Cultivation of Duck Hepatitis Virus in Tissue Culture.* (26465)

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A viral disease of ducks characterized primarily by hepatitis was first described by Levine and Fabricant(1). The viral agent and course of the disease have been studied by several investigators(2-6). Incentive to study this virus has been provided by the economic importance of the condition to the duck industry and possible relationship with viral hepatitis in other species.

Investigation of the virus of duck hepatitis (DHV) has led to attempts to cultivate the agent in other hosts. The virus multiplies readily in embryonated chicken eggs to which it has been adapted(1). Pollard and Starr (7) attempted to propagate the virus in chicken embryo explants and trypsinized chicken embryo cell cultures. The virus multiplied in explant cultures, but persisted through only 3 passages in trypsinized cell cultures.

The reported success in growing trypsinized liver cells in tissue culture(8,9) suggested a promising host for the virus. This paper reports results of cultivation of duck hepatitis virus in liver tissue culture cells.

Materials and methods. Virus. DHV strain R85952(3) which had been passed serially 18 times in embryonated chicken eggs was used for this study. Allanto-amniotic fluid from embryos which died following viral inoculation served as a source of virus. Virus was stored at –20°C.

Viral assay and identification. Viral titer of allantoamniotic fluid and tissue culture material was determined by infectivity for 8-day-old embryonated chicken eggs. Identification of virus was established by neutralization with duck serum known to contain DHV neutralizing antibodies. Gel diffusion precipitation tests(10) utilizing anti-DHV rabbit serum served to identify virus and provided a semiquantitative measure of viral content.

Tissue culture. Livers of 16-day-old chicken embryos were removed aseptically, minced with scissors, and trypsinized at 39°C with 0.25% trypsin (Difco 1:250) in Earle's saline (ES). Cells were washed, once in cold ES and once in nutrient medium, and 2 ml of a 1.5% suspension of cells in nutrient medium were placed in 16 × 150 mm rubber stoppered tubes. Nutrient medium consisted of 30% inactivated sheep serum, 10% chicken embryo extract or 15% beef embryo extract, 50 mg/ml dihydrostreptomycin sulfate and 100 units/ml penicillin added to Eagle's medium. Tubes were incubated at 37°C in a stationary rack positioned at a 5° angle.

Inoculation of tissue culture with virus. When cells had formed a confluent sheet (about 4 days) they were inoculated with virus. Nutrient medium was removed and 2 ml of fresh medium and 0.2 ml of viral suspension were added. Tubes were incubated without a change of medium for 6 days, at which time they were frozen at –20°C.

Cytopathologic study. Cells were observed daily for signs of abnormality. Coverslips were removed periodically from tubes and cells were stained by the May-Grünwald-Giemsa technic. Stained cells were examined microscopically for signs of cytopathogenic effects (CPE).

Results. Cell cultures derived from chicken embryo livers were composed of at least 3 cell types: fibroblasts, epithelial-like cells and polygonal cells with small, round nuclei. The latter cells were very similar histologically to hepatic cells and tended to grow in islands surrounded by the other cell types (Fig. 1). Polygonal cells characteristically contained lipoidal inclusions, and numerous mitotic figures could be observed. Fibroblasts overgrew other cell types and, if

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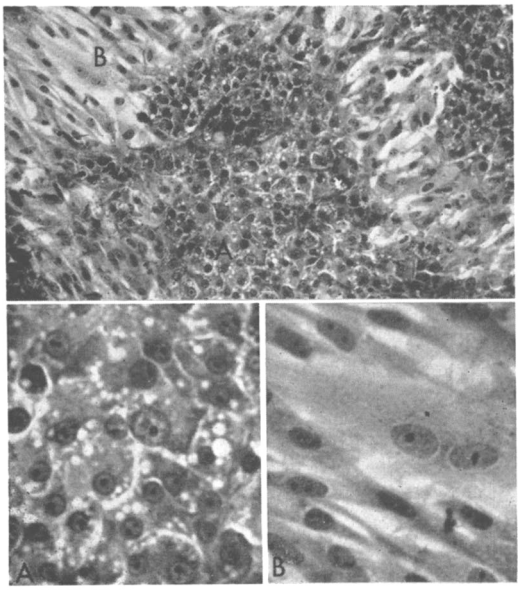

FIG. 1. Uninoculated chicken embryo liver tissue culture. Note islands of "hepatic" cells (A) surrounded by cells with fibroblastic and epithelial morphology (B). May-Grünwald-Giemsa stain. Magnification 100× and 300× respectively.

their multiplication was not restricted, excessive growth caused peeling of the cell sheet. Embryo extract incorporated in nutrient medium served to restrict fibroblast proliferation.

Assay of each serial passage of virus in tissue culture demonstrated a decrease in viral titer from passages 1 to 6 and a moderate rise in the 7th passage as shown in Table I. Although titer of virus dropped considerably from that in allanto-amniotic fluid it is quite obvious that virus did multiply in tissue culture medium (See Discussion).

TABLE I. ID_{50} Titers of Tissue Culture Virus for Embryonated Chicken Eggs.

	T.C. passage						
	1	2	3	4	5	6	7
Titer*	$10^{3.3}$	$10^{2.5}$	$10^{1.6}$	$10^{1.9}$	$10^{1.7}$	$10^{0.9}$	$10^{2.9}$

* Calculated by method of Reed and Muench (11).

Gel diffusion precipitin test demonstrated presence of virus (Fig. 2). Observable lines of precipitate developed within 12 hours with viral passages 1, 2, 6, and 7. Faint lines of precipitate formed with passages 3, 4 and 5 after reservoirs were refilled several times with antigen and incubation was continued for 24 to 36 hours. Controlled tests demonstrated the specificity of antigen present in tissue culture material.

Microscopic examination of liver and stained tissue culture cells failed to reveal CPE. No changes in character of cells or their multiplication were seen during passage of the duck hepatitis virus.

Discussion. Limited multiplication of DHV was demonstrated in chicken embryo liver tissue culture cells. Multiplication would have been necessary for virus to be present in terminal passages since dilution and decay of virus at 37°C would have reduced the amount of virus below the threshold of detection. Pollard and Starr(7) have reported a decrease in titer between 10^2 to $10^{5.5}$ fold in several tests when the virus was incubated at 37°C for 21 days. The 42-day incubation period, taken together with a total dilution factor of 10^7 over 7 passages, would have totally removed original input virus. The marked drop in viral titer during the first few passages followed by an increase in the 7th passage suggests that the virus was becoming adapted to tissue culture cells. Earlier development of lines of precipitate with the gel diffusion precipitation test in initial and terminal passages was also probably related to viral concentration thereby indicating multiplication of virus.

Since marked histopathologic changes are seen in infected chicken embryos and ducks (4), failure of virus to induce cytopathologic changes in tissue culture cells was not expected. However, mouse hepatitis virus(12), as well as a number of other viruses(13), can

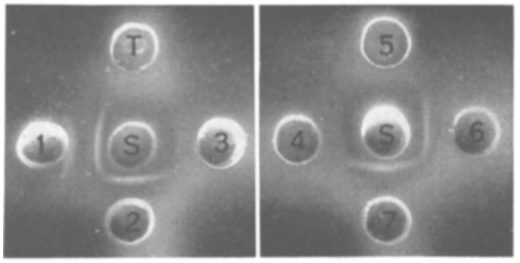

FIG. 2. Gel diffusion precipitation with duck hepatitis virus from various tissue culture passages. Numbers indicate passage level, T represents uninoculated tissue culture, and S stands for rabbit anti-duck hepatitis serum.

multiply in tissue culture without visible CPE. Pollard and Starr(7) also were unable to detect histopathologic changes in chicken embryo explants that supported multiplication of DHV. Apparent lack of CPE may be a reflection of a low number or percentage of infected cells rather than an indication of viral multiplication without CPE. Observed titers could conceivably have been produced by release of a few hundred infective virus particles from as few as 10 cells per ml, a number virtually undetectable among the far larger number of cells in each tube.

Summary. Duck hepatitis virus was passed serially 7 times through monolayer chicken embryo liver cell cultures. Multiplication of virus was demonstrated by assay in embryonated chicken eggs and by gel diffusion precipitin test. Microscopic examination of stained tissue culture cells failed to reveal CPE.

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Cultivation *in vitro* of Adult Human Skin and Oral Mucosa on Gelatin Film.* (26466)

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Successful cultivation of human skin *in vitro* has been reported by numerous investigators(1-8), the majority of whom employed the plasma clot technic with small fragments of skin obtained from either living donors, or from cadavers. Plasma clot may present certain disadvantages for immunological, nutritional(9) and viral studies(10). Evans and Earle(9) have used perforated cellophane as a substitute for plasma clot for growth of some tissues. It would appear, however, that perforated cellophane is not a satisfactory substrate for primary cultivation of adult human skin(4). Attempts in this laboratory to cultivate single specimens of adult human skin from 9 donors with perforated cellophane were unsuccessful. Puck *et al.*(7) have used the method of trypsinization for successful growth of cells from fresh adult skin and other human tissues. Excision of 20 to 50 mg of skin(11), however, is not always feasible when repeated fresh specimens, particularly from the same area, are desired for cultivation. Ehrmann and Gey have reported growth of a number of cell strains and fresh explants of human tissue on transparent collagen gels(12) and Enders(13) has

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