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Method for Establishing Human Cells in Culture: Susceptibility to Poliomyelitis after Prolonged Cultivation.* (20423)

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The application of tissue culture technics to the study of mammalian viruses has received renewed impetus since Enders and his associates showed that the viruses of poliomyelitis would multiply in cultures of human tissues (1). Quantitative studies have been greatly restricted by the existing tissue culture technics for virus propagation, which usually depend on the explantation of fresh tissue for each virus passage. It would obviously be of great value, therefore, to have established cell strains susceptible to viral infection. Of the established cell cultures, the "L" strain (Earle) has been shown to be susceptible to infection with the virus of Rous sarcoma(2), and to pseudo-rabies and herpes simplex(3). Recently, Scherer, Syverton, and Gey(4) have described a strain of cells susceptible to poliomyelitis. The technics described by Earle for the initiation of cell strains on glass or cellophane surfaces have not been widely used. The purpose of this communication is to describe a simple technic for establishing human cells in mass culture and to show how such cell strains may be maintained. These cultured cells appear to retain their initial susceptibility to viral infection.

Methods. The standard technic for cultivation of tissue in roller tubes has been employed. The lower two-thirds of an 18 x 150 mm culture tube is coated with 0.05 ml chicken plasma. The desired number of tissue fragments are placed on the side of the tube and 0.05 ml chick embryo extract (EE 50) is added and mixed with the plasma. The plasma-extract mixture is then rolled over the surface of the tube, allowed to drain for a few seconds, and the excess is removed, leaving the tissue embedded in a very thin clot. The tube is allowed to stand for 5-30 minutes and

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then 3 ml of nutrient fluid consisting of 85% Fischer's V-614 solution(5), 10% horse serum and 5% EE 50 are added and the tube stoppered and incubated at 37°C. The nutrient fluid is changed at intervals determined by the decrease in pH from the initial value of 7.6 to approximately 7.0, usually every 2 or 3 days. After one to 10 days, new growth appears in the clot around the explanted fragments. The time depends on the type of tissue and its previous treatment. When growth is well established, the original explants are dislodged and removed with the fluid when the latter is changed, usually within 3 to 4 weeks. By this time, the surface of the tube is covered with a thin sheet of cells. Where lysis of the plasma coagulum has occurred, cells grow freely over the glass. The initial cell population is subcultured by dislodging the cells with a platinum wire bent to fit the curvature of the tube. This procedure yields a suspension containing large masses of cells. These cell masses are largely broken up by drawing the fluid into a serologic pipette and then forcefully expelling it. The pooled suspension from several tubes is centrifuged at 175 g for 10 minutes and the supernatant is discarded. The cells are suspended in a small volume of EE 50 and added to tubes containing chicken plasma in the manner already described. Microscopic examination reveals that single cells and small clumps of cells were carried over and are now embedded in the very thin plasma coagulum lining the culture tube. After the first hour of incubation, the cells have assumed their characteristic appearance and can be seen migrating from the small cell masses. Multiplication begins promptly. Cells which have been subcultured in this manner for 4 to 6 months continue to grow well.

Examination of the original culture tube reveals that many individual cells and small sheets of cells remain on the inner surface. If nutrient fluid is added to the tube, these cells will multiply, and within a short period of time the tube is again lined with cells. These cells, which have grown directly on the glass, can be dislodged as already described and further treated by repeated expulsion from a pipette, or by vigorous shaking, to yield a suspension that contains primarily single cells or small masses (2-12 cells) with an occasional larger fragment. These suspensions may be centrifuged and the cells resuspended in EE 50 and planted in a thin plasma coagulum, or the cells may be planted directly on glass, as described by Shannon and Earle(6). In the latter case, there is a longer interval between planting and the initiation of growth; also, many of the larger cell clumps do not adhere to the glass and are lost when the fluid is changed. After each successive harvest, a sufficient number of cells are left behind on the glass to restore the cell population. In this way, a *mother* tube may serve as a source of cells over an indefinite period. In our experience, the effect of repeated harvesting from a single tube has been to increase the tendency of cells to grow from isolated cells on the tube wall, or from increasingly smaller clumps, with the ultimate effect of reducing the size of the clumps in the cell harvest. This factor influences the efficiency of transplantation of cells to glass, as has been noted by Earle, Waltz, and Shannon(7).

Results. Skin-muscle cells of human embryonic origin which were cultivated for 6 months in the manner described were tested for their susceptibility to the cytopathogenic effect of the MEF_1 strain of poliomyelitis virus. The cells obtained from 6 mother tubes were suspended in 25 ml of nutrient fluid. After removing the larger cell clumps from the suspension, it was dispensed in 1 ml volumes into 13 x 150 mm tubes. The tubes were stoppered, inclined at an angle of approximately 5°, and incubated in stationary racks. After 3 days, when cell growth was well established, the nutrient fluid was replaced with Mixture 199 of Morgan, Morton, and Parker(8), and 0.1 ml of the appropriate dilution of virus suspension was added to each tube. The viral suspension used in this experiment consisted of the fluid phase from a culture of MEF_1 poliomyelitis virus(9) in uterine tissue. The tubes were placed in the roller device and incubated at 37°C. After 3 days the cultures were examined for evidence of cytopathogenic effects. The results are recorded in Table I, and indicate that a very small amount of virus is capable of in-

TABLE I. Susceptibility of Human Embryonic Skin-Muscle Cultures to the MEF_1 Strain of Poliomyelitis.

Virus dilution (log)	0	1	2	3	4	5	6	No virus
Results	2/2*	2/2	2/2	3/3	3/3	2/3	1/3	0/3

* Denominator represents number of tubes inoculated with 0.1 ml virus suspension; numerator, the cultures showing degeneration in 3 days.

ducing cytopathogenic effects in a cultured strain of skin-muscle fibroblasts.

The thin clot technic has been successful with all the tissues so far studied, including adult human uterus and testicle, and embryonic skin-muscle, liver, lung and kidney. It appears, however, that for the optimum growth of each cell strain that may be studied, the nutritional requirements, the pH range and other factors will need to be established. Thus, for example, embryonic skin-muscle fibroblasts seem to be more easily maintained in roller tubes than in stationary cultures, whereas testicular and uterine cells may be maintained under either condition. Also, skin fibroblasts appear to maintain undiminished vigor in nutrient fluid containing chick embryo extract, whereas uterine fibroblasts seem to do better in beef embryo extract. On the other hand, it appears that while fibroblasts of testicular origin can be cultured initially in fluid containing chick embryo extract, their rate of growth eventually declines and can be restored to normal by substitution of beef embryo extract.

Summary. A technic is described by which human tissues may be established readily *in vitro* either on glass or in thin plasma clots. Susceptibility to infection with the MEF_1 strain of poliomyelitis is retained after prolonged cultivation.

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L Type Cultures Isolated from Streptococci.*† (20424)

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Pleuropneumonia-like colonies and larger colonies of similar structure corresponding to L type colonies of various bacteria grow usually in cultures made from the human mouth, especially from tooth scrapings, if bacterial growth is inhibited by penicillin. The observations to be described originated from an effort to determine the origin of these colonies and their eventual connection with bacteria. Numerous colonies of various types of bacteria were picked from anaerobic mouth cultures and were tested for the production of L forms. None of the bacteria produced L type colonies similar to those of the oral pleuro-

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