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Poliomyelitis Virus in Tissue Culture VI. Use of Kidney Epithelium Grown on Glass.* (20710)

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Tissue culture methods have become essential to the conduct of research with poliomyelitis virus and indeed with certain other viruses as well(1). For this purpose rolled or stationary test-tube cultures of tissue have been chiefly used in which the tissue fragment is imbedded in a plasma clot. This paper reports a simplification of the technic for preparing cultures, in that the tissue fragment can be made to stick on glass in the absence of plasma and clotting solution (chick embryo extract or thrombin). The new cells then grow out directly on the glass surface. Before the culture is used, the tissue fragment, easily removed to leave behind a sheet of newly grown cells, can be transferred to seed another culture tube. A variety of tissues from man, monkeys, and mouse have been cultured on glass without plasma. However, this paper will deal exclusively with the culturing and use of monkey kidney tissue.†

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Materials and methods. The technic of preparing test tube cultures of monkey tissue in plasma as used in this laboratory has been described in detail(4,5). Similar conditions have been followed in the present experiments in preparing the plasma-clot test-tube cultures, (hereafter called "plasma tubes"). Test tube cultures prepared in the absence of plasma are called "dry tubes", and their preparation is given in detail below.

Complete medium for all cultures, in the presence or absence of plasma clot, consisted of 9 parts of mixture No. 199(6) and 1 part bovine serum.‡ The No. 199 was made up in

† Epithelial and fibroblastic cells derived from human cancer and capable of growth on glass are also sensitive to poliomyelitis virus(2,3). For certain purposes where one does not want a cell line of cancerous origin, or a cell which requires human serum for its growth, or a cell line which may suddenly be lost because of mold or other contamination, the use of primary explants from readily available normal tissue offers advantages.

Earle's salt solution containing 2.20 g of $NaHCO_3$ per liter. Penicillin and streptomycin were added so that the final concentration in the complete medium was 100 units and 100 μg per ml respectively. All tubes contained 1 ml of complete medium, and were incubated at 36°C in a stationary position(5). When in 10-14 days an epithelial sheet of cells had grown out, the complete medium was removed, the tubes were washed with 1 ml of Earle's salt solution (to remove poliomyelitis virus inhibitory substances present in bovine serum), and to each tube there was added 0.09 ml of maintenance medium consisting of 1 part of No. 199 and 1 part of Earle's salt solution. The cultures were then ready for use with virus. In many experiments, the original tissue fragments were shaken loose after the outgrowth was sufficiently luxurious, and they were transferred together with the original complete medium to a new test tube. During the manipulation, the outgrowth from the tissue remained attached to the wall of the tube. The original tube was now washed and maintenance medium added as indicated above. The subculture containing the original fragments and fluid was fortified with 0.25 to 0.5 ml of fresh complete medium, and incubated also at 36°C in a stationary position. The Brunhilde (Type 1) and Y-SK (Type 2) poliomyelitis viruses in their 11th and 21st tissue culture passages were employed. Tubes were read every 24 or 48 hours for 6 days for their reaction to virus (cytopathogenic effect). *Test tubes* (16 x 150 mm) are immersed overnight in "7 x" detergent solution§ diluted 1 to 10 with tap water. The following morning the tubes are taken from the detergent solution and placed in groups of 170 in stainless steel boxes (256 mm x 156 mm x 156 mm deep) with the open ends at the top. The tubes are scrubbed with a test tube brush while they are in the box. As an aid in rinsing the tubes, a wire grid cover is employed. With the cover in place the box and tubes are flooded with tap water. The tubes are rinsed 10 times in tap water and 3 times in distilled water. A paper liner is fitted into the cover and the box and tubes are autoclaved at 15 lb for 30 minutes, and then dried at 105°C for 3 hours. In the afternoon these tubes are placed in a 45°C incubator for the following day's production of cultures. *Cultures in "dry" tubes.* Soon after its removal from a cynomolgus or rhesus monkey, the kidney is minced in a widemouth tube with 2 volumes of Earle's solution, using conventional sharp scissors, or Bard-Parker replaceable-blade scissors. The kidney is reduced to fragments of about 1 mm, which are transferred to a flask and washed by repeated addition of salt solution and decantation of the turbid supernate, using about 300 ml of salt solution per kidney. The washed fragments are kept under 50 ml of salt solution at 4°C until needed, but for not longer than 3 days. One pair of kidneys from a young monkey (5 to 7 lb) will provide tissue for 2000 primary culture tubes. Warm tubes from the box are placed on a rack in groups of 24. A narrow line of kidney fragments is streaked into the lower third of the tubes, with the minimum amount of fluid necessary to make the streaking possible. An uncalibrated pipette with a small rubber bulb is used to take up enough tissue from the flask at one time to prepare a full rack of 24 tubes. The tissue-containing tubes are then transferred into another box. With 2 persons working together the preparation of 170 tubes in this manner takes about 5 minutes. During this time there is a drop in temperature of the tubes from the original 45°C. Whatever this may amount to, the technic is not so sensitive that the adhesion to the tubes or growth potential of the tissue varies discernibly in the first or last tubes made in each box. When the 170 tubes have all been placed in the second (transfer) box, the sterile top from the first box is also transferred to it. These tubes remain at room temperature for about 5 minutes while the next box is being prepared. After this time, they are transferred to a refrigerator (4°C) where they remain for 30 minutes. 1 ml of complete medium is then added by an automatic hand pipette, care being exercised not to direct the fluid on the tissue fragments. The tubes are

‡ Bovine serum was obtained from Microbiological Associates, Bethesda, Md. It was passed through a bacteriological filter and then stored and shipped in the frozen state.

§ Obtained from Linbro Chemical Co., New Haven, Conn.

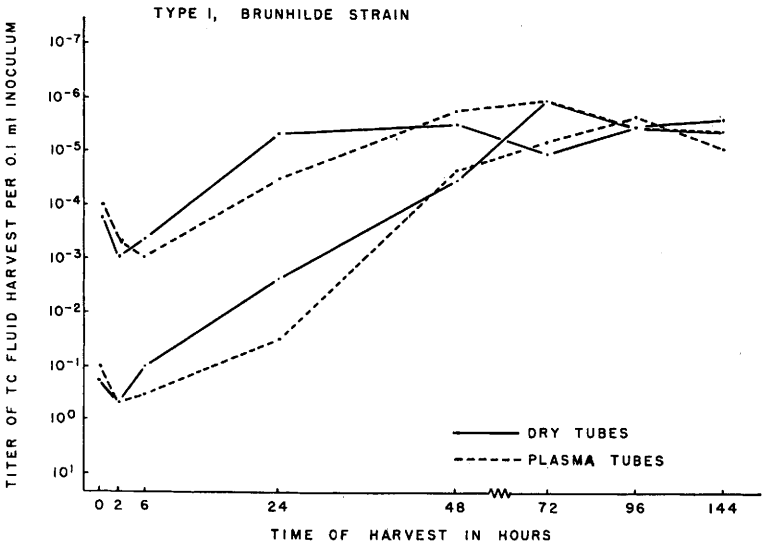

FIG. 1. Growth curves of the Brunhilde Strain (Type 1) in kidney epithelial cells grown in presence and in absence of a plasma clot.

quickly stoppered after addition of medium, and transferred to a new box similar in size to those described above. The storage box has a strip of board taped across the front lower edge to provide the desired tilt to the tubes (about 5°). A row of small nails with 4 mm heads driven into the back of the box 16 mm apart and 12 mm from the bottom of the box keeps the first row of tubes in position and facilitates the storage of the upper rows. Each box firmly holds 145 tubes. Each alternate tube is pulled out about ½ inch to reduce excessive tilting of the upper rows (which would otherwise result from the diameter of the stopper being larger than that of the tube). The tubes are left *undisturbed* at 36°C for 10 to 14 days, at which time clear luxuriant sheets of epithelial cells averaging 10 mm in mean diameter appear. Not every tissue fragment gives rise to a sheet of cells, for usually 2 to 4 cellular sheets are formed in each tube. The percentage of usable tubes (having cellular sheets of 8 mm or greater) varied from 70 to 100%.

Virus development in plasma and dry tubes. A comparison of the capacity of the two types of culture tubes to support virus growth was made by inoculating groups of each with high and low dilutions of Types 1 and 2 poliomyelitis virus and harvesting the tissue-culture fluids at intervals over a period of 6 days. The harvested fluids were frozen at −20°C. When all of the fluids had been collected they were titrated simultaneously, using serial tenfold dilutions. The Brunhilde strain of Type 1 was titrated at the time of inoculation and yielded a TC_{50} of 10^{-6} per 0.1 ml of inoculum in plasma tubes and $10^{-5.8}$ in dry tubes. The Y-SK strain of Type 2 was similarly titrated at this time and had a titer of 10^{-5} in plasma tubes and $10^{-5.3}$ in dry tubes. The stock viruses were arbitrarily diluted to give about 100,000 and 100 TC_{50} doses for Brunhilde and 10,000 and 10 TC_{50} doses for Y-SK virus. Intervals of 2, 6, 24, 48, 72, 96, and 144 hours were chosen for harvesting. Eight tubes of each type, plasma and dry, were taken at each collection period for each of the inoculated series, making a total of 64 tubes for each harvest. The tissue-culture fluids from the 8 tubes of each set were pooled into 2 groups of 4 tubes each. Each dilution in the titration series was inoculated into 4 culture tubes which were then followed for 6 days. The cytopathic changes usually became apparent in 2 to 3 days with large doses of virus and in 4 to 5 days with low doses. As shown in Fig. 1 and 2, there appeared to be no significant difference between the curves obtained with dry and plasma-clot tubes nor between those for the

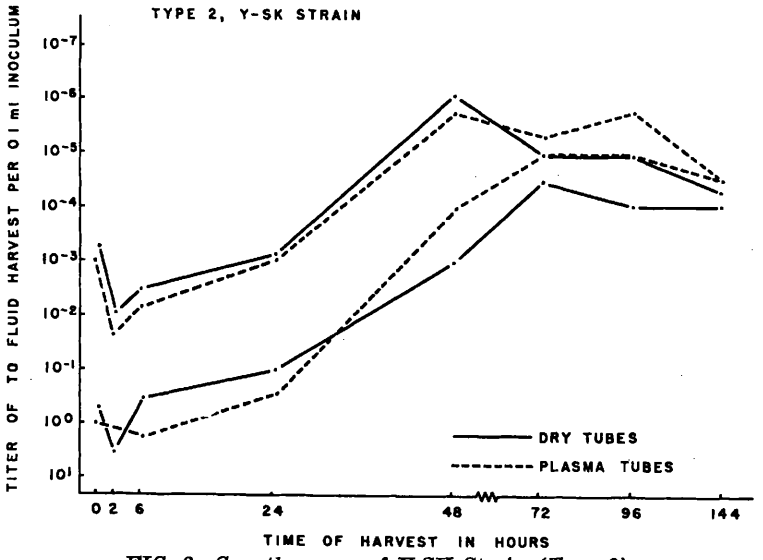

FIG. 2. Growth curves of Y-SK Strain (Type 2).

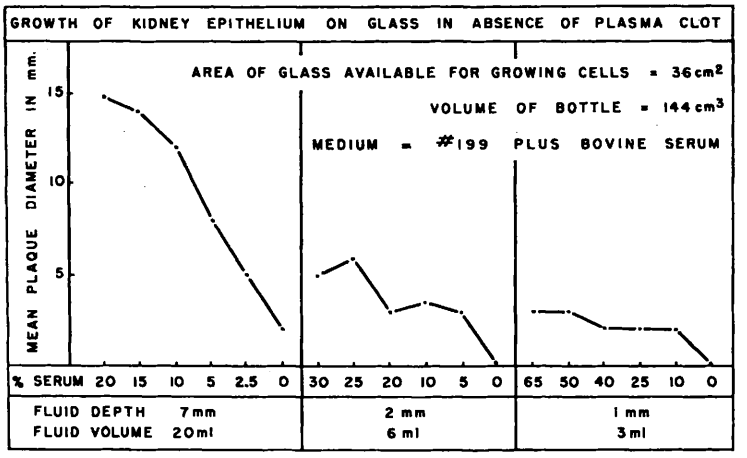

FIG. 3. Effect of serum concentration and fluid volume (or depth) on growth of kidney epithelial cells on glass.

2 strains of virus employed. The initial drop in titer was approximately 90%. Peak titers were reached in 24 to 48 hours in tubes receiving the high virus dose, whereas 72 hours were required for the more dilute viruses to attain corresponding titers. Cytopathic changes occurred at about the time the peak titers were observed. The highest titer observed was in the Y-SK series where the 48 hours sample rose to concentrations of 10^{-6} per 0.1 ml, in both dry and plasma clot tubes. When low concentrations of virus were used as inocula, virus could not always be detected in the standard 0.1 ml inoculum, even when undiluted fluids were tested. Therefore, in order to test for virus in such fluids, the sample being tested was used to replace the complete medium in the culture tubes of the test. In this way 1 ml of inoculum was introduced into the culture. If 2 of 4 tubes thus inoculated became positive, the titer was expressed as 10^{1}.

Dry technic in bottle cultures. The same technic employed in making dry tubes can be used in growing plasma-free cultures in bottles.

TABLE I. Growth of Tissue Following Transplantation from 12- to 14-Day-Old Cultures Prepared in "Dry" Tubes.

Group	Medium in transplanted tubes	Total No. of tubes with primary outgrowths	Secondary outgrowth of epithelial cells: Good growth No.	Good growth %	Min growth No.	Min growth %	% with growth	No growth No.	No growth %	Day of reading secondary culture
A 1	Transfer from original tube	82	18	22	23	28	50	41	50	4
2	Original + 0.25 ml fresh medium	46	36	78	2	5	83	8	17	4
3	Original + 0.5 ml fresh medium	92	74	80	6	7	87	12	13	4
B 1	Transfer from original tube	120	86	72	6	5	77	28	23	7
2	Original + 0.3 ml fresh medium	110	90	82	0	0	82	20	18	7

The amount of tissue needed varies with the size of the bottle. For example with a bottle having a flat side of 36 sq. cm., 4 or 5 drops of minced kidney suspension are placed on the inside wall and spread about with a pipette. The bottle is stoppered and turned to allow excess fluid to drain from the tissue. After 5 minutes at room temperature and ½ hour at 4°C, the complete medium is added to cover the tissue with about 7 mm of fluid. The growth rate of monkey kidney tissue in bottles parallels that of test tube cultures. Fig. 3 illustrates a typical experiment. Bovine serum was an important constituent of the medium; concentrations of 10%, when used with No. 199, were satisfactory for good outgrowth. Of the three depths of fluid studied in the experiment, that of 7 mm was found to give the best growth. Subsequent experiments have shown that with deeper layers of fluid (up to 20 mm) growth may be enhanced even further. Prior to inoculation of bottle cultures, tissue fragments are manually shaken loose from the wall of the bottles. The fragments and the media in which they are suspended are transferred to another sterile bottle and incubated further at 36°C. The original bottle is rinsed thoroughly with salt solution to wash out the bovine serum, and maintenance medium (equal parts of No. 199 and Earle's salt solution) is added to cover the tissue with about 7 mm of fluid. Bottles with confluent growth inoculated with 10,000 to 100,000 TC_{50} doses of virus yielded harvests 24 to 48 hours later with titers of $10^{-5.5}$ to $10^{-6.5}$, and at times even 10^{-7} per 0.1 ml. This has occurred with strains of all three types: Brunhilde and W-S (Type 1), Y-SK (Type 2) and Leon (Type 3). The fragments which have been transferred to the second bottle react differently from fresh fragments. They settle spontaneously in the presence of the complete medium, attach, and, apparently being predisposed to grow, reach satisfactory proportions in 6 to 8 days. The fragments may then be used for a second transfer, but not beyond this point because of their greatly reduced size. The original medium is transferred along with the fragments. However, it has been advisable on the second transfer to recharge it with ¼ to ½ volume of fresh medium. Additional experiments on the re-use of kidney fragments in the production of test tube cultures is given below.

Cultures made from transplanted fragments. Several thousand secondary cultures, and even tertiary cultures, have been prepared by the technic outlined under *Methods*. The addition of ¼ to ½ volume of fresh medium usually increases the number of useful tubes obtained (as in Groups A1, A2, and A3 of Table I) but this is not always true (as in Groups B1 and B2). The yield of tubes in the secondary cultures is of the same order of magnitude as in the primary cultures, about 75 to 90%. Simultaneous titrations of a number of virus samples of all 3 types were made in primary and secondary cultures, and there was no significant difference in the observed results (Table II).

Discussion. Culturing fragments of kidney tissue on glass without a plasma clot has many advantages over the technic with plasma. Cultures can be prepared more easily and more rapidly. The clotting system of plasma and chick embryo extract is no longer needed, nor

‖ Dr. Emanuel Eylan, Fellow of the World Health Organization, on leave from the Ministry of Health, Jerusalem, Israel, actively participated in these experiments during a brief visit to New Haven.

TABLE II. Replicate Titrations of Virus in Primary, Secondary, and Tertiary Epithelial Outgrowths.

Virus		Titer of virus (negative logarithm) in indicated culture		
		Primary	Secondary	Tertiary
Type 1. Br.	TC 11 A	6.0	6.0	
	B	5.5	6.0	
	C	5.8	6.0	
	D	5.5	6.0	5.5
2. Y-SK	TC 21 A	6.0	5.5	
	B	5.5	6.0	
	C	6.3	5.5	6.0
	D	5.8	6.0	
3. Leon	TC 58	6.0		5.5

is the antitryptic factor which is used to prevent lysis of the clot(7). Kidneys from a single monkey can yield 2000 primary cultures and at least 1000 secondary cultures. Such cultures grow readily when held stationary so that roller drums are unnecessary. The production of virus in large quantities, as required for vaccine production, or other purposes, is possible using sheets of epithelial cells of normal monkeys, bathed in protein-free media at the time of virus inoculation. Proteins from the kidney fragment itself or from the plasma clot are less apt to contaminate the virus yield. Although several workers have shown that virus can be grown in suspensions of kidney fragments without added protein, in our hands the epithelial outgrowth not only is more sensitive to smaller amounts of virus, but yields virus of higher titer. Finally, sheets of epithelial cells grown on glass in the absence of plasma by the present technic may offer certain advantages in observing virus-induced plaques(8).

Summary. 1. Monkey kidney epithelial cells can be cultured on glass without a plasma clot. The kidney fragments are induced to adhere to the wall of the test tube by preheating of the tube (45°C), and partial drying after introduction of tissue. These cultures compare well with cultures prepared in plasma clots, and respond as readily to the introduction of poliomyelitis virus. A great advantage is realized in the simplicity of preparation of these cultures, and their incubation in stationary racks. Exclusive of preliminary preparations and subsequent stoppering of the tubes, two trained workers can prepare well over 1000 cultures in 1 hour. About 2000 such cultures can be provided from a single monkey. 2. Bottle cultures can be prepared by the same technic, and have yielded poliomyelitis virus titers of about 10^{-6} per 0.1 ml inoculum. Titers in tubes or bottles reach their maximum in 24 to 72 hours depending upon the dose of virus inoculated. 3. After a culture has grown to satisfactory dimensions, original tissue fragments may be shaken off and transferred to new vessels. The fragments settle and produce comparable outgrowth in a shorter period of time than in the original culture.

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