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Propagation of Mouse Polyoma Virus in a Strain of Mouse Mammary Tumor Cells *in vitro*. (25692)

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The mouse parotid tumor virus(1,2) designated as the S.E. polyoma virus(3), has stimulated much interest because of its oncolytic effect when grown in tissue cultures and its ability to produce multiple tumors in mice and hamsters(4-6). Development of hemagglutinating(7), complement - fixing(8), and mouse antibody production(9) procedures and plaque technics(10-12) provided simple tools for quantitation detection and study of this virus. In the present work tissue cultures of an established cell line of mouse mammary tumor have been used to support growth of polyoma virus, and observations on *in vitro* assay of the virus in these cells are reported.

Materials and methods. Virus. The 1956-8C strain of polyoma virus* used here had been cultured through 5 passages in mouse kidney cells. Virus stocks are prepared by inoculating 8 oz. bottles† of adult ICR mouse kidney cultures, grown by methods like those of Youngner(13), with 0.5-1.0 ml of undiluted polyoma virus infected tissue culture fluid. The supernatant was saved at each medium renewal and stored at –20°C. When cytopathic changes were nearly complete, cells were collected with the medium and pooled with earlier supernatants. *Tissue culture medium.* "Lactalplus medium," routinely used consists of lactalbumin hydrolysate(14) in modified Earle's salt solution(15), supplemented with vitamins(16), and other components (Table I). To promote cell growth, this medium was further supplemented with 10% calf serum; for maintenance and for viral infectivity titrations, 1% horse serum and $NaHCO_3$ in final concentration of 2.2 g/l were used. All media components were passed through Selas‡ filters under 5 lb pressure of 5% CO_2 in air. Vitamins and glutamine were prepared at 100 times the desired concentration, frozen at –20°C and incorporated into the medium immediately before use, as were penicillin, 100 μ/ml; streptomycin, 50 μ/ml; and neomycin, 20 μ/ml. *Tissue cultures* were prepared from a strain of cells of mouse mammary tumor (MT) originating spontaneously in a C3H mouse and maintained by serial passage in tissue culture for 7 months.§ Stock

* Kindly supplied by Dr. S. Stewart and Dr. B. Eddy, Nat. Inst. of Health, Bethesda, Md.

† Neutraglas serum bottle #14250, Kimbal Glass Co.

‡ Microporous porcelain, .02 and .03 porosity. Selas Corp. of America, Phila., Pa.

§ We are indebted to Dr. Crile Doscher, of Metropolitan Hosp., N.Y.C., and John Turpanjian of these laboratories for establishment and maintenance of this cell line.

TABLE I. Lactalplus Medium.

Ingredient	g/l	Ingredient	mg/l
NaCl	7.0	PABA	.1
KCl	.4	d-Biotin	1.0
$CaCl_2$	.2	Choline	1.0
$MgSO_4\ 7H_2O$	.2	Folic acid	1.0
$NaH_2PO_4H_2O$	.2	Nicotinic acid	1.0
$NaHCO_3$	1.1	Nicotinamide	1.0
Glucose	2.5	Pantothenic acid	1.0
Lactalbumin hydrolysate	5.0	Pyridoxal	1.0
		Riboflavin-5-PO_4	.1
L-cysteine	.05	Thiamine	1.0
Asparagine	.03	Ascorbic acid	50.0
Sodium pyruvate	.10		
L-glutamine	.2		
Phenol red	.001		

cells were grown in 8 oz culture bottles in the growth medium described and subcultured once a week. Experimental cultures were trypsin-dissociated cells from stock cultures resuspended in growth medium. Leighton tubes‖ having a flattened area of 12 x 40 mm near the base, each containing a glass coverslip and 16 x 150 mm tubes were inoculated with 1 ml (approximately 250,000 cells) of cell suspension. Tubes were stoppered and incubated in a nearly horizontal position at 37°C for 48 hours, then renewed with 1 ml of maintenance medium and used. Forty-eight-hour tube cultures of MT cells infected with 0.1 ml of 10^{-1} dilution of stock virus were incubated at 37°C with equal number of uninfected controls. At 2-day intervals the cultures were examined microscopically for cytopathic changes, and 4-8 were selected from both infected and uninfected groups for electron microscopy. From some the fluids were removed, pooled and centrifuged at 500 x g for 15 minutes. From others, the cells were scraped from glass with a 2-5 mm perforated cellophane, and both cells and fluids were collected. Fluids and fluids containing cells were frozen at −20°C for later hemagglutination and tissue culture infectivity titrations. For later study of cytopathic effects (CPE), cover slips were removed from infected and non-infected Leighton tubes at intervals fixed in Bouin's solution and stained with Harris's hematoxylin. Maintenance medium was renewed every 4 days, and the experiment terminated on 12th day.

Assay of virus. Tissue culture fluids alone were titrated for infectivity in tube cultures of MT cells; fluids containing cells were frozen and thawed 3 times and homogenized in a Ten Broeck grinder before titration. Serial 10-fold dilutions of fluids with and without cells were made in growth medium lacking serum and inoculated in 0.1 ml amounts into each of 2 or 3 cultures. Cultures were held 3 weeks, renewed with maintenance medium each 3 or 4 days, and observed daily for CPE. Titers were calculated by method of Reed and Muench(17) and expressed as number of tissue culture infectious doses ($TCID_{50}$)/0.1 ml. Hemagglutination (HA) tests(18) were performed on heated samples of fluid with and without cells. HA titers were expressed as the highest dilution which caused complete hemagglutination. Hamsters and ICR mice were inoculated within 48 hours of birth with 0.2 ml of original undiluted stock virus and with fluids from cultures infected 8 days.

Electron microscopy. Cells were fixed by replacing supernatant with 1% osmic acid buffered to pH 7.3 and scraping cells from the glass. After a 50% alcohol rinse, they were dehydrated by successive applications of 70, 95 and 100% alcohol, and small portions of sediment were embedded in 75% butyl 25% methyl methacrylate. Sections cut on a Porter-Blum microtome with glass knives and picked up on carbon-filmed specimen grids, were observed with an RCA EMU3C microscope.

Results. Light microscope observations. CP effects in infected MT cultures during 12 days incubation were noted in Table II. Uninfected cells varied from polygonal to spindle-shaped and, where less crowded, showed connecting protoplasmic processes. Nuclei were large and prominent; giant cells and multinucleated cells were sometimes seen (Fig. 1). CPE seen earliest 6 days after infection was characterized by scattered necrotic cells, somewhat rounded, shrunken, and with densely staining nuclei. Cytoplasm around many of these nuclei appeared fragmented, and no intact cell wall could be seen. Subsequent ratings of CPE were based primarily on increasing number of necrotic cells and cellular debris. Ten days after infection, fragments of densely staining cells and of destroyed cells peppered the culture. Some ap-

‖ Microbiological Assoc., Bethesda, Md.

TABLE II. Correlation of Cytopathic Effects with Tissue Culture Infectivity and Hemagglutination Titers of Polyoma Virus on Mouse Mammary Tumor Cells *In Vitro*.
(Original inoculum 4.5 $TCID_{50}$)

Days incubation	CPE	Tissue culture infectivity titers*		Hemagglutination titers†	
		Fluid only	Fluid + cells	Fluid only	Fluid + cells
2	0	4.0	4.0	0	0
4‡	0	4.5	4.5	0	0
6	<+	4.5	5.0	1:16	1:256
8‡	2+	5.0	6.0	1:256	1:1024
10	2-3+	5.5	6.5	1:256	1:2048
12	4+	5.5	6.5	1:512	1:2048

* Tissue culture infectivity titers expressed as log 10 of $TCID_{50}$/0.1 ml.
† Hemagglutination titers expressed as highest dilution causing complete hemagglutination.
‡ Culture fluid renewal.

parently unaffected cells could also be seen (Fig. 2). After 12 days of infection, most cells appeared necrotic and disintegrating.

Virus HA and tissue culture titrations. Infectivity titrations in tissue culture and results of HA tests on tissue culture fluids from infected cultures, Table II, clearly show that polyoma virus multiplied and that highest levels of virus production and release began around 8 days after infection. Titrations of centrifuged fluids and fluids containing disrupted cells show also that a significant amount of the virus produced remains associated with cells and is not released immediately into the medium. This observation supports that made by Sachs on polyoma-virus-infected mouse embryo cells(19). Virus production appears to accompany cytopathic changes observed under light microscope. Failure of fluids from 2-day and 4-day cultures to give positive HA results is not clearly understood. Positive HA results were obtained with tissue culture fluids beginning 6 days post-infection, *i.e.*, with early cytopathology and probably early virus production. Tissue culture titrations, however, revealed presence of virus (possibly entirely that introduced with original inoculum) in both 2-day and 4-day culture fluids.

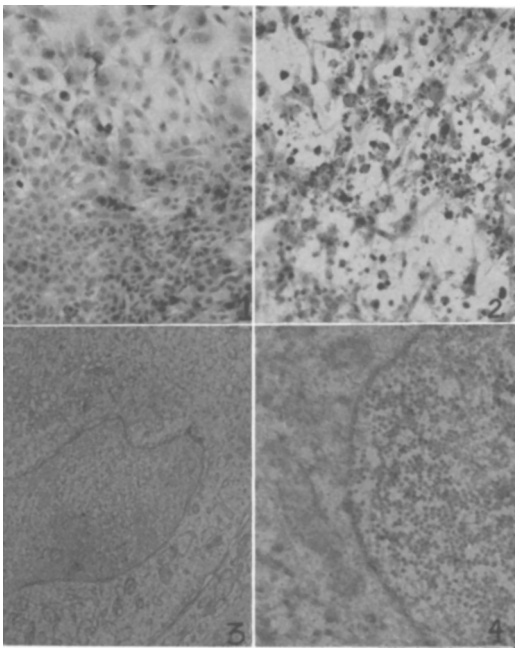

FIG. 1. Uninfected mouse mammary tumor cells incubated 12 days. Harris' hematoxylin stain. × 87.

FIG. 2. Mouse mammary tumor cells 10 days after infection with polyoma virus. Harris' hematoxylin stain. × 87.

FIG. 3. Portion of uninfected mouse mammary tumor cell showing large irregular nucleus with marginated chromatin and small mitochondria. × 5280.

FIG. 4. Portion of cell 12 days after infection showing concentration of virus particles within nucleus and necrotic state of cytoplasm. × 12,820.

Electron microscope observations. In uninfected cells, relatively large irregular nuclei could be seen. Chromatin was unevenly distributed, tending to concentrate near the nuclear membrane. The cytoplasm contained many small mitochondria generally ellipsoid in shape (Fig. 3). Six days after infection the cytoplasmic components of some cells began to show loss of structure and, in some thin sections, virus-like particles could be seen in the enlarged nucleus. Eight days after infection the majority of cells were infected; particles were numerous in the nuclei, and occasionally a few were seen in the cytoplasm. Whether these latter emerged from the nucleus is not

known. The intranuclear particles appeared to increase rapidly with a concomitant necrosis of the cytoplasm. About 12 days after infection in those cells which had not been lysed, virus particles practically filled the nucleus, but showed no tendency to form geometric arrays (Fig. 4). They averaged approximately 30 mμ in diameter, and no internal structure has been seen.

Animal inoculations. On the whole, tumor production in hamsters and mice with this strain of virus has been disappointing. Out of 4 hamster litters (20 animals) inoculated with original stock virus, only 7 animals survived weaning of which 2 developed a solid subcutaneous tumor mass at site of inoculation at 2 months of age, and one developed multiple neoplasms and heavy ascitic fluid at 3 months of age. Out of 4 litters (18 hamsters) inoculated with tissue culture fluid collected from cultures 8 days after virus infection, 9 animals survived weaning. Two of these developed solid tumor masses at site of inoculation at 2.5 months of age, and one developed tumor of heart and liver at 3.5 months. Out of 8 ICR mouse litters (48 animals) inoculated with original undiluted stock virus and tissue culture fluids collected 8 days after virus infection, 31 animals survived weaning. Of these, 3 developed multiple tumors (parotid and mammary gland, heart and liver) at 4 months, and 2 developed parotid gland tumors only at 4.5 months. No animals receiving uninfected tissue culture fluids have developed tumors after 4.5 months.

Discussion. This strain of MT cells is particularly useful for tissue culture titrations of materials infected with polyoma virus. Infected cultures can be maintained in a satisfactory condition for 3 weeks or more in medium containing low concentrations of serum —a factor of some importance because of inhibiting properties of serum on this virus(11). Usefulness of a milk-adapted strain of P388 mouse lymphoma cells for studies on polyoma virus has been noted(20). Primary cultures of whole mouse embryo or adult mouse kidney generally used for this purpose may be less desirable because of the possible introduction of latent mouse viruses. Uninfected control cultures of the MT cell line have not shown cytopathic effects due to polyoma virus infection as sometimes observed in control tubes of primary whole mouse-embryo cultures. Moreover, separately prepared batches of tube cultures from stock MT cell strain gave satisfactorily uniform results on titrations of virus activity in contrast to irregular results sometimes observed with primary mouse-embryo cultures(9).

That polyoma virus CPE on tissue culture cells is accompanied by virus production agrees with the report of Eddy *et al.*,(3) who demonstrated that only fluids from infected tissue cultures which showed cytopathic changes produced tumors in hamsters. The relatively low incidence of tumors induced by these preparations is presumably due to the considerable number of serial passages of virus in tissue culture and accords with the findings of Drs. W. P. Rowe and S. E. Stewart.¶ Neutralization tests in tissue cultures of MT cells with sera from several mice and hamsters selected from our stock animals did not indicate presence of polyoma antibodies. Three different tissue culture passages of this strain of polyoma virus, one representing 7 passages in MT cells, one representing 4 passages in mouse kidney cultures, and one representing 5 passages in whole mouse-embryo cultures were completely neutralized in hemagglutination-inhibition assay(21) by a sample of polyoma virus antiserum kindly supplied by Dr. W. P. Rowe.

Summary. 1) An established cell line originating from a spontaneous mouse mammary tumor is a convenient host in which to propagate and to assay polyoma virus *in vitro*. Cytopathic effects of the virus were first observed in cultures 6 days after infection and progressed to complete destruction of all cells by 12th day. Increasing cell destruction was accompanied by rise in tissue culture infectivity and hemagglutination titers. 2) Observations with electron microscope revealed presence of virus in infected cells beginning 6th day after infection. Virus particles were observed in the nucleus and occasionally in the cytoplasm. Particles averaged 30 mμ in diameter and did not show an internal structure.

¶ Personal communication.

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Electrolytes in Denervated and Reinnervated Muscle. (25693)

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Since the work of Hines and Knowlton(1) on ion changes in the denervated muscle it has not been clear whether the decrease of K ions in the denervated muscle is the result of a decrease in intracellular K concentration(2) or is only proportional to diminution of the muscle phase itself and to increase in relative amount of intercellular collagen phase(3). The content of extracellular ions Na and Cl increases after denervation when expressed in mequiv. Na and Cl/100 g fat-free dry solid (FFDS)(1,2,3). However, ion changes in denervated muscles have not been evaluated in absolute units, *i.e.*, total amounts of Na and Cl ions present in whole normal and denervated muscles have not been compared. Such an evaluation makes it possible to study the relationship between extra- and intracellular muscle phases in denervation atrophy. Moreover, if ion composition of the denervated muscle is changed, it is very important to know whether and how it returns to normal in the course of reinnervation. These aspects are the subject of this report.

Methods. Experiments were performed on male rats of 150-200 g body weight. Ion composition was studied in extensor digitorum longus following section of peroneal nerve. For reinnervation studies, denervation was induced by crushing the sciatic nerve; this procedure leads to reinnervation of the muscle and restitution of function about 14 days after nerve crushing(4). For these experiments the anterior muscle was used. Na and K ions were determined by flame photometry, Cl ions by modification of Sanderson's method(5). Non-collagenous protein nitrogen was extracted from the muscle with 0.1 N NaOH, precipitated with TCA, and after Kjeldahlisation was determined by Conway's micromethod(6).

Results. Decrease in non-collagenous protein nitrogen occurs in denervated muscle at the same rate as the decrease in K content