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Growth of Laboratory and Naturally Occurring Strains of Eaton Agent in Monkey Kidney Tissue Culture. (26114)

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There has been renewed interest in the agent recovered in the embryonated egg from patients with atypical pneumonia by Eaton *et al.*,(1). Until recently considerable controversy has surrounded the role of this agent in respiratory illness. Studies carried out in the past few years, however, have provided evidence that Eaton agent is associated with human respiratory disease. Thus, 80 to 95% of patients with cold agglutinin positive atypical pneumonia were found to develop fluorescent stainable antibody for the agent (2,3). In addition, a controlled epidemiologic study in military recruits indicated that Eaton infection was associated with febrile upper respiratory illness as well as atypical pneumonia(4). Approximately 10% of children hospitalized with lower respiratory tract illness in Washington, D.C. developed a serologic response to this agent(5).

‡ The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

Early studies were hindered by the difficulty of working with an agent whose only index of activity was the production of lung lesions in a portion of, but not all, inoculated hamsters or cotton rats(1,6,7). Application of the fluorescent antibody technic resulted in a more quantitative assay system and permitted demonstration of specific antigen in the bronchial epithelium of the experimentally infected chick embryo(8). Employing this technic it was possible to recover a limited number of strains from patients with pneumonia by serial amniotic egg passage(3, 8,9). This procedure, however, is difficult, involved, and clearly limits the quantity of epidemiologic information which can be obtained. Simpler laboratory technics are needed to facilitate the recovery and identification of the agent before extensive field studies necessary to an understanding of the natural history of infection are possible.

This report will describe preliminary studies which indicate that Eaton agent propagates in several different types of tissue

culture. In addition, it will be shown that naturally occurring strains of the agent can be recovered in monkey kidney tissue culture.

Materials and methods. Eaton agent. The egg adapted laboratory strain designated FH was kindly supplied by Dr. C. Liu(8). This strain was passaged 4 times in yolk sac entodermal cultures at 7 to 9-day intervals in this laboratory, as described in a companion communication(10). A pool of the 5th, 7th, and 9th day harvests of the 4th passage constituted the inoculum used to initiate passage series in other culture systems.

Fluorescent antibody technic. This technic has been described previously(5). Chick embryo lung sections or tissue culture cover slips were routinely fixed in acetone at room temperature for 10 minutes. Human serum containing antibody to the Eaton agent was then placed on the slide and allowed to incubate at 37°C for 30 minutes. The slides were thoroughly washed in 3 changes of buffered saline (pH 7.2) following which goat anti-human globulin conjugated with fluorescein was then added. After 30 minutes at 37°C and 3 washes in buffered saline the tissue sections or cover slips were covered with buffered glycerol (pH 7.0) and viewed by UV microscopy.

Tissue culture. Rhesus monkey kidney and HEP-2 roller tube cultures, obtained from Microbiological Associates, were washed 5 times with Hanks' solution to remove antibiotics present in the growth medium. The cultures were maintained with Eagle's medium (containing double the usual quantity of amino acids and vitamins) and 5% inactivated chicken serum. Penicillin at a concentration of 2000 units per ml was the only antibiotic added to this fluid medium. Chick kidney cultures were prepared as described by Maassab and maintained with Eagle's medium and 5% chicken serum(11).

Titration. Tissue culture fluid or cell suspension was diluted in 4-fold steps in Eagle's medium with 5% chicken serum. One-tenth ml was then inoculated amniotically into each of ten 13-day-old embryonated eggs. After 6 days incubation at 35°C the surviving embryos were harvested and the thorax quick frozen in a CO_2 alcohol bath. Lung sections were prepared in the frozen state and then tested with a 1:20 dilution of a potent atypical pneumonia convalescent serum (titer of staining antibody = 1:320) for presence of specific antigen in the bronchial epithelium as described above. A minimum of 5 eggs were examined before a dilution of material was considered noninfective.

Collection of specimens. Throat swabs from patients with pneumonia were immersed in trypticase soy broth with 0.5% bovine albumin without antibiotics. The fluid was then quick frozen in a glass-sealed ampule and stored at −60° for 3 to 5 months.

Isolation attempts in eggs. Each of ten 13-day-old embryonated eggs was inoculated amniotically with 0.2 ml of throat swab fluid. After 6 days incubation at 35°C the lungs and trachea were removed from surviving embryos and ground in trypticase soy broth with 0.5% bovine albumin—2 ml per embryo. This suspension was then inoculated amniotically into ten 13-day eggs. After 3 such passages the lungs were quick frozen and examined for presence of specific antigen by the fluorescent antibody technic.

Isolation attempts in tissue culture. Six monkey kidney cultures were inoculated with 0.2 ml of throat swab fluid at the same time eggs were injected. Cultures were maintained at 35°C. Culture fluid was removed on the 7th and 11th days and stored at −60°C; fresh culture fluid was then added to the tubes. On the 15th day the monkey kidney cells and fluid were combined with the stored fluids and 0.2 ml of the mixture was inoculated into each of 10 eggs. After 6 days incubation, the lungs of the surviving eggs were harvested and tested for presence of specific antigen as described previously.

Results. Chick kidney cultures. Chick kidney cultures were 7 days old when inoculated with the 4th yolk sac culture passage of the FH strain. Fluid harvested on the 31st day of the 1st chick kidney passage contained Eaton agent, as shown by sub-inoculation into 13-day eggs. This represented a $10^{7.0}$ dilution of the yolk sac culture inoculum when fluid changes over the 31-day period were considered. Twelfth day fluid from the 1st passage was used to initiate a 2nd

TABLE I. Multiplication of Eaton Agent in Monkey Kidney Tissue Culture.

Time after inoculation	Reciprocal of egg infectious doses/0.1 ml of fluid or cell suspension at indicated time		
	Exp. 1	Exp. 2	
	Inoculum = 16 egg doses	Inoculum = 1000 egg doses	
	Culture fluid	Culture fluid	Culture cells*
8 hr†	0‡	0	0
1 day	0	0	0
3 days	0	4	4
5 "	4	16	
7 "	4	256	
10 "	256 or >	1024	
15 "	256 or >	16384	16384

* Monkey kidney cultures were washed 3 times, the cells suspended in the same quantity of culture fluid used for maintenance—1.5 ml—and cell suspension frozen and thawed twice.

† Cultures were washed 3 times with 1.5 ml of Hanks' solution 6 hr after inoculation following which 1.5 ml of maintenance medium was added to each tube.

‡ A minimum of 5 inoculated eggs was tested before a specimen was considered negative.

passage in chick kidney culture. Fluid harvested on the 7th day of the 2nd passage contained $10^{1.8}$ egg infectious doses of the agent. This represented a $10^{-7.6}$ dilution of the yolk sac culture inoculum. These findings suggest that the agent replicated in this culture system.

Specific antigen, however, was not visualized when infected cover slip preparations were tested by the fluorescent antibody technic. These tests were performed with a 1:10 dilution of an atypical pnumonia convalescent serum which had a staining titer of 1:320 to 1:640 with infected chick embryo lung sections. No cytopathic effects were observed.

HEP-2 culture. The 4th yolk sac culture fluid was inoculated into HEP-2 cultures and the agent was recovered after 31 days at 35°C, representing a $10^{-8.4}$ dilution of the inoculum. A 2nd successful passage was carried out in HEP-2 cells. No cytopathic effects were observed.

Monkey kidney. A passage series in rhesus monkey kidney culture was initiated with 4th yolk sac passage material. At the end of 3 serial transfers at 7 to 10 day intervals, culture fluid contained $10^{4.2}$ egg infectious doses, representing a $10^{-14.2}$ dilution of the yolk sac culture inoculum. This indicated that Eaton agent replicated in monkey kidney tissue culture.

Growth of the agent in monkey kidney was then studied in a more detailed manner since the level of infectivity attained in this system was higher than that observed in yolk sac, chick kidney, or HEP-2 cultures. The results of 2 growth curve experiments are shown in Table I. In these experiments the cultures were washed 6 hours after inoculation to reduce the quantity of unadsorbed infectious material. Employing this procedure it was possible to demonstrate an eclipse phase which lasted 1 to 3 days, depending on size of the inoculum. A high level of infectivity did not develop until the 10th to 15th day.

Tissue culture cells were thoroughly washed, then frozen and thawed twice in Exp. 2 to assay infectious material associated with the cell. The data suggest that Eaton agent was not retained in association with the cell for a long period since early in infection and later at time of peak production the quantity of infectious material associated with cells was the same as that found in the fluid medium.

Although Eaton agent multiplied to a level of $10^{4.2}$ egg infectious doses in monkey kidney cells, specific antigen was not visualized by means of indirect immunofluorescence employing a potent human serum at a 1:10 dilution. Infected tissue culture cover slips also were fixed by other methods (acetone at −60°C for 2 hours, ethanol at room temperature, and ethanol at −60°C for 2 hours) which permitted staining of chick embryo lung antigen but specific fluorescence was not observed. Cytopathic effects were not seen in infected cultures. Elementary bodies or inclusions were not seen when infected cover slips were stained with Macchiavello or Giemsa stain. (The tests were performed through the courtesy of Miss E. B. Jackson).

Recovery of the agent in monkey kidney culture. An opportunity to test the efficacy of monkey kidney cultures for recovery of naturally occurring strains of Eaton agent

was afforded during an epidemiologic study of infection among marine recruits(4). Throat swab fluids from individuals with pneumonia who developed fluorescent stainable antibody during convalescence were inoculated into monkey kidney culture. Recovery of an agent in culture was determined by sub-inoculation of the combined 7, 10, and 15-day fluids into eggs which were tested for antigen by the fluorescence technic. Fourteen strains were recovered in culture from 17 patients with pneumonia. One tissue culture isolate was passaged twice more in monkey kidney cells and on the 13th day of the 3rd passage the fluid medium contained $10^{3.0}$ egg infectious doses, representing a $10^{10.4}$ dilution of the original throat swab fluid. This suggests that the isolate propagated in monkey kidney cells and that the positive result in eggs did not represent mere persistence of the agent in the culture fluid.

Although throat swab fluids were inoculated simultaneously into tissue culture and eggs only 6 specimens were suitable for comparative study because of the problem of bacterial contamination during amniotic passage. All of the 6 specimens were positive in tissue culture whereas only 5 yielded an isolate when tested in eggs, suggesting that the culture system was as sensitive as the hen's egg to naturally occurring strains of the agent.

Strains. Three of the tissue culture isolates were compared among themselves as well as with the FH strain to determine if antigenic differences existed. Sections of lung from chick embryos infected with the various strains were employed in tests with the acute and convalescent sera of the individuals from whom the isolates originated. The 3 tissue culture isolates were similar and did not differ from the FH strain recovered by Liu (Table II)(8).

Discussion. The results of this study suggest that the Eaton agent multiplies in chick kidney, HEP-2, and monkey kidney tissue cultures. Growth of the agent has also been demonstrated in yolk sac entodermal cultures (10). Of some interest was the demonstration of an eclipse phase during replication in monkey kidney cells. This property suggests that the agent should be classified as a virus according to a recent definition proposed by Burnet(12). Large size (180 to 250 mμ) of the Eaton agent and its sensitivity to the antibiotics streptomycin and aureomycin, however, poses some difficulty in regard to such a classification(6,13,14,15). A similar difficulty exists among the psittacosis-lymphogranuloma agents, a group which is biologically but not antigenically similar to the Eaton agent.

TABLE II. Antigenic Relationship of Strains Recovered in Tissue Culture to F.H. Strain of Eaton Agent.

Convalescent phase serum*	Reciprocal of fluorescent antibody titer with indicated strain			
		Strains recovered in tissue culture		
	F.H.	P.I. 646	P.I. 725	P.I. 898
Parris Island #646	40	40	40	40
" " 725	80	40	40	160
" " 898	160	160	80	160

* Acute phase serum gave negative (<1:10) results when tested with all 4 strains.

The purpose of this investigation was to develop simpler technics for recovery and identification of the agent, to facilitate epidemiologic studies. This aim was only partially realized. It was shown that monkey kidney tissue culture was as sensitive to naturally occurring strains of Eaton agent as was the hen's egg. One egg passage and immunofluorescent examination of chick embryo lung sections were still necessary, however, to determine presence and quantity of infectious material in the culture system. Attempts to visualize specific antigen in infected monkey kidney cells were unsuccessful, presumably because of an insufficient concentration of antigen. Efforts in this direction as well as attempts to obtain a culture system in which cytopathic effects occur are clearly indicated and are continuing.

The 3 isolates from 1959 selected for characterization were found to be antigenically similar and to resemble the FH strain recovered in 1954(8). Previous studies indicated that the FH strain was indistinguishable from strains recovered in 1943, 1944, 1954, 1955, 1956, and 1958(2,3). These findings suggest that the Eaton agent has a stable antigenic configuration.

Summary. An egg adapted strain of Eaton agent multiplied in rhesus monkey kidney tissue culture. Antigen concentration within the cells was insufficient to permit visualization by immunofluorescence. An eclipse phase was demonstrated during replication in monkey kidney cells. In a simultaneous test monkey kidney culture was found to be as sensitive as embryonated eggs for recovery of naturally occurring strains of Eaton agent. Fourteen strains were recovered in tissue culture from 17 patients with pneumonia.

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Growth of the Eaton Agent of Primary Atypical Pneumonia in Chick Entodermal Tissue Culture.* (26115)

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The Eaton agent, associated with primary atypical pneumonia, has had a limited range of known susceptible host cells(1,2). Although pneumonia may appear after intranasal inoculation of hamsters or cotton rats, its irregularity renders precise experiments difficult. Liu(3) developed a dependable, although cumbersome, laboratory method of demonstrating the agent by using its infectivity for bronchial epithelium of the embryo chick in conjunction with indirect fluorescent antibody staining. It is clear that acquisition of additional information about this agent depends to a great extent upon improved methods of study.

The investigation described here was undertaken with the thought that cultures of entodermal cells taken early from chick embryos might prove to be susceptible to this agent since it can infect an entodermal derivative in the older embryo. The methods used followed closely those described for cultivation of rickettsiae and psittacosis group viruses in chick entodermal cell cultures(4,5). The Eaton agent was shown to multiply in such cultures by successfully accomplishing 10 serial passages in one series, and 6 in another.

Materials and methods. The FH strain of Eaton agent, kindly supplied by Dr. Chien

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