus mixture was inoculated into the amnion of chick embryos.

Dilutions of chick embryo tissue of $10^{-2}$ or $10^{-3}$, about 10 to 100 minimal infectious doses, were mixed with serum either undiluted or diluted 1:2 and allowed to stand for 30 to 60 minutes before amniotic inoculation. Six or more embryos were inoculated with each serum-virus mixture, and at the end of 5 days the survivors were tested for the presence of virus by subinoculation of hamsters or cotton rats.

The results of representative experiments with acute-phase and convalescent serum specimens from four patients who had atypical pneumonia are presented in Table IV. Neutralization titers by the direct test in animals are given in

the third column (see also Tables I*a* and II). Of the total of 65 embryos inoculated with acute serum plus virus 30 survived, while of the same number receiving convalescent serum and virus 41 survived, a slight and possibly insignificant difference in mortality. The results presented in the last column indicate that some virus usually was present in the embryos receiving convalescent serum, but on the basis of the number and extent of pulmonary lesions the amount appeared to be less than in the developing eggs receiving virus and acute serum. The results of these experiments were irregular in that neutralization by the convalescent serum did not always occur. Similar irregularities or failure of clear-cut neutralization were noted by Burnett (6) in attempts to neutralize influenza virus in the amnion of chick embryos.

### *Neutralization Experiments with Three Respiratory Viruses from Cotton Rats and Hamsters*

Neutralization of the pneumonia virus of mice by some human sera without change in titer has been noted by Horsfall and Hahn (7). Since the antigenically related pneumonia virus of hamsters (8) has been frequently encountered and a related agent has been found in cotton rats, the possibility of "nonspecific neutralization" of this agent or of some other rodent pneumonia virus by sera from cases of atypical pneumonia was considered.

*Pneumonia Viruses of Hamsters and Mice.*—Neutralization tests in hamsters and mice were done with 2 antigenically related strains of pneumonia virus isolated from the respective species and sera from persons with atypical pneumonia in whom definite increases in neutralizing antibodies to the virus of atypical pneumonia had been demonstrated.

The technique of the neutralization was similar to that for the atypical pneumonia virus except that dilutions of $10^{-2}$ of hamster or mouse lungs (approximately 100 lesion-producing doses) were used and the period of incubation was 1 hour. Hamsters were sacrificed at 8 days and mice at 8 to 12 days after inoculation.

A summary of the results of tests in 7 cases is given in Table V. In cases 11, 13, 17, and 44 no neutralization of the pneumonia virus of hamsters or the pneumonia virus of mice was observed in either the acute-phase or convalescent specimen. In cases 9, 10, and 16 some evidence of neutralization in hamsters and mice was obtained, but no significant differences between the titers of the paired serum specimens were found. In case 16 there appeared to be a slight increase in neutralizing antibodies in hamsters, but this was considered of doubtful significance.

There was no correlation between the presence of neutralizing antibodies for the atypical pneumonia virus and the antibodies to the pneumonia viruses of hamsters or mice. Three human serum specimens (not included in Table V) were found to have neutralizing antibodies for the latter viruses, but none

for the atypical pneumonia virus. The sera of 2 rabbits immunized with the atypical pneumonia virus and having demonstrable antibodies to this agent failed to neutralize the pneumonia virus of mice.

*Attempts to Neutralize the Agent W2.*—The unidentified agent W2 isolated from cotton rats (1, 2) and producing pneumonia in these animals and in ham-

TABLE V

*Neutralization Tests with the Pneumonia Viruses of Hamsters and Mice*

| Case No. | Days after onset | Neutralization* titer (strain De) | Results of intranasal inoculation of hamsters‡ | | | | | Results of intranasal inoculation of mice‡ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Virus and serum dilutions of | | | | Virus and horse serum | Virus and serum dilutions of | | | | Virus and horse serum |
| | | | 1:4 | 1:8 | 1:16 | 1:32 | | 1:4 | 1:8 | 1:16 | 1:32 | |
| 9 | 3 | 4 | 0/3 | — | 4/6 | — | 6/6 | 3/4 | 3/4 | 6/8 | — | 8/8 |
| | 17 | 128 | 1/3 | — | 4/6 | — | | 1/4 | 4/4 | 6/8 | — | |
| 10 | 1 | 4 | 1/3 | — | 4/6 | — | 6/6 | 2/4 | 1/4 | 7/8 | 2/4 | 8/8 |
| | 39 | 128 | 1/3 | — | 3/6 | — | | 1/4 | 2/4 | 4/8 | 4/4 | |
| 11 | 4 | <2 | 3/3 | — | — | — | 3/3 | 4/4 | 2/3 | — | — | 4/4 |
| | 32 | 8 | 3/3 | — | — | — | | 4/4 | 2/3 | — | — | |
| 13 | 10 | <2 | 3/3 | — | — | — | 3/3 | 6/8 | 2/4 | 3/4 | — | 8/8 |
| | 210 | 4 | 3/3 | — | — | — | | 7/8 | 3/4 | 3/4 | — | |
| 16 | 6 | 2 | 2/3 | 4/6 | — | 4/6 | 6/6 | — | 3/4 | — | 3/4 | 4/4 |
| | 13 | 32 | 0/3 | 3/6 | — | 4/6 | | 1/4 | — | — | 3/4 | |
| 17 | 6 | <4 | 3/3 | — | 3/3 | — | 3/3 | 3/4 | — | — | — | 4/4 |
| | 41 | >64 | 3/3 | — | 3/3 | — | | 2/4 | — | — | — | |
| 44 | 4 | <4 | 3/3 | — | — | — | 3/3 | 4/4 | — | — | — | 4/4 |
| | 42 | >8 | 3/3 | — | — | — | | 4/4 | — | — | — | |

* See Tables I, I*a*, and II.

‡ Tests in hamsters and mice done with antigenically related strains of pneumonia virus isolated from the respective species.

Final virus dilutions 0.5 per cent ($5 \times 10^{-3}$) all experiments.

sters was not neutralizable by any of the convalescent sera from 15 patients having atypical pneumonia. Undiluted sera from cases 2, 3, 4, 9, 10, 11, 13, 16, 17, and 44 were tested with dilutions of $10^{-2}$ or $10^{-3}$ of hamster or cotton rat lung containing the agent W2, and no evidence of neutralization in either species of animal was obtained. To eliminate the possibility that higher dilutions of virus might be neutralized, acute-phase and convalescent sera at a constant dilution of 1:2 or 1:4 were tested with decreasing dilutions of the

virus. Negative results with sera of 2 patients are shown in Table VI and no evidence of neutralization was found with sera of 3 other patients tested in a similar manner. The sera of 3 rabbits immunized against agent W2 and having specific neutralizing antibodies for this virus failed to neutralize the atypical pneumonia virus.

*Attempts to Neutralize Agent W3.*—This agent was found only in cotton rats and did not produce pulmonary lesions in hamsters, consequently it could have affected the results of neutralization tests only in the former species. Attempts to neutralize this agent with convalescent sera from cases 4, 17, and 44 (Tables I, II, and V) gave negative results.

The remaining known respiratory agent W1 from hamsters was not neutraliazable by homologous immune rabbit serum (1) and apparently was only an

TABLE VI

*Failure of Neutralization of Agent W2 from Cotton Rats*

| Case No. | Serum | Titer | Pulmonary lesions* after intranasal inoculation of serum and virus diluted | | |
|---|---|---|---|---|---|
| | | | $2 \times 10^{-3}$ | $4 \times 10^{-4}$ | $8 \times 10^{-5}$ |
| 17 | Acute convalescent | <4 | 3,2,2 | 1,1,0 | 1,0,0 |
| | | 64 | 2,2,2 | 1,1,0 | 1,0,0 |
| 44 | Acute convalescent | <4 | 3,2,0 | 2,1,0 | 0,0,0 |
| | | >8 | 2,1,0 | 1,0,0 | 2,2,0 |
| Broth | | | 3,2,2 | 2,1,0 | 1,1,0 |

* Lesions graded as in Table IV.

infrequent cause of the appearance of pulmonary lesions in hamsters after intranasal inoculation of broth or normal chick embryo tissue.

## DISCUSSION

In the preceding papers (1, 2) evidence was presented that an agent having the properties of a filterable virus could be passed in chick embryos primarily inoculated by the amniotic route with filtered suspensions of sputum or lung tissue from patients with atypical pneumonia. The production of pulmonary lesions in hamsters or cotton rats, used as a means of demonstrating the virus in the embryos, could be attributed to one of the following modes of action: (1) pulmonary infection of the animals with the chick embryo virus; (2) the action of a toxic agent on the lung tissue of the animals; (3) the activation of a latent respiratory virus in both cotton rats and hamsters by an agent in the chick embryo passages. No similar infective, toxic, or activating agent was found in comparable passages of normal chick embryo tissue.

The data presented in this paper indicate that patients with atypical pneumonia develop in their sera an antibody which prevents the appearance of pulmonary lesions in hamsters or cotton rats inoculated with mixtures of convalescent serum and infected chick embryo tissue. This development of neutralizing antibodies does not occur in cases of pneumonia caused by the viruses of influenza A or the psittacosis group, or in bacterial pneumonias.

Aside from the observation that, in general, the agent producing pulmonary lesions behaved more like a virus than a toxin, the only evidence that the neutralization was not antitoxic was obtained in experiments in which the convalescent serum and virus were inoculated into the amnion of chick embryos with subsequent passage to hamsters or cotton rats. These experiments tended to be inconclusive because of the irregularity of neutralization of viruses in the amnion of chick embryos and the possibility of some carry-over of the human antibody in the chick embryos. Under the conditions of the experiment, however, the virus, unless it did multiply in the amnion, would have been diluted beyond the range for production of pulmonary lesions in animals.

The third hypothesis, activation of a latent virus, seems improbable because of the failure to demonstrate any significant increase in neutralization titer for the most common virus-like respiratory agents of hamsters or cotton rats with sera from patients having atypical pneumonia. In order to explain the results on the basis of this hypothesis, it would be necessary to assume that one of these agents was neutralized on activation from the latent state, but not after passage and direct inoculation in hamsters or cotton rats. Furthermore, cross-immunity tests by reinoculation of recovered animals and by neutralization with immune rabbit sera have indicated a lack of antigenic relationship between the agents from animals and the atypical pneumonia virus passed in chick embryos.

Only about half of the patients with atypical pneumonia who had increases in neutralizing antibodies also showed cold agglutination and agglutination of the indifferent streptococcus No. 344. It has been suggested that the latter two reactions, which often occur in parallel (5), tend to appear in the more severe cases (5, 10). The absence of cold agglutinins apparently does not exclude infection with the virus described. Neither does positive cold agglutination with pneumonia necessarily indicate infection with this virus, since cold agglutinins were found in 3 influenzal pneumonia patients having only increases in antibodies for the virus of influenza A, and in 4 persons with pneumonia of undetermined etiology. The possible existence of antigenically dissimilar strains of the virus of atypical pneumonia as in influenza A and B should be kept in mind.

Streptococcal agglutinins were found infrequently in cases without specific increases in neutralization titer for the virus of atypical pneumonia. This seems largely to exclude the possibility that there are two forms of atypical

pneumonia, one caused by an indifferent streptococcus and one by a virus. The indifferent streptococcus may be a secondary invader or symbiont with the virus in some cases of the disease. It is not unlikely, however, that the agglutination of this organism may be analogous to the Weil-Felix reaction with *proteus* OX 19 in typhus. It may result from the presence of a common antigen in the streptococcus and the virus or from a heterogenetic antibody response not necessarily related to common antigenic factors.

## SUMMARY

Significant increases in neutralizing antibodies were demonstrated in 42 of a total of 69 persons with a clinical diagnosis of primary atypical pneumonia. Detailed titrations of virus-neutralizing antibodies in a representative group of 28 patients are presented. Increases of four- to 64-fold were demonstrated. Acute-phase titers were 4 or less in 83 per cent and convalescent titers were 16 or over in 86 per cent of these cases.

Only about half of the number of patients having increases in neutralizing antibodies also developed cold agglutinins and agglutinins for the indifferent streptococcus No. 344.

Patients from the Eastern United States as well as those from the Pacific Coast were shown to develop virus-neutralizing antibodies.

Patients with pneumococcal pneumonia and pneumonias caused by influenza virus type A or viruses of the psittacosis group did not have significant increases in neutralizing antibodies for the virus of atypical pneumonia. Cold agglutinins appeared in 3 cases of type A influenzal pneumonia.

Sera from persons with atypical pneumonia, when tested against the 3 most prevalent respiratory viruses isolated from cotton rats and hamsters, failed to neutralize these agents or showed no significant change in neutralization titer.

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