Propagation of Poliovirus in Chick Embryo Cell Cultures. I. Cultivation of 3 Virus Types. (23313)

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Cells grown in culture are in certain instances susceptible to infection by a particular virus even though the tissue from which the cells were derived is not capable of being infected in the living animal(1,2). The results of the present study reveal another instance of this biological difference between cells *in vitro* and *in vivo*. We have been unsuccessful in numerous attempts to establish strains of Type 2 poliovirus in the chorioallantoic membrane (CAM) of chick embryos. However, strains of cells derived from this membrane and grown *in vitro* were readily infected with not only Type 2 poliovirus, but also representative strains of Types 1 and 3. The virus had a cytopathogenic effect and multiplication occurred.

Materials and methods. Cell cultures. CAMs from 10-day chick embryos were cut into 1-2 mm pieces, washed in Hanks' solution (H) and mixed with chick embryo extract (CEE)*, 0.1 ml per membrane. The CEE had been centrifuged during preparation at 4000 rpm for 30 minutes and frozen twice before use. About 0.1 ml suspended tissue and 1 ml frozen and thawed chicken plasma (CP)* were introduced into each 4 x 13 cm culture bottle. When clotting had occurred, 10 ml of 40% centrifuged human serum and 60% H ($HS_{40}H_{60}$)† was added. This medium was kept at 4°C for at least 2 weeks before use. The cultures were incubated at 36°C. Every 2-3 days when the medium had become acid, 5 ml medium was removed and replaced with 5 ml $HS_{40}H_{60}$. The first transfer was made when the sheet of cells began to break up due to partial release of fibrin. The medium was removed and to each bottle was added 10 ml 1% Bacto trypsin in H. After 2 hours at 36°C the contents were centrifuged and the sediment washed twice with H. The sediment was resuspended in H, 2 ml per original bottle. The tissue fragments were allowed to settle for 10 minutes when the supernate, containing chiefly individual cells, was removed and centrifuged. The sediment was resuspended in $HS_{40}H_{60}$, approximately 2 ml per bottle depending on the concentration of cells. This was transferred in 1 ml amounts to Leighton tubes containing fibrin clots on the flat areas. While establishing the strains, cultivation and transfer in tubes were the same as with the bottles except that volumes were 1/10 as large. These CAM cells were used for virus studies after 5 or more transfers when the cultures were free of tissue fragments and infection produced uniform cytopathic changes. As the cells grow, the fibrin clot becomes somewhat loosened from the glass. To prepare cultures for virus inoculation, the clot was teased off leaving cells on the glass. These were fed and inoculated when sufficient growth had occurred. The fibrin, containing most of the cells, was digested with trypsin and the cells transferred to fresh fibrin clots. *Virus studies.* The poliovirus strains employed were Mahoney, Type 1; Y-SK, Type 2; and Saukett, Type 3. These were obtained through the courtesy of Dr. Jerome T. Syverton, University of Minnesota. Stock suspensions consisting of centrifuged maintenance solution from infected HeLa cell cultures were stored at −17°C until used. CAM cell cultures were rinsed 3 times with H before inoculation. To each culture tube were then added 0.9 ml of a mixture of 10% chicken serum and 90% Scherer's maintenance solution (CHS_{10}-MS_{90})* and 0.1 ml virus suspension consisting of stock or fluid from a previous passage of virus in CAM cells. Virus titrations were performed by similar inoculations of cultures of HeLa or CAM cells using dilutions of virus

* Obtained from Microbiological Associates, Washington, D.C.

† Obtained from the Carver Foundation, Tuskegee, Ala.

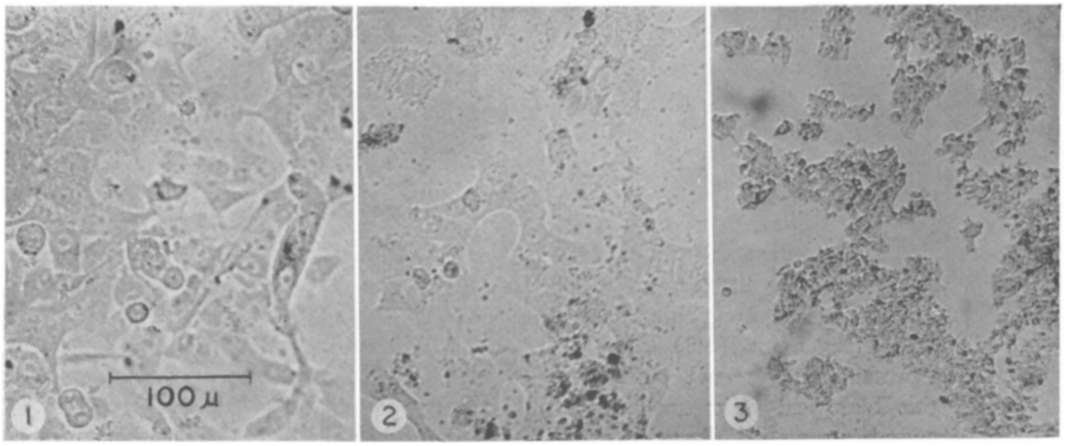

FIG. 1. Control CAM cells. Phase contrast.
FIG. 2. CAM cells showing partial degeneration 2 days after inoculation with poliovirus, Type 2.
FIG. 3. Similar cells showing degeneration at 4 days.

suspensions in $CHS_{10}MS_{90}$. Infection was assumed if there was +++ destruction of cells and no degeneration in the uninoculated controls. Tissue culture infectious doses were calculated by the method of Reed and Muench(3). Identification of the cytopathogenic agents was accomplished by neutralization tests. Virus suspensions, diluted 1:10, were mixed with equal volumes of polio type-specific monkey immune serum, 1:50, for 1 hour at 27°C and then used in 1.0 ml amounts to inoculate HeLa cells.

Results. CAM cells. Strains of CAM cells were established 3 times. All gave apparently identical results. More than 30 transfers have been made of one strain with no observed change in susceptibility to infection with poliovirus. Early transfers of CAM cells contain many spindle-shaped and round cells that are markedly granular. After some 5 transfers the proportion of spindle-shaped cells diminishes and polygonal cells become prominent, growing in small sheets (Fig. 1). The cytoplasm of these cells is almost clear. Occasional cells have narrow processes that are frequently 3 times as long as the diagonal of the angular cells. A few round cells persist for at least 30 transfers. Time-lapse cinephotomicrographs have not revealed movement or changes in shape of the round cells. Some of the polygonal cells, however, show active pinocytosis and locomotion without marked change in shape.

Infection with poliovirus. The rate of cytopathic change is related to the amount of virus introduced. At first the long processes are withdrawn; the cells become rounded and somewhat granular (Fig. 2). Cinephotomicrographs reveal sudden cytoplasmic vesiculation of some cells which may be due to increased intracellular pressure caused by contraction of the cell membrane and extravasation of cytoplasm through a rupture of the membrane. Later all motion ceases and masses of granular cells with ill-defined boundaries remain (Fig. 3).

Multiplication of poliovirus. Cultivation of poliovirus in CAM cells apparently could be continued for many passages or indefinitely. The titer was lower than that of the original seed virus which had been grown in HeLa cells. This may be due in part to incomplete adaptation of the virus, in spite of 20 passages in the case of strain Y-SK, or to an inherent quality of the CAM cells. Each passage produced a 10-fold dilution of the original inoculum. At the 10th passage the seed virus had been diluted 10^{-10} and at the 20th passage 10^{-20}; yet the titer was only about 2 log units below that of the seed virus, proving that multiplication occurred (Table I). Proliferation of poliovirus was not dependent on the presence of human serum as preliminary studies have shown that the virus

TABLE I. Tissue Culture Titrations of Poliovirus Propagated in Cells Cultured from Chick Embryo Chorioallantoic Membranes.

Test cells	Virus strain	Immunologic type	Seed virus	No. of passages in CAM cells				
				5	8	10	18	20
HeLa	Mahoney	1	$10^{-4.5}$	$10^{-3.8}$	$10^{-3.8}$	$10^{-3.2}$		
	Y-SK	2	$10^{-5.2}$		$10^{-3.3}$		$10^{-2.5}$	$10^{-2.8}$
	Saukett	3	$10^{-5.3}$	$10^{-4.9}$	$10^{-3.6}$	$10^{-3.5}$		
CAM	Mahoney	1			$10^{-3.0}$			
	Y-SK	2			$10^{-3.2}$		$10^{-3.0}$	$10^{-3.1}$
	Saukett	3			$10^{-3.5}$			

multiplies also in a strain of CAM cells grown exclusively in mixtures of H, CEE and chicken serum ultrafiltrate. In numerous neutralization tests, type-specific immune serum uniformly gave complete protection and so verified that the cytopathogenic agent in each instance was poliovirus.

Discussion. A fundamental biological difference between certain cells in the intact animal and cell strains derived from them in tissue culture is emphasized by the results of this study. At present no explanation can be offered for the fact that even though poliovirus generally fails to multiply in the unaltered CAM, the strains used infect, multiply in and destroy CAM cell cultures. Reports have appeared on unsuccessful attempts to cultivate poliovirus in chick embryo tissue *in vivo*(4) and *in vitro*(1,5). Roca-Garcia, Moyer and Cox(6) established strain MEF_1 in chick embryos by yolk sac inoculation following passage in suckling hamsters and we (7) obtained multiplication of the Lansing strain for a brief period in cortisone-treated embryos; but these appear to be rare instances of *in vivo* cultivation and in marked contrast to the ready cultivation of poliovirus in CAM cell cultures.

CAM cells may be valuable for cultivation of attenuated strains of poliovirus for human immunization. After establishing a strain of cells apparently free from any other virus, there would be greater security against contaminating virus than when recourse is made to fresh tissue as in the present use of monkey kidney cells. Conversion of the virus to a strain virulent for humans might be less likely to occur in CAM cells than in human or monkey cells as the CAM cells are derived from a different zoological class.

Summary. Cell cultures were prepared from the chorioallantoic membrane of chick embryos. After 5 transfers these were tested for susceptibility to infection by representative virulent strains of the 3 immunologic types of poliovirus. Multiplication was observed until the series were terminated after 10 or 20 passages. Neutralization tests confirmed the identity of the viruses after passage. The susceptibility of these cells to infection with poliovirus is in marked contrast to that of the tissues from which they were derived.

The authors wish to express their appreciation to Mr. Malcolm Parker for the cinephotomicrographs and Fig. 1 and 2; to Mr. Alex Gravesen for Fig. 3.

1. Enders, J. F., *Viral and Rickettsial Infections of Man,* 1952, Chap. 6, p. 133-134, Thomas M. Rivers, Editor, J. B. Lippincott Co., Philadelphia.

2. Evans, C. A., Byatt, P. H., Chambers, V. C., and Smith, W. M., *J. Immunol.,* 1954, v72, 348.

3. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

4. Enright, J. B., and Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 541.

5. Sabin, A. B., *Bull. N. Y. Acad. Med.,* 1957, v33, 17.

6. Roca-Garcia, M., Moyer, A. W., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 519.

7. Dunham, W. B., and Ewing, F. M., *J. Bact.,* 1953, v65, 224.

Received April 24, 1957. P.S.E.B.M., 1957, v97.