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Virus Growth in Tissue Culture Fibroblasts. II. Coxsackie Virus (Group B) in Cultures of Mouse Fat Tissue. (19971)

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(Introduced by Icie G. Macy.)
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One of the distinguishing features of the B group of Coxsackie viruses is their ability to produce histopathologic changes in adipose tissues of infant mice(1-3). Although virus has been recovered from these tissues, this does not constitute proof that the virus has specifically infected the cells found in embryonal fat tissues(2,3). Current tissue culture technics offer an opportunity to demonstrate: a) whether a group B Coxsackie virus can be propagated in adipose tissues *in vitro* and b) whether there is a viral susceptibility relationship between the cells of the host tissue and fibroblasts derived from it. In this demonstration we have determined the growth patterns and cytopathogenic effects of a group B type 1 Coxsackie virus in tissue cultures of interscapular fat pads of newborn mice as well as in cultures of fibroblasts derived from this tissue.

Materials and methods. Tissue cultures. A modification of the Porter roller flask method(4) utilizing clotted plasma was employed. The nutrient fluid consisted of 10% chick embryo extract and 30% horse serum incorporated in Hank's balanced salt solution. The initial pH of the nutrient medium was adjusted to 7.4-7.6 with 1.4% (isotonic) $NaHCO_3$. Phenol red in a final concentration of 0.002% was used as indicator. To the medium were added 100 units of penicillin and 100 micrograms of streptomycin per milliliter to control bacterial contamination. Two drops of chicken plasma were placed on cover slips cut to provide for insertion easily into a Porter flask. With a curved-tip pipette 2 tissue explants were taken up in 2 drops of nutrient fluid and added to the plasma on each cover slip. Following coagulation of the plasma, 2 cover slips, each containing 2 embedded tissue fragments, were each placed on the opposite flat surfaces of a Porter flask. They adhered firmly after the flat inner surfaces of the flasks were wet with nutrient fluid. After adding 1 ml of nutrient medium the flasks were tightly stoppered, placed in a rotating drum (⅕ rpm) modified to hold the Porter flasks, and incubated at 36°C. For routine maintenance of the cultures, 3 times each week the nutrient fluid was replaced and lysis of the plasma clot was repaired with fresh plasma. To divide cell colonies the cover slips were removed from the flasks and the fibroblastic outgrowths dissected into portions approximately 1.0 x 1.0 mm for replantation. *Source of cultured cells.* One-half millimeter fragments were prepared from the interscapular fat pads of newborn mice. Four explants and their resulting cellular outgrowths were designated primary culture passages. When colonies reached maximum size they were dissected and portions were transferred to other flasks for the second and third passages. After they had attained their maximum sizes colonies of the primary culture passages contained a central area consisting of tissue carried over from the animal host and a surrounding 2-3 mm band of outgrowth consisting of fibro-

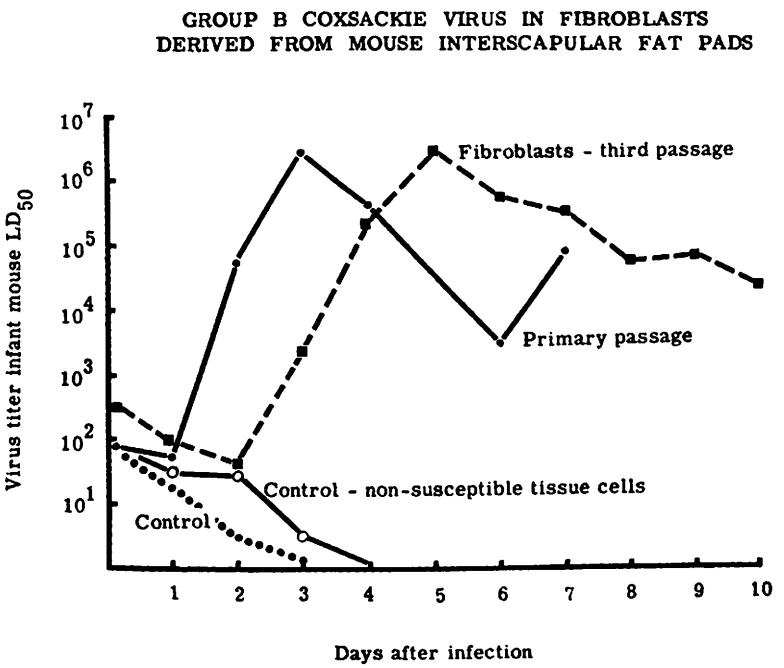

FIG. 1. Growth patterns of group B Coxsackie virus in primary cultures and third passage fibroblasts of mouse interscapular fat pad tissue.

blasts. By the third passage few, if any, of the original cells of the animal host were present and only fibroblasts could be observed microscopically. Although the cultures cannot be said to consist wholly of fibroblasts, we must assume that if other types of cells were present they would be so greatly outnumbered by fibroblasts that the latter must have played the major role in producing the relatively high concentrations of newly propagated virus. For cytological observations, the cover slips containing the colonies were removed from the flasks, washed with balanced salt solution, fixed with Bouin's solution, and stained with Harris' hematoxylin.

Virus. The Connecticut 5 strain of Coxsackie virus, classified as Group B type 1(5), was obtained from the American Type Culture Collection. The virus was received in infected mouse brain in 50% glycerin and was passed once in infant mice before seed preparations were made. For seed preparations, the mice were skinned, eviscerated and the carcasses ground and suspended in 20% dilution in balanced salt solution. Of a 10^{-2} dilution of this suspension, 0.03 ml was inoculated subcutaneously into 2-day-old mice. When the mice became sick or showed paralysis (4 to 5 days after infection) they were skinned, eviscerated and the carcasses were washed in balanced salt solution, ground and suspended in 20% dilution in balanced salt solution containing 500 units of penicillin and 100 μg of streptomycin per ml. The suspension was centrifuged at 3,000 rpm for 30 minutes and the supernatant fluid was placed in ampules, glass-sealed, and stored in a dry chest until used. *Inoculation of tissue cultures.* The seed preparations of virus were diluted to 10^{-3} in nutrient fluid so that the virus concentration in a 1 ml inoculum was approximately 100 infant mouse LD_{50}. When tissue cultures were 5 to 7 days old the nutrient fluid was removed and flasks containing 4 colonies each were infected with 1 ml of fresh nutrient fluid containing the desired virus concentration, then incubated at 36°C. Culture fluid samples, 0.25 ml, were withdrawn daily and replaced with fresh nutrient fluid to maintain a 1 ml level in the culture flasks. *Titration of viruses.* The samples of virus-infected culture fluids were diluted in tenfold steps with balanced salt solution immediately after removal from the cultures and each dilution was placed

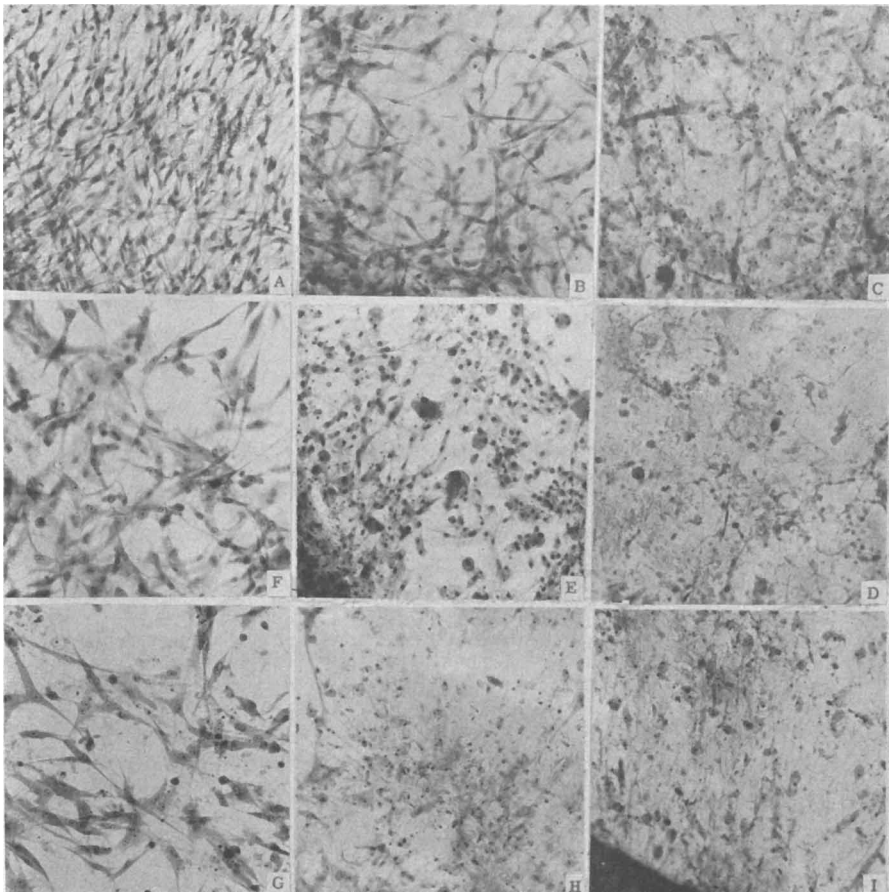

FIG. 2. Normal and virus-infected fibroblasts in cultures of mouse interscapular fat pad tissue. Hematoxylin, × 150. A. Primary culture, 12 days old, uninfected. B. Primary, infected 3 days. C. Primary, infected 5 days. D. Primary, infected 7 days. E. Primary, infected 9 days. F. Third passage fibroblasts, 9 days old, uninfected. G. Third passage, infected 3 days. H. Third passage, infected 5 days. I. Third passage, infected 7 days.

in an ampule, glass-sealed, and stored in a dry ice chest. This procedure allowed for the inoculation of the various dilutions of virus into 2-day-old mice as litters became available. Of each dilution of virus, 0.03 ml was inoculated subcutaneously into each of 7 2-day-old infant mice. The mice were observed for 2 weeks and 50% lethal endpoints were determined by the method of Reed and Muench (6).

Results. Growth patterns of virus in primary cultures. A series of roller flasks, each containing 4 tissue explants, were incubated for 5 days. By this time the fibroblastic outgrowth had formed colonies approximately 4 mm in diameter. The cultures were inoculated with 1.0 ml of nutrient fluid containing $10^{1.88}$ LD_{50} of virus (10^{-3} dilution of stock virus). Two series of control flasks were inoculated similarly: one contained clotted plasma and nutrient media without cells; the other contained a primary culture of cells from a non-susceptible host, the chick embryo (7).

Fig. 1 presents a typical growth curve of virus in primary cultures of mouse fat pad tissue as demonstrated by infant mouse titrations of tissue culture fluids. After a brief lag the virus titer rose rapidly, reaching a peak of $10^{6.42}$ mouse LD_{50}. Thereafter, the titers gradually decreased through the seventh day following inoculation. Viable virus in the control flasks rapidly disappeared, none remaining after 2 days in the flask without cells

or after 4 days in cultures of non-susceptible tissues. The rapid loss of active virus from control flasks, particularly from cell-free controls, has occurred consistently in this laboratory and has been observed with other viruses (8).

Cytopathogenic effects of virus in primary cultures. Although gross evidence of virus infection of the cells was indicated by the failure of the pH to drop as low as that of uninoculated cultures, microscopic observation revealed a direct cytopathogenic effect of virus on the fibroblasts, particularly in stained preparations. The effect was observed first on the third day after infection when the titer of propagated virus was maximal. Fig. 2 shows the progressive cytopathogenesis of the virus on the fibroblastic outgrowth 3, 5, 7 and 9 days after infection and a 12-day-old uninoculated parallel control culture. Three days after inoculation evidence was present of increased granulation of the cells and a few nuclear remains could be observed after fixation and staining. After 5 days necrosis of the fibroblasts had progressed so far that few healthy cells could be observed in the masses of cellular debris. Seven days after inoculation most of the fibroblasts had been destroyed and the outgrowths were almost completely acellular. By the ninth day another cell type was evident in increased numbers (Fig. 2E). These cells appeared to be healthy and unaffected by virus. At that time a secondary growth of fibroblasts had appeared, but whether these were subsequently affected by virus was not determined.

Growth pattern of virus in third passage fibroblasts. Six-day-old cultures with colony diameters of approximately 4-6 mm were inoculated with $10^{2.5}$ mouse LD_{50} of virus as described for primary cultures. The representative growth curve in Fig. 1 demonstrates that after an initial decrease, the culture fluids began to increase in virus titer on the third day, reaching maximal titers of $10^{6.4}$ mouse LD_{50} 5 days after infection. During the next 9 days the virus titers decreased slightly.

Cytopathogenic effect of virus in third passage fibroblasts. As in primary cultures, evidence of viral destruction of fibroblasts was observed as early as 3 days following infection (Fig. 2G). In contrast, however, the early cytopathogenic effect occurred 2 days before the maximal titer of virus was observed in the extracellular fluid phase of the culture, but at a time when newly propagated virus could be detected. Progressive destruction of the fibroblasts continued through the fifth and seventh days after infection of the cultures (Fig. 2H, I). Fig. 2F shows the cells of a 9-day-old, uninoculated control culture.

Discussion. The ability of group B type 1, Coxsackie virus to propagate in cultures of adipose tissues of newborn mice suggests that the virus has this ability in the intact host where inflammatory and necrotizing lesions of embryonal fat lobules is a distinctive characteristic(3).

It is evident that in tissue culture the virus was growing in "fibroblasts" derived from the fat tissues. The exact origin of the fibroblasts in these cultures cannot be determined or whether in primary cultures the virus multiplied in the fat cells themselves.

Morphologically, tissue culture fibroblasts are spindle-shaped cells connected with branching cell processes to form a network. Although fibroblasts cultivated from a variety of tissues from a single animal species may be indistinguishable in appearance, the question whether some may be dedifferentiated forms of more mature cells remains unanswered.

The observations with Coxsackie virus parallel, to some extent, those of Scherer and Syverton(9), who showed that poliomyelitis viruses multiply and produce cytopathogenic effects in fibroblasts isolated from monkey testicular tissues. The authors' experiments with influenza A virus(8) indicated that fibroblasts derived from susceptible tissues of the chick embryo do not support the growth of that virus. Herpes simplex virus, which has a broader affinity for different cell types, was grown readily in fibroblasts derived from susceptible tissues of the chick embryo(8). A study of the capacities of different viruses to infect and cause cytologic changes in various cultured tissues and in fibroblasts derived from those tissues might explain some of these problems of virus trophisms and fibroblast origin.

Summary. The ability of a strain of Coxsackie virus, group B type 1, to propagate in cultures of mouse interscapular fat pad tissue and in fibroblasts derived from that tissue was investigated. The virus multiplied in primary cultures containing both explanted host cells and outgrowing fibroblasts, as well as in cultures of fibroblasts carried through 3 tissue culture passages. In each instance the growth of virus was accompanied by progressive cytopathogenic effects which continued until nearly all of the fibroblasts were destroyed.

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Variables in Agglutination and Lysis of Human Red Cells by NDV. (19972)

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An hemolysin associated with the Newcastle disease virus particle has been described by Kilham(1) and also by Burnet and Lind(2). Both groups of workers found that chicken red cells were hemolyzed more readily than human red cells. This paper reports the hemolysis of human O-type red cells by the vaccine strain of NDV and outlines the variables affecting the agglutination and lysis of these cells. Freshly harvested virus did not agglutinate or lyse fresh human red cells but previously frozen virus did both. Furthermore, fresh human red cells which were not agglutinated by fresh virus were agglutinated after storage of the red cells at 4°C for 5-7 days.

Materials and methods. Virus. The Blacksburg or Hitchner(3) strain of NDV which is currently used as a commercial live vaccine for newly hatched chicks, was furnished by Dr. Herald Cox of Lederle Laboratories. This virus had several passages in chick embryos at our laboratory and is referred to here as the Vaccine Strain(4). In contrast to other strains, the vaccine virus causes a transient agglutination of chicken red blood cells and the reaction is inhibited by cold(4). Pools of virus were prepared by allantoic inoculation of 11-day-old embryos and the allantoic fluid was harvested after 2 days of incubation at 35°C. *Human O-type red blood cells.* In most of the experiments, the red cells were from one individual. Blood was obtained by intravenous puncture and put into a large volume of chilled 0.85% NaCl buffered at pH 7.2 with 1% 1/15 M phosphate, without any citrate or anticoagulant. The red cells were washed 3 times with buffered saline and a 1% cell suspension was used in the experiments. *Glassware.* Since the persistence of slight amounts of detergents may give irregular results in the hemagglutination tests(5), no lysol or detergents were used in our laboratory for sterilizing or cleansing glassware. All glassware was cleansed by overnight immersion in chromic acid cleaning solution. Following that, the pipettes and tubes were washed 7 times in running tap water and twice

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