ROLE OF EATON AGENT IN DISEASE OF LOWER RESPIRATORY TRACT EVIDENCE FOR INFECTION IN ADULTS

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The aetiology of primary atypical pneumonia has been the subject of considerable debate for the past 20 years. Much of this controversy has centred in the agent isolated by Eaton, Meiklejohn, and van Herick in 1944. This agent could be maintained by serial amniotic passages in chick embryos, and its presence was demonstrated by induction of pneumonic lesions in cotton-rats and hamsters inoculated intranasally with suspensions of the chick-embryo tissues. Eaton and his associates also demonstrated that approximately 50% of the sputum or throat-washing specimens from patients with primary atypical pneumonia produced similar lung lesions in hamsters or cotton-rats inoculated intranasally. The specimens which produced the highest rate of pneumonic lesions in animals were those collected during the first few days of illness from patients who developed cold agglutinins. Neutralization tests in animals with paired sera from a limited number of patients suggested that neutralizing antibody increases were found most often in patients who also showed significant rises in cold agglutinins and in agglutinins for *Streptococcus MG*.

In 1957 Liu developed his unique indirect fluorescent antibody technique for the agent isolated by Eaton *et al.* in 1944. Liu studied the 1944 isolate, as well as eight additional strains which he isolated from cases of primary atypical pneumonia in the Boston area, and another strain isolated from the lung of a patient who died in 1943. The nine strains isolated by Liu from cold-agglutinin-positive patients were shown to be antigenically similar to each other and to the 1944 isolate of Eaton *et al.* Later, Liu *et al.* (1959), using the indirect fluorescent antibody test, studied three additional groups of 89 patients with non-bacterial pneumonia and found 67-90% showed rises to the Eaton agent. The development of fluorescent stainable antibody for the Eaton agent paralleled neutralizing antibody for this agent as measured in cotton-rats. This finding provided additional evidence that the individuals who developed

fluorescent stainable antibody for the Eaton agent were indeed infected with this agent or an antigenically related agent.

The present investigation, also employing the indirect fluorescent antibody technique, was undertaken to re-examine the role of the Eaton agent in primary atypical pneumonia. Patients who developed cold agglutinins and/or *Streptococcus MG* agglutinins during their convalescence from pneumonia were selected to represent the experience of various years from 1947 to 1959 in various geographical localities. An association of a positive serological response for the Eaton agent in such a diversified group of patients would further strengthen the suggested role of this agent in atypical pneumonia. Individuals with atypical pneumonia unassociated with cold agglutinins or *Streptococcus MG* agglutinins were also studied in order to estimate the potential importance of the Eaton agent in such illness. Evidence that infection with the Eaton agent had occurred concurrent with illness was sought by attempts to recover this agent from the sputum or throat washings of selected patients who developed a positive serological response in the fluorescent antibody test. Such evidence is of some importance, since it has been suggested that the development of fluorescent stainable antibody during convalescence may represent a non-specific response (Dingle and Jordan, 1959).

MATERIALS AND METHODS

Strains of Eaton Agent.—The Mac strain of the Eaton isolated from human lung in 1944 in California by Eaton *et al.* (1945) was kindly furnished by Dr. Eaton. The F. H. strain isolated from sputum (Liu, 1957) was received through the courtesy of Dr. Liu. Two additional new strains of the Eaton agent recently isolated at the National Institutes of Health from pneumonia patients were also included in the study.

Preparation of Pools of Eaton Agent. Inoculation and Harvest of Chick Embryos

All strains of the Eaton agent used were cultivated in 13-day-old white leghorn or Rhode Island Red chick embryos. Using the transillumination technique for amniotic inoculation described by Enders and Habel (1956), 0.1-0.2 ml. of a 10% suspension of infected chick-embryo lung diluted 1:2-1:5 in trypticase soy broth with 0.5% bovine albumin (T.S.B.) was inoculated into each egg. The lung suspensions were maintained in a crushed-ice bath during the time interval required for inoculation. Incubation was at 35° C. for five to six days and the embryos were harvested without previous chilling. For the preparation of a pool of the Eaton agent only the lungs and the lower portion of the trachea were collected from each embryo and ground in 2 ml. of T.S.B. per embryo. The suspension was immediately ampouled in 0.5-2-ml. amounts in glass phials, sealed, and stored in a dry ice-chest (-70° C.) as a stock pool of the agent.

Each passage was checked for bacterial sterility by inoculating thioglycollate broth tubes and blood-agar plates, and 6 to 10 embryos were sectioned and tested for the presence of the agent by the indirect fluorescent antibody test described in a subsequent section. In addition, a passage of the newly prepared stock virus was made into 10 to 12 13-day-old chick embryos to determine the capacity of the pool to produce

subsequent infection of chick embryos. The stock pool was further tested by its ability to induce pulmonary consolidation and pneumonia in intranasally inoculated cotton-rats (*Sigmodon hispidus*) and DBA/2JN mice. Only 20 to 25% of these chick-embryo lung pools of the Eaton agent were satisfactory, and there was a wide variation in the numbers of positive embryos found in any individual pool.

Preparation of Fluorescein-Labelled Anti-Human Globulin

Anti-human globulin sera were prepared by immunizing rabbits with human gamma-globulin (poliomyelitis-immune globulin (Lederle)). The first immunization consisted of a mixture of equal volumes of Freund's complete adjuvant (Difco) and gamma-globulin given in 10 0.1-ml. amounts intradermally. A three-weeks "rest" period was followed by a second immunization consisting of an equal mixture of gamma-globulin and Freund's incomplete adjuvant given in 10 0.1-ml. intradermal doses. This was followed by monthly 1-ml. intradermal injections of gamma-globulin without adjuvant. In some cases a satisfactory anti-gamma-globulin titre was obtained after two months. Other animals required a more lengthy immunization series.

These sera were labelled with fluorescein isothiocyanate by the method described by Marshall *et al.* (1958). Specifically, 5 ml. of serum was mixed with 25.1 ml. of phosphate-buffered saline, pH 7, chilled in a crushed-ice bath, and adjusted to pH 9 with 3.4 ml. of M/2 carbonate buffer; 16.8 mg. of fluorescein isothiocyanate was added and conjugation was allowed to proceed with constant agitation on a magnetic stirrer at 0-4° C. for 18 to 24 hours. The conjugate was then dialysed against phosphate-buffered saline, pH 7, for three to four days to remove free fluorescein isothiocyanate. The fluorescein-labelled serum was absorbed twice with monkey-kidney powder and twice with chicken-liver powder prior to use for fluorescent staining to eliminate non-specific staining properties. The absorbed conjugates were kept at 4° C. for five to seven days or at -20° C. for five to six weeks without a change in their staining properties. The tissue powders were prepared by acetone extraction using standard methods (Coons and Kaplan, 1950).

Indirect Fluorescent Antibody Test for Eaton Agent

The indirect fluorescent antibody test developed by Watson (Weller and Coons, 1954), with its practical application as a serological diagnostic test for primary atypical pneumonia, has been described by Liu (1957) and Liu *et al.* (1959) and was performed in this laboratory according to their methods. In brief, frozen sections of chick-embryo lungs were cut in a cryostat, mounted on glass microscope slides, fixed in acetone for 10 minutes, and allowed to dry. Only those chick-embryo lungs that showed intense fluorescent staining which was specific for the Eaton agent in several bronchioles or air sacs in both the right and the left lung lobes were used. Serial sections were cut and stored at -20° C. One embryo produced 200 to 900 sections (4-6 μ thick). The sections were satisfactory for use in the test for three to four months, though they were usually used in two to three days.

A 1/10 dilution of the convalescent serum of the paired sera under study was used for a preliminary screening test. The 1/10 serum dilutions and all

subsequent dilutions were made in guinea-pig complement diluted 1/10 in veronal-buffered saline, pH 7. The section was covered with the diluted serum and allowed to react at 37° C. for 30 minutes. The serum was rinsed off and the slide immersed in phosphate-buffered saline, pH 7. The slide was then wiped dry except in the area of the chick-embryo lung section, which was then covered with the fluorescein-labelled anti-human conjugate and plated at 37° C. for 30 minutes. The final step consisted in washing the slide for 10 minutes in buffered saline and mounting the section under a coverslip with buffered glycerol, pH 7. The slides were then examined for intensity of specific fluorescence in the bronchial epithelial areas at a magnification of 125, using a microscope with an ultra-violet-light source.

The intensity was graded at 1+ to 4+. Sections with no fluorescence in the bronchial epithelial areas were recorded as negative (<10) and no further testing of these sera was performed. The slides were read without knowledge of the serum-donor's clinical diagnosis. A positive control serum was titred in each test and any deviation in the serum titre invalidated the test. A convalescent serum specimen which produced any degree of fluorescence in the screening test was then titred simultaneously with an acute-phase specimen from the same patient. Twofold serum dilutions were used in all tests. The highest serum dilution giving a 1+ staining was taken as the endpoint of the antibody titration. A fourfold increase in antibody titre from the acute to the convalescent specimen was regarded as significant.

Cold Agglutinins and Streptococcus MG Agglutinins

The techniques for the cold agglutination and *Streptococcus MG* tests were those described by Feller (1948).

Complement Fixation

Allantoic fluid, amniotic fluid, and tissue-culture fluid antigens for members of the myxovirus group were prepared as described by Cook *et al.* (1959). The method of preparation of the yolk-sac antigen for Q fever was that of Smadel (1956), for psittacosis that of Meyer and Eddie (1956), and for L.G.V. that of Rake (1956). The preparation of adenovirus antigen has been described by Rowe *et al.* (1955), *Coxsackie B* by Beeman *et al.* (1952) and respiratory syncytial virus (Long strain) by Chanock *et al.* (1957). The Bengsten modification of the complement-fixation test, using 2 full units of complement, 4 units of antigen, and overnight fixation at 4° C., was employed for most tests. The technique of this test has been described in detail by Cook *et al.* (1959). In occasional tests, using Q fever or psittacosis antigens, water-bath fixation at 37° C. for one hour was employed.

Haemagglutination Inhibition

Haemagglutination inhibition tests for the prevalent strains of influenza were used for screening the cold-agglutinin-negative serums. The technique employed was that of Hilleman and Werner (1953).

Animals

The National Institutes of Health line of DBA/2JN mice was used and the cotton-rats were purchased from the University of Michigan. The volume of the intranasal inoculum for the mice varied from 0.05 to 0.1 ml.,

while the inoculum for the rats was 0.5 ml. A group of control animals were inoculated with formalized saline with each group of test animals to check for the presence of latent respiratory agents.

Isolation Attempts

Attempts to isolate Eaton agent were made with two sputum specimens from cases of clinically diagnosed primary atypical pneumonia from 1956 and 1958. The specimens had been stored without antibiotics at -70° C. Eight throat washings from patients with clinically diagnosed viral pneumonia in 1959 who had significant fluorescent antibody rises to the Eaton agent were also examined for the presence of the agent.

The sputums and throat washings were ground in T.S.B. with 2,000 units of penicillin/ml. and inoculated amniotically into six 13-day-old chick embryos, intranasally into four DBA/2JN mice and two cotton-rats, and into tissue cultures of chick-embryo mince, chick-embryo lung, hamster kidney, monkey kidney, chicken kidney, kitten lung, human kidney, and HEP-2 cells. The lungs and trachea of the chick embryos were harvested six days after inoculation and carried through two additional amniotic passages in chick embryos. These embryos were then tested for the presence of the Eaton agent by the indirect fluorescent antibody test, using a 1/20 dilution of a known positive serum which gave definite staining at a 1/320 dilution. The mice and rats were sacrificed 12 to 13 days after inoculation and the lungs were immediately scored for the presence of pneumonia and/or consolidation. The tissue cultures were maintained for 10 to 20 days, and observed for cytopathogenic effect, for haemadsorption by the technique of Vogel and Shelokov (1957), and, when feasible, for fluorescence, using the indirect technique.

RESULTS

Cold-agglutinin-positive or Streptococcus-MG-positive Viral Pneumonia in Adults

A retrospective study was undertaken to correlate the occurrence of cold agglutinins and/or *Streptococcus MG* antibody rises and antibody rises to the Eaton agent in patients with a clinical diagnosis of primary atypical pneumonia. In this survey sera from pneumonia patients in many different geographical areas, both in the United States and in Europe, were tested for evidence of infection with the Eaton agent. Many of the sera were obtained from the Walter Reed Army Institute of Research serum files. The sera from 1947 were kindly supplied by Drs. Floyd Denny and John Dingle. Tables I and II give the results of these tests. Twenty-two patients had antibody rises to the Eaton agent, two had antibody but failed to show a fourfold rise in the fluorescent antibody test, while two were negative for the Eaton agent. These sera were drawn in nine separate years, and antibody rises were demonstrable in every year except 1948, where only one serum pair was available. Eleven of the atypical pneumonia patients showed a rise from <10 to $320+$. Six patients showed lower levels of antibody development, while five who possessed antibody in the acute-phase serum had fourfold or greater fluorescent antibody rises. The acute serum specimen of one of the patients who possessed antibody but who did not develop a fourfold rise in antibody was obtained on the 10th day of disease.

Table II shows the correlation of cold agglutinins, *Streptococcus MG* agglutinins, and the Eaton agent antibody rises in clinically diagnosed primary atypical pneumonia in adults. Approximately 85% of the individuals with rises of cold agglutinins and/or *Streptococcus MG* agglutinins developed a rise of

TABLE I.—Development of Antibody to Eaton Agent in Cold-agglutinin and/or Str.-MG-Agglutinin-positive Atypical Pneumonia in Different Localities and in Different Years

Date	Location	Age and Sex	Day Disease	Reciprocal of Antibody Titre		
				Cold Aggl.	Str. MG	Eaton Staining Antibody
1947 Oct.	Cleveland, Ohio	32 M	{ 6 13	<4 256	<10 20	160 2,560
Nov.	" "	23 F ^A	{ 7 13	4 256	<10 10	<10 320
Nov.	" "	45 F	{ 6 13	<4 512	<10 10	<10 640
Nov.	" "	27 M	{ 7 14	<4 1,024	<10 80	<10 640
Dec.	" "	41 F	{ 8 15	<4 512	<20 20	<10 640
1948 Dec.	New Jersey	17 M	{ 14 23	<2 32	—	<10 10
1953 July	Washington (State)	— M	{ 3 21	<2 128	16 32	<10 80
Nov.	District of Columbia	43 M	{ 3 63	<2 2	<8 16	<10 80
1954 May	" "	30 F	{ 4 23 33	<2 2 —	<8 32 —	<10 40 —
Nov.	" "	24 M	{ 6 23 41	<2 8 2	<8 16 8	<10 — 320
1955 July	" "	36 M	{ 4 24 47	<2 64 —	<8 64 —	<10 — 160
Sept.	" "	22 M	{ 10 25	<2 4	<8 64	<10 320
Oct.	New York	23 M	{ 5 12	32 32	16 64	<10 320
Oct.	Texas	29 M	{ 4 17	<2 64	<8 8	40 160
Mar.	Newfoundland	24 M	{ 3 13 30	<2 2 4	<8 8 8	<10 160 —
1956 Jan.	District of Columbia	23 M	{ 2 30	<2 2	<8 32	<10 320
Aug.	Italy	26 M	{ 9 24	<2 64	<8 256	<10 640
Oct.	District of Columbia	— F	{ 9 23	<2 64	<8 128	<10 320
1957 Nov.	Oklahoma	22 M	{ 10 16 40	<2 256 4	<8 16 16	80 — 160
Dec.	" "	22 M	{ 6 11 25	<2 2 32	<8 8 16	10 80 —
1958 Jan.	District of Columbia	24 F	{ 7 14	<2 8	<8 64	10 320
Jan.	Kentucky	23 M	{ 5 30	<2 8	<8 8	<10 20
July	New York	31 M	{ 9 36	<2 2	<8 32	<10 10
Aug.	Virginia	33 M	{ 6 33	<2 256	<8 8	40 80
1959 Jan.	South Carolina	18 M	{ 3 16	<2 8	<8 8	<10 320+
Feb.	" "	17 M	{ 4 16	<2 64	<8 8	40 320+

TABLE II.—Correlation of Rises in Cold-agglutinin, Str. MG, and the Eaton Agent Antibody in Primary Atypical Pneumonia

Presence of Indicated Antibody		No.	No. with Eaton Antibody
Cold Agglutinins	Str.-MG-Antibodies		
+	+	14	13
—	+	5	4
+	—	7	5

fluorescent stainable antibody for the Eaton agent. A single outbreak or epidemic in which several agents are widely disseminated can often lead to a spurious association of one of the agents with the illness in question. Broader experience, however, with numerous outbreaks or epidemics will often establish the identity of the agent responsible for this illness. Therefore it is important to note that the high level of association of cold agglutinins and/or *Streptococcus MG* antibodies with fluorescent antibody rises to the Eaton agent was maintained over a 12-year period and was observed for patients studied in many different geographical locations, thus minimizing the possibility of such dual infections. These findings confirm and extend the observations reported by Liu *et al.* (1959). When the sera in Table I were tested in complement-fixation tests for antibody rises to influenza A, B, or C, mumps, para-influenza 1 (HA-2), para-influenza 2 (CA), para-influenza 3 (HA-1), adenovirus, L.G.V., and Q fever no serological evidence of infection with these agents was found.

Aetiologically Undiagnosed Pneumonia in the Paediatric Age-group

Previous studies utilizing the cold-agglutinin technique indicated that an appreciable proportion of children developed such antibodies during convalescence from primary atypical pneumonia (Garrow, 1958). It was of interest to determine if the Eaton agent appeared to play a part in viral pneumonia in the paediatric age-group. One hundred and ten children with infection of the lower respiratory tract were studied as part of a continuing collaborative study with the Virus Laboratory of Children's Hospital, Washington, D.C., designed to determine the role of viral agents in childhood respiratory disease. Adenoviruses or myxoviruses were not recovered by standard isolation procedures, and these children failed to develop complement-fixation antibodies for influenza A, B, or C, para-influenza 2 (CA), para-influenza 1 (HA-2), para-influenza 3 (HA-1), respiratory syncytial virus, or adenovirus antigens.

Eighteen (16%) showed at least a fourfold rise to the Eaton agent by the indirect fluorescent antibody test. Eight of these serum pairs which showed high-level rises to the Eaton agent were also tested in complement-fixation tests with L.G.V. and Q fever antigens with negative results. Only one (2.4%) of 42 children whose respiratory illness was associated with para-influenza 1 (HA-2), para-influenza 2 (CA), para-influenza 3 (HA-1), Asian influenza A, or adenovirus, by virus isolation or serology, showed an antibody rise to the Eaton agent. In a group of 64 children with no respiratory illness no fourfold rises to the Eaton agent were noted. The study extended for 16 months, from October, 1957, to January, 1959. The second paper of this series, Chanock *et al.* (1960), will present the detailed serological results, epidemiological data, and clinical information on these children.

Cold-agglutinin- and *Streptococcus-MG*-negative Viral Pneumonia in Adults

Liu *et al.* (1959), employing cross-adsorption techniques, failed to demonstrate a relationship between fluorescent stainable antibody for the Eaton agent and cold agglutinins or *Streptococcus MG* agglutinins. Furthermore, the occurrence of fluorescent antibody rises to the Eaton agent in pneumonia patients who were negative or failed to show fourfold antibody rises

in cold agglutinins or *Streptococcus MG* antibodies has been noted (Liu *et al.*, 1959; Johnson *et al.*, 1960). It was of interest, therefore, to determine how frequently antibody rises to the Eaton agent developed in a series of 69 cold agglutinin- and *Streptococcus - MG* - negative adult patients with pneumonia. Sera from these patients were submitted to the Walter Reed Army Institute for Medical Research over a period of eight years, from 1952 to 1959. Haemagglutination inhibition or complement-fixation tests were negative for Q fever, psittacosis, adenovirus, and the prevalent strains of influenza A and B. Eighteen (26%) developed fourfold or greater rises to the Eaton

TABLE III.—Pattern of Fluorescent Antibody Rise for the Eaton Agent in Cold-agglutinin Negative, Str.-MG-Negative Pneumonia

No. with Indicated Pattern	Pattern of Fluorescent Antibody Development Acute Serum Titre → Convalescent Serum Titre
4	<10 → 10
2	<10 → 40-80
12	<10 → 320+
3	10 → 40-80
1	40 → 320+
Total 22	

agent. An additional four individuals showed antibody patterns of <1:10 to 1:10. The patterns of antibody responses in this series are shown in Table III. It will be seen that the level of antibody developed is not unlike that observed in the cold agglutinin- and *Streptococcus-MG*-positive group. It is apparent from the results of this study that antibody rises to the Eaton agent are not uncommon among patients with pneumonia who fail to develop cold agglutinins or *Streptococcus MG* agglutinins.

Isolation of Strains of Eaton Agent

Material for isolation attempts was available from one patient who developed a rise in cold agglutinins and from nine patients who did not develop cold agglutinins or *Streptococcus MG* agglutinins. Both new strains of the Eaton agent were isolated from sputum specimens by three amniotic passages in 13-day-old chick embryos. No isolates were obtained from the eight throat-washes examined. The presence of the agent was detected by the use of the indirect fluorescent antibody test using a known positive serum as an indicator of infection. One of the strains (R.J.) from a cold-agglutinin-negative viral pneumonia was also isolated in DBA/2JN mice. Pulmonary consolidation was noted in three-quarters of the animals inoculated. Identification of the mouse isolate was accomplished by passage of the mouse lungs into chick embryos which contained specific areas of fluorescence when tested by the indirect fluorescent antibody technique. The strain from the cold-agglutinin-positive case (L.P.) was not recovered from mice. It was not possible to recover an agent from the lungs of cotton-rats inoculated with the two positive sputum specimens. No evidence of consolidation or pneumonia was seen in these animals, and passages of the lungs into chick embryos failed to result in the recovery of the R.J. strain, the L.P. strain, or any additional strains.

Differences in the ability of strains to propagate in mice and cotton-rats may represent important biological properties or they may be an expression of the number of infectious particles present in the isolation specimens. After three or more passages in chick embryos both strains were capable of producing pneumonia in cotton-rats and mice.

Heretofore all strains of the Eaton agent have been shown to be antigenically indistinguishable (Liu *et al.*, 1959). It was important to determine if the newly isolated strains were also antigenically similar to the strains isolated by Eaton and Liu. Table IV presents the results of reciprocal fluorescent antibody tests in which serial sections of chick-embryo lungs infected with the F.H. strain, the L.P. strain, or the R.J. strain were used to titrate acute and convalescent serum specimens

TABLE IV.—*Antigenic Relationship of Three Strains of the Eaton Agent by Indirect Fluorescent Antibody Technique*

Post-infection Human Serum*	Reciprocal of Fluorescent Antibody Titre with Indicated Strain of the Eaton Agent†		
	F.H.	L.P.	R.J.
Wm.‡	320	320	320
L.P.	640	640	640
R.J.	1,280	640	1,280

* Acute-phase serum gave negative (<1:10) results when tested with all three strains of the Eaton agent.

† Individual strains of the Eaton agent were inoculated into 13-day chick embryos and harvested on day 19. Two to three areas of 4+ fluorescence were visible in the bronchial epithelium when tested with a 1:40 dilution of Wm. serum in guinea-pig complement.

‡ Human serum used to standardize test throughout entire study period.

from patients L.P. and R.J. and the serum used to control the staining reactions with the F.H. strain. The new strains appear to be antigenically similar to each other and to the older F.H. strain. Since the 12 strains of the Eaton agent isolated from 1943 to 1958 were similar by the fluorescent antibody technique, it would appear that a major antigenic variation has not occurred during this time interval.

Attempts to establish the F.H. or the Mac strain in tissue culture have failed to date in every instance. Tissue cultures of chick-embryo mince, chick-embryo lung, chick kidney, hamster kidney, monkey kidney, human kidney, kitten lung, and HEP-2 cells were employed with many combinations of media pH, incubation periods, and serial passage. It was hoped that newly isolated strains which have not been passed extensively in eggs or animals might propagate in tissue culture. Unfortunately this was not the case. No evidence of the Eaton agent could be demonstrated in any of the above tissue-culture systems inoculated with the sputum specimens which yielded positive results in embryonated eggs (R.J. and L.P. strains). Evidence of a specific Eaton agent effect was sought by observation of the tissue cultures for cytopathic effects, haemadsorption, and the indirect fluorescent antibody technique.

Specificity of the Fluorescent Antibody Test for Eaton Agent

Further evidence for the specificity of the fluorescent antibody test for the Eaton agent in human respiratory disease was sought when sera from adults infected with para-influenza 1 (HA-2) (Reichelderfer *et al.*, 1958), para-influenza 3 (HA-1) (Kapikian *et al.*, unpublished data), Asian influenza A (Jap-305-57) (Bell *et al.*, 1957), or adenovirus (Ward *et al.*, 1955; Bell *et al.*, 1956) from human volunteer studies were examined for antibody rises to the Eaton agent. All of these individuals developed complement-fixation antibody titres of 1:64 or greater to the virus inoculated, and at least a fourfold increase in specific antibody titre. Table V summarizes the results of the fluorescent antibody tests. Of the 13 individuals studied, 38% had antibody for the Eaton agent but no rises were observed.

TABLE V.—*Lack of Fluorescent Antibody Rise to Eaton Agent in Adults Infected with Para-influenza 3 (HA-1), Para-influenza 1 (HA-2), Asian Influenza A, or Adenovirus*

Virus Infection	No. Tested	No. with Antibody Rise	No. with Antibody but No Rise
Para-influenza 3 (HA-1) ..	4	0	2
1 (HA-2) ..	5	0	2
Asian influenza A	2	0	1
Adenovirus	2	0	0
Total ..	13	0	5 (38%)

Fourteen serum pairs from cases of Q fever and six serum pairs from psittacosis patients (three of these serum pairs were received from Dr. K. F. Meyer) also failed to show antibody rises to the Eaton agent in the indirect fluorescent antibody test.

Prevalence of Fluorescent Antibodies to Eaton Agent in a General Population Group

Studies currently in progress designed to determine the role of the Eaton agent in respiratory disease in military recruits stimulated a serological survey to determine the frequency of Eaton antibody in various age-groups. This information is of importance in establishing a general baseline for comparative studies involving other populations and other time intervals. Serum specimens obtained in 1957 from a group of persons in the upper middle-income bracket living in Montgomery County, Maryland, were tested at a 1/10 dilution for the presence of Eaton antibody. Table VI presents the results of this

TABLE VI.—*Frequency of Fluorescent Antibody to Eaton Agent in Persons in Upper Middle Income Brackets in Montgomery County, Maryland, 1957*

Age in Years	No. of Persons Tested	No. with Eaton Agent Antibody	Percentage Positive
0-2	20	0	0
3-10	20	3	15
11-19	20	3	15
20-24	5	2	40
25-29	15	6	40
30-39	20	4	20
40-49	20	4	20
50-59	17	3	18
60-69	15	0	0

survey. By 3 years of age 15% of the individuals tested were positive for Eaton antibody and the percentage of individuals with antibody remained at least at this level until the age of 59. The greatest prevalence of antibody to the Eaton agent occurred in the 20-29-year-old age-group, but the differences in different age-groups are not significant. Liu *et al.* (1959) have shown that antibody levels for the Eaton agent decrease rapidly after three to four months, but no information is available on the time interval required for conversion to a negative antibody status. Antibody to the Eaton agent has been shown to be present, however, for a year or longer after the illness (Liu *et al.*, 1959; Johnson *et al.*, 1960). For this reason it is not possible to interpret the data in terms of age-specific infection rates. The data are consistent with Eaton's (1950) observation that primary atypical pneumonia may affect any age-group but that there is some evidence for a diminished susceptibility of those over 30 years of age.

Seasonal Incidence of Infection with Eaton Agent

Some respiratory agents (influenza A, B) have a seasonal incidence, show periodic antigenic shifts, and occur in epidemic spurts, while others—para-influenza 1

(HA-2) and para-influenza 3 (HA-1)—have been shown to occur in every month of the year and have not appeared to exhibit major antigenic variations (Chanock, unpublished data). The Eaton agent appears to be similar in these respects to the latter group. In Table VII, where the antibody rises to the Eaton agent have been grouped by months, it can be seen that they could be demonstrated in every month of the year with the exception of April, when only one specimen was tested.

TABLE VII.—Seasonal Incidence of Fluorescent Antibody Rises to the Eaton Agent

Status		Month												Total All Mths.
		J	F	M	A	M	J	J	A	S	O	N	D	
Cold-agglutinin and/or Str.-MG-positive	No. tested	4	1	1	0	1	0	3	2	1	4	6	3	26
	No. positive	4	1	1	0	1	0	3	2	1	4	6	3	26
Cold-agglutinin and Str.-MG-negative	No. tested	10	14	4	1	2	7	5	4	6	5	8	3	69
	No. positive	5	5	0	0	1	3	1	1	3	1	1	1	22
Total	No. tested	14	15	5	1	3	7	8	6	7	9	14	6	95
	No. positive	9	6	1	0	2	3	4	3	4	5	7	4	48

DISCUSSION

A serological study of adult patients with atypical pneumonia who developed cold agglutinins and/or *Streptococcus MG* agglutinins indicated that the development of antibody to the Eaton agent was a fairly consistent finding. Of 26 such patients, 22 developed antibody for the Eaton agent demonstrable by the indirect fluorescent antibody technique. Such positive serological responses to the Eaton agent were observed for patients whose illness occurred during various years from 1947 to 1959 and in various geographical areas. These findings minimize the possibility of a spurious association of the syndrome of cold agglutinin- and *Streptococcus-MG*-positive atypical pneumonia with an antibody rise for the Eaton agent. Positive serological responses to the Eaton agent, however, were not limited to adult pneumonia patients who developed cold agglutinins and/or *Streptococcus MG* agglutinins. Twenty-six per cent of the pneumonia patients studied from 1952 to 1959 who failed to develop cold agglutinins or *Streptococcus MG* agglutinins and whose illness could not be attributed to influenza A or B, adenovirus, psittacosis, or Q fever also developed a serological response for the Eaton agent.

Additional evidence associating infection leading to the development of Eaton antibody and respiratory illness was obtained in a controlled study of aetiological undiagnosed lower respiratory disease in infants and children. Antibody rises to the Eaton agent were observed almost exclusively in the patients whose illness was aetiological undiagnosed, rarely among patients whose respiratory disease was associated with myxovirus or adenovirus infection, and not at all among infants or children without respiratory illness. The detailed findings and a discussion of these results will be presented in the second paper of this series.

Present evidence indicates that the antibody to the Eaton agent as measured by the fluorescent antibody technique is a specific one. Thus, adults infected with para-influenza 3, influenza A. or adenovirus who developed high levels of homologous antibody failed to develop antibody to the Eaton agent. Similarly, persons infected with psittacosis virus or *Coxiella burnetii* failed to develop antibody for the Eaton agent, confirming the

findings of Eaton (1950) and Liu (1957). In addition, adult pneumonia patients who developed antibody for the Eaton agent failed to develop antibody for influenza, para-influenza, adenovirus, psittacosis, or *Coxiella burnetii*.

Agents immunologically indistinguishable from the Eaton agent were recovered in embryonated eggs from 2 of 10 patients with pneumonia who developed antibody to this agent. This observation confirms the findings of Eaton and of Liu, and suggests that infection with this agent occurred concurrently with pneumonia. This interpretation is also at variance with the suggestion that the development of antibody to the Eaton agent represents a non-specific response (Dingle and Jordan, 1959). The total number of isolations of the Eaton agent is sufficiently limited, however, so that acceptance of this interpretation must await more extensive experience with specimens obtained from carefully controlled studies involving persons with and without respiratory illness.

Liu demonstrated that the nine strains he recovered from patients with primary atypical pneumonia were antigenically indistinguishable from each other and from the prototype strain of Eaton. The two isolates recovered in this investigation were indistinguishable from one of Liu's strains. The failure to detect antigenic variation among strains of the Eaton agent recovered from 1943 to 1958 is consistent with the sporadic occurrence of cold-agglutinin positive pneumonia in the civilian population and the persistent rather than epidemic occurrence of primary atypical pneumonia positives in a military population now under surveillance (Chanock *et al.*, unpublished data).

SUMMARY

Considerable serological evidence for the association of the Eaton agent and viral pneumonia with or without cold agglutinins or *Streptococcus MG* antibody rises was obtained by use of the indirect fluorescent antibody test. In two instances serological rises to the agent were substantiated by isolation of a strain of the Eaton agent from the sputum of the patient. Antigenic variation and non-specific reactions could not be demonstrated. Prevalence of fluorescent antibody to the Eaton agent in a general population group suggests that it is a widespread and common infection which may occur in all age-groups. Infection with the agent may occur in any month of the year.

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TRANSIENT AMINO-ACIDURIA IN SEVERE POTASSIUM DEPLETION

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Severe potassium depletion can produce gross clinical disturbance with weakness, paralysis, and mental confusion. The condition can arise in primary renal disease (Schwartz, 1955) and also from extrarenal causes (Black, 1957). Confusion arises because potassium loss from extrarenal causes can result in renal dysfunction with albuminuria and impaired ability to concentrate or excrete an alkaline urine (Relman and Schwartz, 1956).

Urinary amino-acid loss has been described in the nephrotic syndrome (Blainey, 1954; Tegelaers and Tiddens, 1955; Stanbury and Macaulay, 1957), and in the tubular nephropathies (Milne, Stanbury, and Thomson, 1952; Bickel, Smallwood, Smellie, and Hickmans, 1953).

The case reported here showed, in association with potassium depletion, gross amino-aciduria which disappeared on correction of the potassium deficit.

Case Report

A 72-year-old woman was admitted to hospital as an emergency with the sudden onset of confusion and weakness

12 hours previously. A history was obtained from her relatives of cough, sputum, and breathlessness for 12 years, with intermittent ankle oedema for four weeks. Albuminuria had been noted in hospital seven years previously (acute bronchitis), but not one year previously (pneumonia). The patient had been taking digitalis since 1951 and had had 5 g. of chlorothiazide over the five days prior to admission. Diet was adequate and included two cooked meals with first-class protein and green vegetables daily. She had had no diarrhoea nor did she use purgatives.

On examination she was confused and drowsy. The mouth temperature was normal, the respiratory rate was 18 a minute, and she had a regular pulse rate of 66 a minute, with occasional extrasystoles. The arterial blood-pressure was 170/90 mm. Hg, and her heart was slightly enlarged, with, on auscultation, an apical grade II systolic murmur which had been noted in 1951. A few scattered rales were heard at the lung bases and there was slight pitting oedema of the ankles. Apart from her abnormal mental state, clinical examination of the central nervous system showed nothing abnormal, and no abnormality was found in any other system.

Extensive routine haematological and biochemical investigation showed no abnormalities except in the levels of serum electrolytes, which were Na, 105 mEq/l.; K, 3.8 mEq/l.; and Cl, 73 mEq/l. There was no respiratory acidosis present as judged by arterial CO₂ content and pH, and liver-function tests and oral glucose tolerance were normal.

Chest radiography suggested that the lungs were emphysematous and that the left ventricle was enlarged. The first electrocardiogram showed prolongation of the Q-T interval and prominent U waves. The urine showed no albumin or sugar and no formed elements at any time, but the specific gravity was fixed between 1009 and 1011 for at least the first 17 days after admission. The main abnormalities are recorded in Table I.

TABLE I.—Findings During Potassium Depletion and After its Repletion

Features	During Potassium Depletion	After Treatment ¹	Comments
Serum Na (mEq/l.) ..	105	130	Progressive shortening of Q-T c. and disappearance of U waves. Lead V ³ used throughout R.B.C. chloride and sodium both fell with treatment
" K " ..	3.8	4.2	
" Cl " ..	73	100	
E.C.G.: Q-T c. sec. ..	0.47	0.41	
U waves ..	Prominent	Absent	High intracellular chloride taken to indicate acidosis ⁴
R.B.C. potassium mEq/l. R.B.C. ²	78 (Day 1)	99 (Day 35)	
Muscle intracellular potassium ³ mEq/l. intracellular water	155.6 (Day 1)	175.8 (Day 37)	
Muscle intracellular potassium ⁴ mEq/kg. dry weight	324.0 (Day 1)	433.0 (Day 37)	
Muscle intracellular acidosis	Present	Absent	See Table II
Urinary excretion of potassium	< Intake	≈ Intake (Day 35)	
Amino-aciduria ..	Significant (Day 11)	Normal (Days 32, 33, and 85)	
Maximum specific gravity of urine	1.010	1.016	
Albuminuria ..	Nil	Nil	Over a four-hour period
Creatinine clearance, ml./min.	75 (Day 10)	53	
Clinical condition ..	Confused, weak, paranoid	Mentally clear and co-operative; no weakness	

¹ Day 43 unless otherwise stated.

² Mean normal value = 98.2 mEq/l. R.B.C. (Flear, Hughes, and Quinton, 1958).

³ Mean normal value for intracellular potassium = 154.4 mEq/l. intracellular water (Crampton, Flear, and Matthews, 1960).

⁴ Mean normal value = 329.7 mEq/kg. dry weight (Crampton *et al.*, 1960).

⁵ Inulin space taken as measure of extracellular space (Crampton and Flear, 1958).