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IN CHICK ENTODERMAL TISSUE CULTURE

RESEARCH REPORT

MR 005.09-1200.03 Report No. 3

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FRANCIS B. GORDON
Bacteriologist
Naval Medical Research Institute

ALICE L. QUAN
Bacteriologist
Naval Medical Research Institute

M. KATHERINE COOK
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

ROBERT M. CHANOCK
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

HERNON H. FOX
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland

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SUMMARY

The Eaton agent of primary atypical pneumonia was carried through two series of 10 and 6 passages respectively, in chick entodermal cell cultures, indicating growth of the agent in this cell system. Cytopathic effects were not seen and fluorescent antibody staining of the infected cells by the indirect method was negative. The agent was demonstrated in fluids from the infected cultures by subinoculation to chick embryos and fluorescent antibody staining of lung sections.

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INTRODUCTION

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 ten serial passages in one series, and six in another.

MATERIALS AND METHODS

The FH strain of Eaton agent, kindly supplied by Dr. Chien Liu, was used. For the first tissue culture inoculation a preparation of infected chick embryo lung and trachea was subjected to a partial purification procedure previously used for chick embryo yolk sacs infected with rickettsiae or psittacosis viruses (4). The method includes digestion of the crude emulsion with trypsin, sedimentation of the infective particles by centrifugation, resuspension in Hanks' balanced salt solution (HBSS), and further clarification by addition of bovine plasma albumin to 0.6 percent concentration, and celite to 10 percent, followed by light centrifugation. The supernatant, containing the infectious particles, is relatively free of tissue components.

The entodermal cell cultures were prepared from explants from the blastoderm of 4-day chick embryos, and grown on circular coverslips in flat-bottomed tubes (4). The medium consisted of HBSS, 75 percent, and chicken serum, 25 percent; it was used in a volume of 0.5 ml. per tube. Inoculation was accomplished by replacing with a similar medium to which the infective preparation was added in 10 percent dilution. Incubation was at 36°-37° C. Two hundred units of penicillin per ml. were incorporated with the medium in the first 5 passages of Series A; the last 5 passages contained no antibiotic. Penicillin at 2000 units per ml. was employed throughout for the 6 passages of Series B. Ten cultures were routinely used for each passage and uninoculated controls were regularly included.

The fluorescent antibody technique has been described in detail previously (6). Chick embryo lung sections or tissue culture coverslips were routinely fixed in acetone at room temperature for 10 minutes. A 1:10 dilution of a 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 convalescent sera used had a staining antibody titer of 1:320 to 1:640. Acute phase (negative) sera from the same patient were also used for control purposes.

RESULTS

Two series of passages were accomplished by transfer of fluid from infected cell cultures. One series was carried out in each of the two laboratories represented, but both were initiated with the same preparation of partially purified infected chick lung.

Series A (10 passages). - The indirect method of staining with fluorescent antibody was applied to both infected and control coverslip cultures from the first 4 passages, taken at various intervals after inoculation, and at the 11th day in the 10th passage. Specific fluorescence was not seen with certainty, presumably due to insufficient concentration of specific antigen in the cells. However, the agent was demonstrated in culture fluid repeatedly throughout the series by infection of chick embryos and detection of the agent in the chick lung by fluorescent antibody staining. Examination of cultures after staining by May-Greenwald-Giemsa revealed no cytopathic effects.

Centrifugation of infective particles onto chick entodermal cells has been found to increase greatly the infectivity of rickettsiae and psittacosis viruses for chick entodermal cells (4). The reported large size of the Eaton agent, 180-250 mμ (2, see also 7), suggested that this maneuver might be advantageous. The first passages included groups of inoculated tubes which were centrifuged at 20° C. for one hour at 1800 g. It was soon found, however, that passage was successful without centrifugation and the practice was discontinued.

The second and third passages were made with fluids harvested on the 6th day, and thereafter with fluids taken on the 10th or 11th day. This was usually preceded by a change of medium on the 5th day. In the final (10th) passage both 5-day and 11-day fluids were tested but only the latter was found infective, suggesting a higher level of infectivity at the latter interval. The 10-fold dilution effected at each passage gives an estimated 10^{-10} dilution of the original inoculum in the final 10th passage fluid. This figure can be further reduced by approximately 5 log units because of the complete fluid change made once in each of 5 passages. Detection of infectivity in the 10th passage indicates clearly that increases of the agent occurred in this culture system.

Series B (6 passages). - These passages were made at 7 to 9 day intervals, and fluid on the infected cultures was frequently changed once or twice in the interim. On the 14th day of the 6th passage the original inoculum had been diluted 10^{-11} by the transfers and fluid changes. Fluid harvested at that time was infective for 13-day embryonated eggs as demonstrated by fluorescent antibody staining of lung sections, indicating that the agent had replicated in the entodermal cell cultures. The agent was still present in fluids removed from cultures at 16-18 days of incubation. Specific antigen, however, was not visualized when infected coverslip preparations were tested by the fluorescent antibody technique and no cytopathic effects were observed.

COMMENTS

Chick embryo lungs infected with the FH strain may contain 10^5 or 10^6 embryo infective doses per 0.2 ml. (3). Although the original inoculum in these series was not titrated, the finding of the infective agent after 10 and 6 passages, in the two series, at estimated dilutions of the original inoculum of 10^{-11} or greater, leaves no doubt that the agent increased in the chick entodermal cell cultures. No titrations of the culture harvests were made and there is no evidence that any degree of adaptation to these cells occurred. The identity of the agent cultured was established by characteristic appearance after fluorescent staining of subcultures in embryo lung with known antisera (convalescent), and the more extensive serologic examination applied to later cell cultures infected by passage from entodermal cells (8). Unfortunately, this method of cultivation in entodermal cells does not provide any improvement in current techniques. However, this first step in propagation of the Eaton agent in cultured cells suggests that better *in vitro* methods can be found.

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