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Demonstration of Eaton's Agent in Tissue Culture.* (26732)

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Many problems of studying the Eaton agent of primary atypical pneumonia have related to its limited host range and the lack of convenient methods to indicate its presence in a test system. The indirect fluorescent antibody procedure was adapted to localization of the agent in the chick embryo by Liu(1). More recently Chanock, *et al.* (2) have demonstrated that propagation in tissue culture systems is possible; they were unable to visualize the agent directly, however, and it was necessary to subculture the tissue culture materials in chick embryos with application of Liu's methods to identify the agent. This report concerns successful direct localization of Eaton's agent in tissue culture. The data to follow amplify the observations of Marmion and Goodburn(3) which have suggested a relationship between the agent and pleuropneumonia-like organisms.

Materials and methods. The Mac strain of Eaton's agent, obtained from Dr. Chien Liu and passaged 8 times in chick embryos in this laboratory, was used in these studies. Rhesus monkey kidney cell flask cultures were obtained from Microbiological Associates, removed from the glass with 0.25% trypsin, and resuspended in growth medium (Hanks' BSS 90%, inactivated calf serum 10%, lactalbumin hydrolysate 0.5%, penicillin 1,000 units/ml). Secondary monolayers of these cells were prepared on 6 × 22 mm

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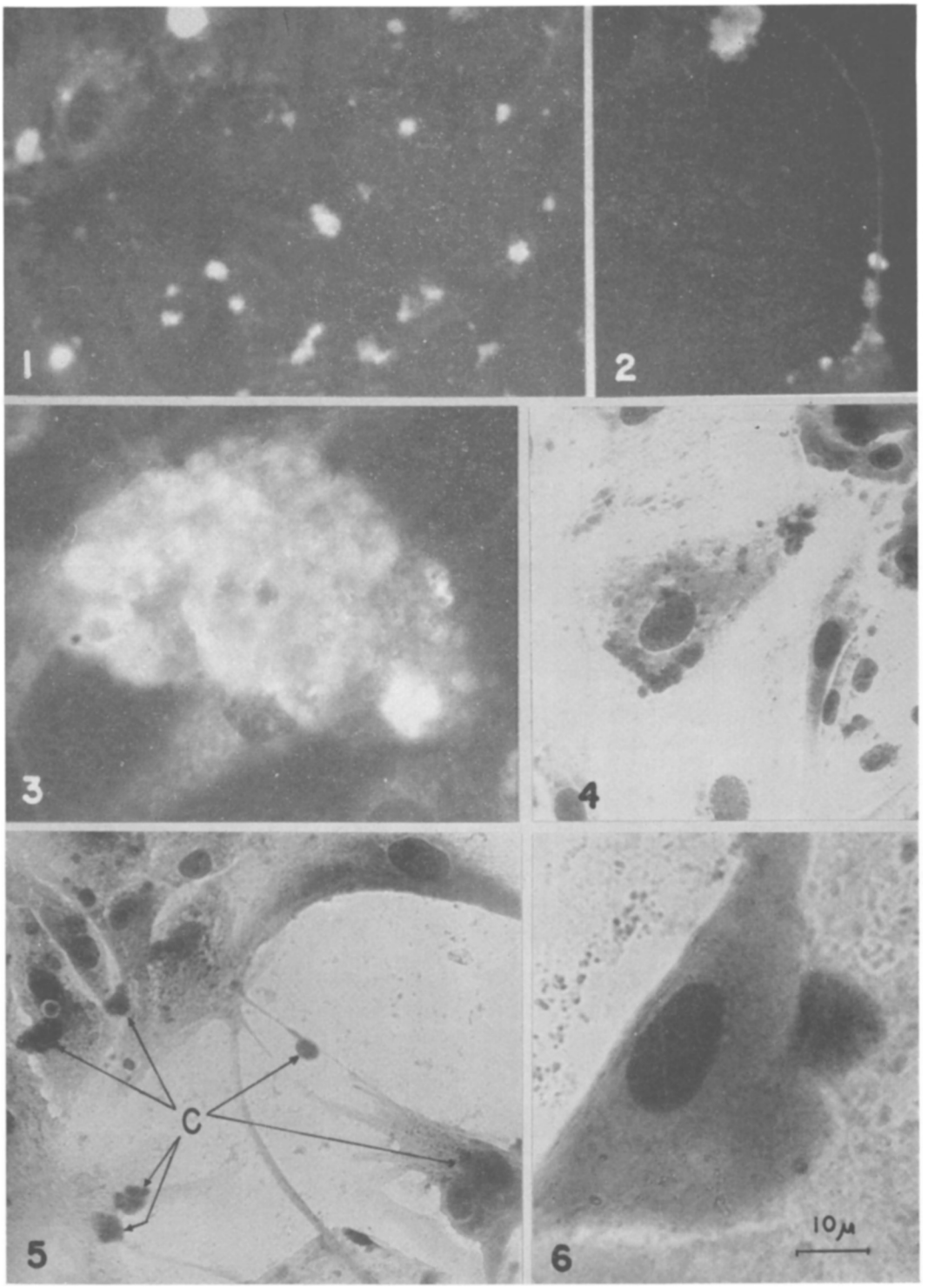

FIG. 1. A secondary monkey kidney cell monolayer 6 days after inoculation with Eaton's agent. Rounded, granular growths are distributed at random over surface of culture. Fluorescent antibody technic. ×430.

coverslips in screw cap tubes, and before inoculation the fluid was changed to a special maintenance medium (Eagle's medium with double amino acids and vitamins 95%, inactivated chicken serum 5%, l-glutamine 0.025%, penicillin 1,000 units/ml, and sufficient $NaHCO_3$ solution to approximate pH 7.4). Infected chick embryo lung suspension was centrifuged 1500 rpm for 10 minutes to clarify, dilutions were prepared in maintenance medium at 4°C, and tubes were inoculated to contain a 10^{-2} final dilution of the stock agent. After various periods of incubation in stationary racks at 32°C the coverslips were harvested, air-dried at room temperature, fixed in acetone for 10 minutes, and stained with either the indirect fluorescent antibody technic as previously described(4) or an intensified Giemsa method(3). Antisera were obtained from a serum collection of primary atypical pneumonia cases occurring in the Cleveland area in 1947-48, or by immunization of rabbits with stock agent as described by Liu(1). Previous titrations using infected chick embryo lung sections (4) revealed that the human convalescent sera contained antibody at dilutions of 1:320-1:640, while the rabbit antiserum had a titer of 5120.

Results. Observations of unfixed cultures. Under the conditions imposed the onset and character of cytopathic effects were variable. The changes consisted most often of the development of plaques of yellowish, granular, refractile cells with ultimate retraction and detachment from the glass. In some experiments these changes could be seen as early as 4 days, while in others they were not apparent until 10 days following inoculation of the cell sheets; at times no specific changes could be seen as late as 14 days, by which time the picture was often confused by the emergence of simian agents. It became apparent early in the course of these investigations that this method of detecting the presence of the Eaton agent was unreliable, and attention was turned to the use of the indirect fluorescent antibody technic.

Observations by means of fluorescent antibody staining. Coverslip cultures were harvested at various times following inoculation, and stained with sera known to contain fluorescent-stainable antibody against Eaton's agent. Antiserum dilutions of 1:10 were prepared in phosphate buffered saline (pH 7.2) and inactivated by heating at 56°C for 30 minutes. Controls for the reaction consisted of the following: 1) infected and uninoculated cell cultures stained with known-negative antisera (human acute or normal rabbit specimens); 2) cultures inoculated with normal chick embryo lung suspension stained with human convalescent serum or rabbit antiserum; and 3) normal and infected cultures stained only with the fluorescein-conjugated goat antirabbit or antihuman globulin solutions.

The changes to be described were seen in infected cultures stained either with human convalescent sera or rabbit antiserum, but not in any of the control systems. Beginning at 2 or 3 days after inoculation, and increasing until destruction or degeneration of the cultures at 14 days, brightly stained, rounded, granular structures were evident. The appearance of an intact culture 6 days after inoculation is illustrated in Fig. 1. Fig. 2 shows an infected monolayer after 10 days' incubation, at which time partial destruction of the cell sheet had occurred. The plane of focus of these structures under high magnification revealed them to be at the surface

FIG. 2. A partially destroyed cell sheet, 10 days after inoculation, with brightly stained structures on edges of cells and upon cytoplasmic processes. ×430.

FIG. 3. A large mass of antigenic material occurring 10 days after inoculation, which is composed of a cluster of smaller rounded units. ×430.

FIG. 4. Companion culture to that in Fig. 1, stained by the Giemsa method. Pink, granular structures are scattered across the surface of the large cell in the center of the field with peripheral aggregation. ×200.

FIG. 5. Companion culture to that in Fig. 2 showing partial destruction and retraction of cell sheet. Clusters of the rounded structures seen earlier are present (C), and all cells in the field now show evidence of infection. Giemsa stain. ×200.

FIG. 6. A single rounded cell bearing a colony on cell wall. The finely particulate nature of the growth is just discernible. Giemsa stain. ×900.

of cells, in intercellular spaces, on the edges of cells at the periphery of the monolayers, and upon long cytoplasmic processes of cells (Fig. 2). They were always in direct association with cells, however. These structures seemed fixed to the cell sheet since they did not wash off during the multiple agitated rinses of the staining procedure. Late in the course of infection much larger aggregates of fluorescing material were present after staining (Fig. 3), but these were composed of clusters of the smaller, rounded units. Cells were frequently seen which contained minute specifically-stained particles; it was impossible to determine if these were intracytoplasmic or on the surface of the cells. Because of the difficulties of visualizing fine detail with the fluorescence microscope, study of companion cultures stained by the Giemsa method was undertaken for further evaluation.

Observations by means of Giemsa staining. Changes comparable to those seen with the fluorescence microscope could be found by examination of infected cultures stained with an intensified Giemsa procedure (Fig. 4 and 5). These changes were not seen in either normal secondary monkey kidney cell cultures or in cultures inoculated with normal chick embryo lung suspension. Study of the alterations with the oil immersion objective revealed that the rounded structures associated with cells were composed of forms near the limit of resolution which stained light pink, and appeared to be predominantly rounded in shape (Fig. 6). Clusters of the minute forms averaged 10 μ in diameter as measured on photographic enlargements with appropriate corrections for degree of magnification, or more directly by use of a calibrated ocular micrometer.

Discussion. It has recently been shown that growth of the Eaton agent is inhibited by gold salts, and that minute cocco-bacilli are present in infected chick embryos in areas corresponding to those containing the fluorescent-stainable antigen(3). This information coupled with the size of the agent (5), range of antibiotic sensitivity(3), and the agent's stability characteristics(6) all are quite similar to those of the family of pleuropneumonia-like organisms (PPLO). The data presented revealed the development of extracellular growths in colonial configuration after inoculation of secondary monkey kidney cell cultures with Eaton's agent. The serological reaction of these colonies was indistinguishable from that of the same stock agent identified in the chick embryo. Carski and Shepard(7) have demonstrated PPLO in tissue culture systems using the fluorescent antibody technic, and their descriptions are strikingly similar to the findings with Eaton's agent reported above. This accumulated evidence strongly suggests that the Eaton agent is a member of, or shares many properties of, the genus *Mycoplasma.*

The methods described make possible experimentation with the agent directly in tissue culture without the need for an additional indicator system. Work is now in progress to evaluate application of these technics to further study of the growth and other biologic characteristics of the agent.

Summary. The Eaton agent was localized in secondary monkey kidney cell cultures by means of fluorescent antibody staining employing either human atypical pneumonia convalescent sera or rabbit antiserum. Morphologic study of material stained by the Giemsa method revealed the occurrence of extracellular growths resembling colonies with an average diameter of 10 micra. These findings plus other characteristics of the agent summarized suggest an intimate relationship to organisms of the genus *Mycoplasma.*

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