STUDIES ON PRIMARY ATYPICAL PNEUMONIA*, ‡

II. Observations Concerning the Development and Immunological Characteristics of Antibody in Patients

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In a previous paper (3), it was reported that the primary atypical pneumonia (PAP) virus can be detected by fluorescent antibody staining of the bronchial epithelium in infected chick embryos. It has also been found that a component in fresh normal human or guinea pig serum enhances the reaction between the PAP virus and the specific antibody in human convalescent sera (2). This serum component has many of the properties of complement. It is heat-labile, is removable by specific immune precipitates, and its activity requires the presence of calcium and magnesium ions. Detailed studies of the enhancing action of this serum factor will be presented in a separate communication (4).

The present paper is concerned with the development and the persistence of antibody in human patients with PAP infection, the relationship of the PAP antibody to the "cold" and streptococcus MG agglutinins, and the use of the fluorescent-staining reaction as a specific serological diagnostic test for PAP.

In 1943, two independent groups, Turner (5) and Peterson *et al.* (6) reported that the sera of patients convalescent from PAP agglutinated human type O cells in the cold and subsequently this was named "cold agglutinin." The cold agglutinin is not always present in patients' sera. Where it occurs, the range reported varies from 20 to 90 per cent (7) and is apparently related to the severity of the illness. In 1945, Thomas *et al.* (8) reported the isolation of a non-hemolytic streptococcus (MG) from the sputum and lungs of patients with primary atypical pneumonia, and found that between 20 and 75 per cent of the patients develop MG agglutinins depending on the severity of the illness. Immunological studies have shown that the streptococcus MG and the

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cold agglutinins are not related (8). Although these two reactions are not specific for PAP infection, they have commonly been used in the laboratory as adjuncts to diagnosis of primary atypical pneumonia for which no specific test was available. The cotton rat neutralization test for PAP virus developed by Eaton and van Herick (9) has not been generally applied owing to the difficulty of finding uniformly susceptible animals, as different breeds of cotton rats may show varying degrees of susceptibility to the PAP virus infection (10).

Materials and Methods

Viruses.—Antibody titrations were usually done on either the Mac or the F.H. strain of PAP virus (3), although any of the 8 strains of PAP virus in our possession may be used for serological diagnosis, for they are all closely related antigenically.

Screening and Titration of PAP Antibody.—Infected chick embryo lungs were screened with a human convalescent serum known to contain PAP antibody. Only those lungs which showed good intensive fluorescent staining for PAP viral antigen in several bronchioles or air sacs were selected for antibody titration. A series of sections from these infected embryos was cut and stored at 4°C. These sections were usually used within a week, although it was found that they could be used after being stored at refrigerator temperature for about 2 weeks. All human sera were inactivated at 56°C. for 30 minutes. Serial 2-fold dilutions starting at $\frac{1}{10}$ were made in 0.85 per cent NaCl solution. To each 0.2 ml. of serum dilution, one drop (about 0.02 ml.) of fresh normal human serum was added from a capillary pipette. The normal serum had been previously tested and shown to be non-reactive for PAP antibody at a dilution of $\frac{1}{5}$. In more recent experiments, serial 2-fold dilutinos of antiserum starting at $\frac{1}{10}$ were made directly in a $\frac{1}{20}$ dilution of fresh normal human serum in veronal buffer saline. This procedure was found to be not only more convenient, but also more precise. The method for staining the sections with fluorescein-labelled antibody has been described (3). In reading the results, slides stained with various serum dilutions were arranged in random order and the examiner graded the intensity of the specific fluorescence present as + to ++++. The highest serum dilution giving a + staining reaction was taken as the endpoint of antibody titration. Screening of sera was done on $\frac{1}{10}$ dilutions, and if no staining was seen, the serum was considered to be negative. One infected embryo lung usually provided 200 to 300 sections (4 to 6 microns thick), enough for many antibody titrations. To give comparable results, acute and covalescent sera from the same patient were always titrated on sections from the same embryo.

Titration of Streptococcus MG and Cold Agglutinins.—The methods used for these titrations have been described by Smadel (11). A culture of streptococcus MG organisms was obtained through the courtesy of Lieutenant Colonel Robert B. Lindberg of the Walter Reed Army Medical Center, Washington, D.C. The MG antigen was prepared in our laboratory according to his instructions. For cold agglutinin titrations, human type O cells were used.

Absorption of Sera.—The methods for the absorption of cold and MG agglutinins are described in the text. For absorption of the sera with animal tissue powder, the method as described for absorption of fluorescent antibody solutions was used (12).

EXPERIMENTAL RESULTS

Choice of Patients.—A group of patients in a boys' private school provided the essential materials for the serological studies. An epidemic of respiratory illness involving 20 patients with clinical and roentgenological findings consistent with primary atypical pneumonia occurred between the end of September, 1952, and January, 1953. Most of these patients also developed cold agglutinin (Table I). From these 20 patients, sera were collected on admission to the infirmary and again 2 and 4 weeks later during convalescence. From three patients,

sputum and throat washings were also collected. The inoculation of these specimens into cotton rats intranasally was followed by the development of pneumonia. However, attempts at that time to isolate a virus by inoculating the sputum into chick embryos failed. Nine pairs of sera were tested against the Mac strain of PAP virus by neutralization tests in cotton rats. They showed a rise of antibody during convalescence. The majority of the sera remained frozen at −20°C. until 1954.

In September, 1954, when it was found that the PAP virus could be detected in the infected chick embryo lungs by means of fluorescent staining, a serological test for PAP infections became available. Therefore all 20 pairs of sera previously collected during the 1952-53 epidemic were tested for the PAP antibody. Subsequently, additional sera were collected from sporadic

TABLE I

Correlation of Positive PAP Fluorescent-Staining Antibody to Positive Cold Agglutinins

Year	Location	No. patients	No. with positive cold agglutinins	Per cent
1952	Exeter, N. H.	20	19/20	95
1953	Cambridge	5	4/5	80
1954–5	Exeter, N. H.	6	5/6	83

TABLE II

Time of Appearance and Titers of Antibody in Sera of Human Patients Convalescent from PAP

Serum collected on days	No. sera	Fluorescent-staining titer*									Positive 10 or >	Negative <10	Percentage of positive
		10‡	10	20	40	80	160	320	640	1280			
1–7	23	19	0	1	2	0	1	0	0	0	4	19	17
8–14	18	5	0	2	1	1	5	2	2	0	13	5	72
15–20	17	1	0	1	2	2	5	2	4	0	16	1	94
21–30	15	0	0	0	0	0	8	2	3	2	15	0	100
31–40	16	0	0	0	1	3	5	3	4	0	16	0	100
>40	16	0	0	1	1	5	5	3	1	0	16	0	100

* Antibody titrations were carried out with the addition of fresh normal human serum.

‡ Reciprocal of serum dilution.

cases of clinical PAP occurring in the same school during 1955 and 1956. At the same time, sputum and sera were obtained from patients in the Boston-Cambridge area. Virus isolations and serological tests were performed with these specimens (3).

Development of Antibody.—The development of PAP antibody was determined by the fluorescent staining technic on a group of 43 patients who had PAP antibody in their convalescent sera and for whom the time of onset of disease was known (Table II and Fig. 1). It can be seen that only 17 per cent of the sera collected during the 1st week of illness had an antibody titer of 1/10 or higher. The percentage of positive sera increased in the 2nd and the 3rd week and all of the sera collected after the 3rd week were positive. It should be noted

that the four positive sera with a titer over 1/10 during the 1st week of illness were collected on the 5th and the 6th day of illness.

Persistence of PAP Antibody.—Sera from six patients were followed periodically for a year or longer (Table III). In two of these patients (W. E. and H. R.), antibody titers started to decline within 4 to 6 months after the illness. By 12 to 18 months after the infection, the drop of antibody in all sera was 4-

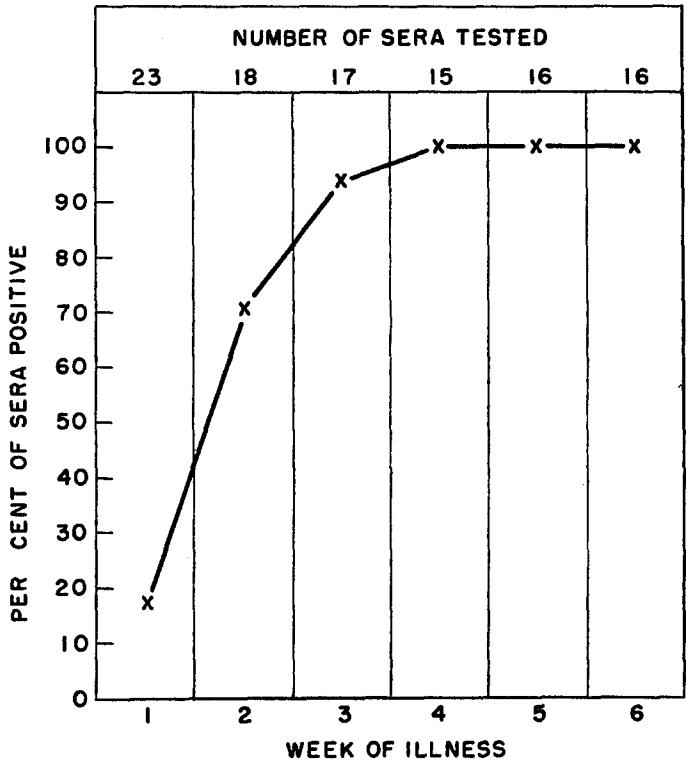

FIG. 1. Development of PAP fluorescent-staining antibody in sera of 43 patients

fold or greater, but still persisted at detectable levels. In four of these patients (A. S., C. P., W. E., and H. R.), both the cold and streptococcus MG agglutinins were also followed. The first two patients had no cold or streptococcus MG agglutinins to start with. In the last two patients, both the cold and streptococcus MG agglutinins had returned to normal levels in the specimens collected 4 to 6 months after the illness.

Comparison of Antibody Response by Neutralization in Cotton rats and Fluorescent Staining.—The sera from nine patients were titrated both by neutralization tests in cotton rats and by fluorescent staining. The neutralization tests were performed in the Spring of 1953 using the Mac strain of virus isolated in

California in 1944 (13). The fluorescent staining was done in 1955 and 1956 on the same sera using both the recently isolated strains and the Mac strain of virus as antigens, since it has been shown that all available strains of PAP virus in our laboratory are antigenically closely related if not identical (3).

As shown in Table IV, the rise of antibody tested both by neutralization and fluorescent staining paralleled each other except in one case (P. H. R.) whose sera did not show a rise of antibody by neutralization test and had only a 2-fold rise of antibody when tested by fluorescent staining. Owing to the small number of cotton rats available, many neutralization titrations could not be carried out to the endpoints.

Serological Diagnosis of PAP Infection.—Sera from 89 patients have been titrated for PAP antibody by the indirect fluorescent-staining method. The results are tabulated in Table V.

TABLE III

Persistence of PAP Antibody in Human Convalescent Sera

Sera collected at	Patients					
	A.S.	C.P.	W.E.	H.R.	M.B.	T.G.
1st wk	<10*	nd‡	nd	nd	<10	nd
2nd to 3rd wk	320	160	>640	640	nd	nd
1 to 3 mos	>160	160	nd	nd	160	80
4 to 6 mos	320	80	80	80	320	80
9 to 12 mos	160	40	40	40	nd	40
12 to 18 mos	80	40	nd	20	40	10

* Reciprocal of serum dilution.

‡ nd, no data.

The first group of 38 patients was from a boys' private school at Exeter, New Hampshire. In 34 of them, there was a definite rise of antibody titer between 8- and 128-fold in the convalescent sera, which made the diagnosis unequivocal. In one patient, the first serum, collected during the 2nd week of illness, had a titer of 1/160 and a second serum collected from him in the 4th week did not show a further rise. However, such a high titer was consistent with a recent PAP infection. The remaining three patients had a titer of 1/20 and 1/40 in their sera collected during the 1st week of illness and subsequent sera did not show more than a 2-fold rise.

In the second group, 30 pairs of sera from patients in Boston and Cambridge, were collected. Some of these sera were from hospital patients while other sera were sent in by local physicians to the Massachusetts State Public Health Diagnostic Laboratory.[1] Most of these patients had pneumonia pre-

[1] We are grateful to Mrs. Joan B. Daniels for making these specimens available.

sumably not of bacterial origin. Of these sera, 20 (67 per cent) had a 4-fold or greater rise of antibody during convalescence when tested by PAP fluorescent staining.

TABLE IV

Comparison of PAP Antibody Titration by Neutralization in Cotton Rats and Fluorescent-Staining Reactions

Patient	Date of sera	Cold agglutinin	Neutralization	Fluorescent staining
F. H.	Dec. 10, 1954	<4	<4	<10
	Dec. 22, 1954	<4	8*	160
	Mar. 16, 1955	<4	8	160
A. S.	Dec. 8, 1954	<4	<4	<10
	Dec. 16, 1954	<4	>32‡	320
	Feb. 3, 1955	<4	16	320
J. R.	Nov. 6, 1952	4	<4	20
	Dec. 4, 1952	<4	>16	640
P. G.	Oct. 28, 1952	<4	<4	<10
	Nov. 24, 1952	16	>16	160
R. W. B.	Oct. 30, 1952	8	<4	<10
	Dec. 4, 1952	64	>16	80
A. E.	Oct. 30, 1952	<4	<4	<10
	Nov. 20, 1952	32	>16	160
P. H. R.	Oct. 30, 1952	<4	4	20
	Nov. 28, 1952	4	<8	40
P. S.	Oct. 30, 1952	<4	<4	<10
	Nov. 13, 1952	32	nd§	nd
	Nov. 28, 1952	8	8	640
T. C.	Oct. 30, 1952	<4	<4	<10
	Nov. 13, 1952	32	nd	nd
	Dec. 1, 1952	4	16	320

* Reciprocal of serum dilution.

‡ Complete neutralization at this dilution; higher dilution not tested.

§ nd, no data.

In the third group, only one serum specimen was collected from each of 21 patients 3 weeks or longer after an illness diagnosed as non-bacterial pneumonia. Two of these patients died. In this group, 14 sera (67 per cent), including the two fatal cases, had PAP antibody at a level of $\frac{1}{10}$ dilution or higher.

As to the specificity of the test, it has been reported (3) from this laboratory that PAP virus does not cross-react with human or rabbit sera known to contain antibody against psittacosis, Q fever, influenza A, influenza B, adenovirus (types 1 to 6), or pneumonia virus or mice.

Relation of PAP Fluorescent-Staining Antibody to the Cold Agglutinin and Streptococcus MG Agglutinin.—Although positive reactions of cold agglutination on streptococcus MG agglutination in human convalescent sera may help to substantiate the diagnosis of primary atypical pneumonia, no experimental data have become available to explain the relation of these antibodies to the disease (5, 6, 8). In one group of 17 patients studied in our laboratory in whom the PAP infection was established by antibody rise and/or positive isolation of virus, 7 did not develop any detectable cold agglutinin. Only 30 per cent of our patients with fluorescent-staining PAP antibody developed

TABLE V

Results of Serological Diagnosis of Human PAP Infections

Sera from	No. of sera per patient	Result		Per cent positive
		Positive*	Negative‡	
Exeter, N. H.	2 or more	35	3	92
Boston-Cambridge	2	20	10	67
Boston-Cambridge	1	14§	7	67

* Positive, a 4-fold or greater rise of antibody titer during convalescence.

‡ Negative, less than a 4-fold rise of antibody titer during convalescence or the antibody titer was less than 1/10 dilution.

§ Antibody titer of the single serum specimen greater than 1/10.

the MG agglutinin. The titers of cold agglutinin usually decline to non-significant levels 4 to 6 weeks after infection (14) and the titers of MG agglutinin may decline during the 7th and the 8th week after infection (8). However, the PAP fluorescent-staining antibody is detectable for a year or longer after the illness (Table III).

To collect additional evidence that cold and MG agglutinins are not related to the PAP antibody, cross-absorption experiments were performed. Convalescent sera from patients who showed high PAP antibody titers and cold or MG agglutinins were diluted to $\frac{1}{5}$ with 0.85 per cent saline. Each serum was divided into two portions. One portion was stored at 4°C. as control, the other portion was absorbed either with washed human type O erythrocytes or with washed heat-killed MG organisms. The absorption was performed by mixing 0.3 ml. packed washed human O cells or MG organisms with 1 ml. of the diluted serum and allowing the tubes to stand at 4°C. for 6 hours with occasional agitation. At the end of 6 hours, the serum was separated by centrifugation and the supernatant was reabsorbed again with 0.3 ml. packed

erythrocytes or bacteria at 4°C. overnight. Finally, the absorbed serum was recovered by centrifugation and tested for cold or MG agglutinins and for fluorescent-staining reaction of the PAP virus. The results are summarized in the Tables VI and VII.

As shown in Table VI, absorption of serum with human O cells at 4°C. removed practically all the cold agglutinin, yet there was no effect on the fluorescent staining of the PAP virus. After the patient's serum was absorbed with

TABLE VI

Relationship of Cold Agglutinin to PAP Antibody

Patient	Absorption	Cold agglutinin	PAP staining
H. R.	None	80*	40
	Human "O" cells	<5	40
T. V.	None	80	160
	Human "O" cells	<5	160

* Reciprocal of serum dilution.

TABLE VII

Relationship of Streptococcus MG Agglutinin to PAP Antibody as Tested by Absorption with MG Organisms

Treatment of aliquots of serum W.E.	MG agglutination	Fluorescent staining on:	
		MG smears	PAP virus
A. Unheated, no absorption	32*	80	160
B. "A" + fresh normal human serum (A. L.)	32	nd‡	320
C. Unheated absorbed 2 × MG	<4	10	40
D. "C" + fresh normal human serum (A. L.)	<4	nd	160
E. Fresh normal serum (A. L.) only	<4	nd	<5

* Reciprocal of serum dilution.

‡ nd, no data.

MG organisms (Table VII), the MG agglutinin and the antibody responsible for the fluorescent staining reaction of MG organisms were reduced. The absorption of unheated serum, W. E., with streptococcus MG apparently removed some of the heat-labile factor which intensifies the staining with PAP virus, thus dropping the observed titer 4-fold in the absence of fresh normal human serum. However, when fresh normal human serum was added to the serum dilutions, the titer was restored to its former level.

To elucidate the relationship between MG agglutinin and the PAP antibody further, two groups of rabbits were immunized to determine if cross-reactions

could be found in the serum (Table VIII). One group was immunized with MG organisms and the other group with PAP-infected chick embryo lung suspensions. It can be seen that rabbits immunized with MG had developed a high agglutinin titer for the organisms but no antibody reactive with the PAP virus. Conversely, rabbits immunized with PAP virus failed to develop

TABLE VIII

Relationship of MG Agglutinin to PAP Antibody as Tested by Immunization of Rabbits

Rabbit sera	MG agglutinin	PAP staining	PAP neutralization
Pre-immunization	<2*	<10	nd‡
Immunized with MG	2048	<10	nd
Pre-immunization	<2	<10	<8
Immunized with PAP virus	<2	>160	>256

* Reciprocal of serum dilution.

‡ nd, no data.

TABLE IX

Effect of Absorption of Human Serum with Tissue Powder on the Fluorescent Staining of PAP Virus

Absorption	Degree of fluorescent staining at:					
	1/20*	1/40	1/80	1/160	1/320	1/640
None	++++‡	++++	+++	++	+	0
1 × chick embryo powder	++++	++++	+++	++	+	±
2 × chick lung powder	++++	++++	+++	+	+	+
2 × mouse liver powder	++++	++++	++	+	±	0

* Serum dilution with fresh normal human serum added.

‡ Degree of specific fluorescent staining:—

++++ maximum intensity

\+ definite but weak

± doubtful

0 no staining

MG agglutinin but developed fluorescent-staining antibody as well as neutralizing antibody against the homologous virus. The data here clearly indicate that the MG agglutinin and the PAP antibody are not related.

Effect of Absorption of Convalescent Serum with Tissue Powder.—It has been reported that some convalescent sera from PAP patients contain non-specific complement-fixing antibody against dissimilar tissue antigens (15). However, absorption of the convalescent serum (Table IX) with chick embryo powder, chick lung powder, or mouse liver powder did not affect the specific fluorescent-staining antibody of the PAP virus.

DISCUSSION

From the evidence presented, it is concluded that among patients clinically designated as having primary atypical pneumonia, there is a distinct entity, associated with the development of cold agglutinin, which is caused by a specific virus. In these patients, the specific antibody against the PAP virus as measured by the fluorescent staining technic appeared during the 2nd and the 3rd week of the illness and persisted for a year or longer. The rise of neutralizing antibody as measured by the neutralization test in cotton rats paralleled the rise of fluorescent-staining antibody. Neither the cold agglutinin nor the streptococcus MG agglutinin was found to be related antigenically to the specific PAP antibody. This was shown clearly by the absorption and animal immunization experiments and also by the fact that the PAP antibody persisted long after the cold and MG agglutinins had returned to normal levels. The fact that a certain percentage of PAP convalescent serum contain the cold and streptococcus MG agglutinins remains unexplained.

At this point, we wish to clarify an observation previously reported (1) that absorption of PAP sera with MG organisms lowered the PAP fluorescent-staining antibody titer. In light of the more recent discovery of the enhancing effect of a serum factor which is likely to be components of complement (4), it is clear now that the agglutination of MG organisms removes this factor rather than the antibody itself from the serum and hence affects the antibody titer. When the titration of serum absorbed with MG organisms was carried out in the presence of fresh normal human serum, the antibody titer was not affected.

The present study was planned to develop a specific serological diagnostic test for PAP infection. An earlier report from this laboratory (3) has shown that the test is specific for PAP and has no cross-reactions with other commonly known agents causing pulmonary infections. Among three groups representing 89 patients with non-bacterial pneumonia which we have studied, 67 to 90 per cent were diagnosed as PAP by the fluorescent-antibody staining technic. The group in which 90 per cent gave positive reactions consisted of patients from three outbreaks of pneumonia in a boys' private school. Each of these patients was seen and diagnosed as PAP by one of the authors (J. T. H.). The other two groups of patients were not as carefully selected. This may account for the difference in favor of a higher percentage of positives in the first group. The etiology of the illness in the patients negative for PAP antibody was not determined. It is likely that the PAP virus may cause milder respiratory infections without the development of pneumonia (9, 16). With a specific serological test available now, it should be feasible to study the epidemiology of this illness by serological survey.

SUMMARY

By using the indirect method of fluorescent staining to study the antibody response in patients with primary atypical pneumonai associated with the development of cold agglutinin, it was found that the PAP antibody developed during the 2nd and 3rd week of the illness, and persisted for over a year, and is not related to the cold and streptococcus MG agglutinins. The development of the PAP fluorescent staining antibody paralleled the neutralizing antibody for the PAP virus as tested in cotton rats. The sensitivity of this specific serological test was indicated by the observation that 67 to 92 per cent of the patients in several outbreaks of PAP showed a rise of antibody titer during convalescence. Absorption of the sera with various tissue powders did not affect the PAP antibody detected by this method.

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