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Received September 9, 1963. P.S.E.B.M., 1963, v114.

SV-40 Induced Proliferation of Tissue Culture Cells of Rabbit, Mouse, and Porcine Origin. (28780)

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The simian vacuolating virus (SV-40) is oncogenic for the newborn hamster(1,2) and induces transformation of hamster(3-6) and human(7-9) tissue maintained *in vitro.* Although several attempts have been made, no oncogenic action of this agent has been demonstrated in the mouse or rabbit(10,11).

To obtain more information about the relationship between transformation *in vitro* and oncogenesis *in vivo,* the effect of SV-40 infection of rabbit and murine, as well as porcine kidney monolayer cultures was studied. In all 3 types of culture a proliferative response occurred which has many similarities to the transformations described with both hamster and human tissue. In this report, transformation is defined as development, in a tissue culture, of a population of rapidly proliferating cells with altered morphology and abnormal growth pattern, with the cells maintaining their abnormal characteristics during prolonged sub-cultivation *in vitro.*

Materials and methods. Virus. SV-40 strain 777 was obtained from Dr. P. Gerber. It was isolated in African green monkey kidney (AGMK) cultures, passed once in the rhesus kidney cell line $LLCMK_2$(12), and thereafter in AGMK. The virus pools used in these experiments were from the 4th and 5th additional AGMK passages, and had infectivity titers of $10^{4.8}$ and $10^{5.3}$ $TCID_{50}$/0.1 ml, respectively. *Tissue Culture.* Cultures were prepared on contract by Dr. T. G. Ward of Microbiological Associates, Inc., Bethesda, Md.; they were prepared by trypsinization of minced kidney fragments and were grown in Eagle's basal medium (BME) with 5% calf serum and antibiotics. Rabbit kidney cultures were prepared from 2-week-old New Zealand white rabbits (Romar Rabbitry, Suitland, Md.). Mouse kidney cultures were prepared from NIH Swiss and C3H/Mai weanling mice. The latter strain is of Bittner origin and was obtained from Dr. Ludwik Gross. Porcine kidney monolayer cultures were derived from embryos.

Tube cultures were used for transformation studies when the cell sheet was fully developed. Each culture received 0.1 ml of undiluted virus; uninoculated cultures served as controls. Maintenance medium for the first rabbit kidney experiment consisted of BME with 5% heated (56°C 30 min.) agammaglobulinic calf serum,* 100 units penicillin, and 100 μg streptomycin. Thereafter, medium NCTC 109 with 10% unheated agammaglobulinic calf serum and antibiotics was used as maintenance medium for all experiments, and as growth medium for cell lines; this medium has been found particularly suitable for rapid transformation of hamster kidney cultures by SV-40(13). Cultures were held in a stationary position at 36°C, and medium was changed twice each week. Samples of fluids from infected cultures were pooled at weekly intervals and stored at −60°C until virus testing. All time intervals stated refer back to the day of virus inoculation.

Tests for SV-40 virus were carried out in

* Agamma Calf Serum—Hyland Laboratories, Los Angeles, Calif.

tube cultures of AGMK. Supernatant culture fluids were assayed for virus by inoculating 0.1 ml of the undiluted fluid into 2 or 3 AGMK cultures. Cell suspensions, containing 2-4 $\times$ 10^5 cells/0.1 ml, were tested for SV-40 infectivity by disrupting the cells by freeze-thawing 3 times and inoculating 0.1 ml of the undiluted extract into 2 or 3 AGMK cultures. All cultures were observed for the characteristic SV-40 cytopathogenic effect for 30 to 40 days. At that time, negative cultures were frozen and thawed 3 times, the fluid-cell mixtures of replicate cultures were pooled, and 0.1 ml used to inoculated fresh AGMK cultures. These were observed for an additional 30 days. Maintenance medium for AGMK cultures consisted of BME with 2% heated agammaglobulinic calf serum, 2 mM glutamine, and antibiotics. Tubes were kept in a stationary position at 36°C and were examined 2-3 times each week.

Transplantation. Serially passaged cells grown in 32-oz. Blake bottles were washed with phosphate buffered saline (pH 7.2), and either dispersed with trypsin or scraped from the glass with a rubber policeman. These cells were then centrifuged at 1,000 RPM and suspended in medium NCTC 109 with 10% agammaglobulinic calf serum. The cells were counted in a hemocytometer but accurate cell counts could not be made because of clumping. Cell suspensions were inoculated into the subcutaneous tissue of the back of isologous or homologous animals.

Results. Rabbit kidney cultures. Five primary monolayer cultures of rabbit kidney inoculated with $10^{4.8}$ $TCID_{50}$ of SV-40 appeared similar to the control cultures for the first 53 days. At this time they consisted of well preserved cell sheets containing both epithelial and fibroblastic calls, but the cytoplasm was granular in appearance. On the 56th day a small focus of new growth of small epithelioid cells was noted in one of the inoculated cultures. Within 10 days the focus enlarged and overgrew the adjacent cells; the new cells piled up on each other, forming a dense multi-layered patch of tissue which was macroscopically visible. Multinucleated giant cells were present at the peripheral portions of the new epithelioid growth. In the central heaped up portion, superficial necrosis and sloughing of cells occurred. Over the next 34 days, 4 additional areas of new growth appeared in this culture and overgrew the surrounding tissue. On the 110th day the cells in this culture were dispersed with trypsin and a cell line established (see below).

Similar changes were observed in one other inoculated culture, the new growth appearing at 67 days. The remaining 3 inoculated cultures degenerated "nonspecifically" from the 90th to the 110th day. No proliferative foci appeared in the 5 control cultures, and nonspecific degeneration occurred from the 80th to the 100th day.

In the second experiment with rabbit kidney cultures, using the same amount of virus but a different maintenance medium (see *Materials and methods*), similar results were obtained. Proliferative foci appeared in 4 of 10 inoculated cultures, the earliest changes occurring at 57 days. By the 80th-100th day most of the tissue in the 5 control cultures had degenerated. It is of interest that inoculated cultures in which proliferative changes did not occur, remained well preserved for over 128 days. By 143 days all cultures had degenerated.

Cell line of "transformed" rabbit kidney cells. As mentioned above, a continuous cell line of rabbit kidney cells was established from a proliferative culture in the first experiment. This line (TRK-1) is currently in its 16th passage. The cell type is predominantly epithelioid, but spindle shaped cells are also present. Mitotic figures are abundant even in fully developed monolayers. The nuclei are pleomorphic with many bizarre forms, and the nucleus-cytoplasmic ratio is increased. Large syncytial giant cells, containing 28-30 nuclei are present within the cell sheet (Fig. 1). There is a tendency for the epithelioid cells to grow atop of one another, forming thickened patches of tissue which are grossly visible. In later passages, cultures consisted almost entirely of these nodular growth centers with little tendency to confluent monolayer formation.

To evaluate the ability of uninfected cultures to be maintained in serial passage, control cultures from the same experiment were

trypsinized at the time the infected cells were passed. These control cultures could be maintained for only 2 passages. Rabbit kidney cells from fresh monolayer cultures, passed at 1-2 week intervals, survived for only 5 serial passages.

Approximately 5×10^5 "transformed" cells at the 6th passage level were inoculated

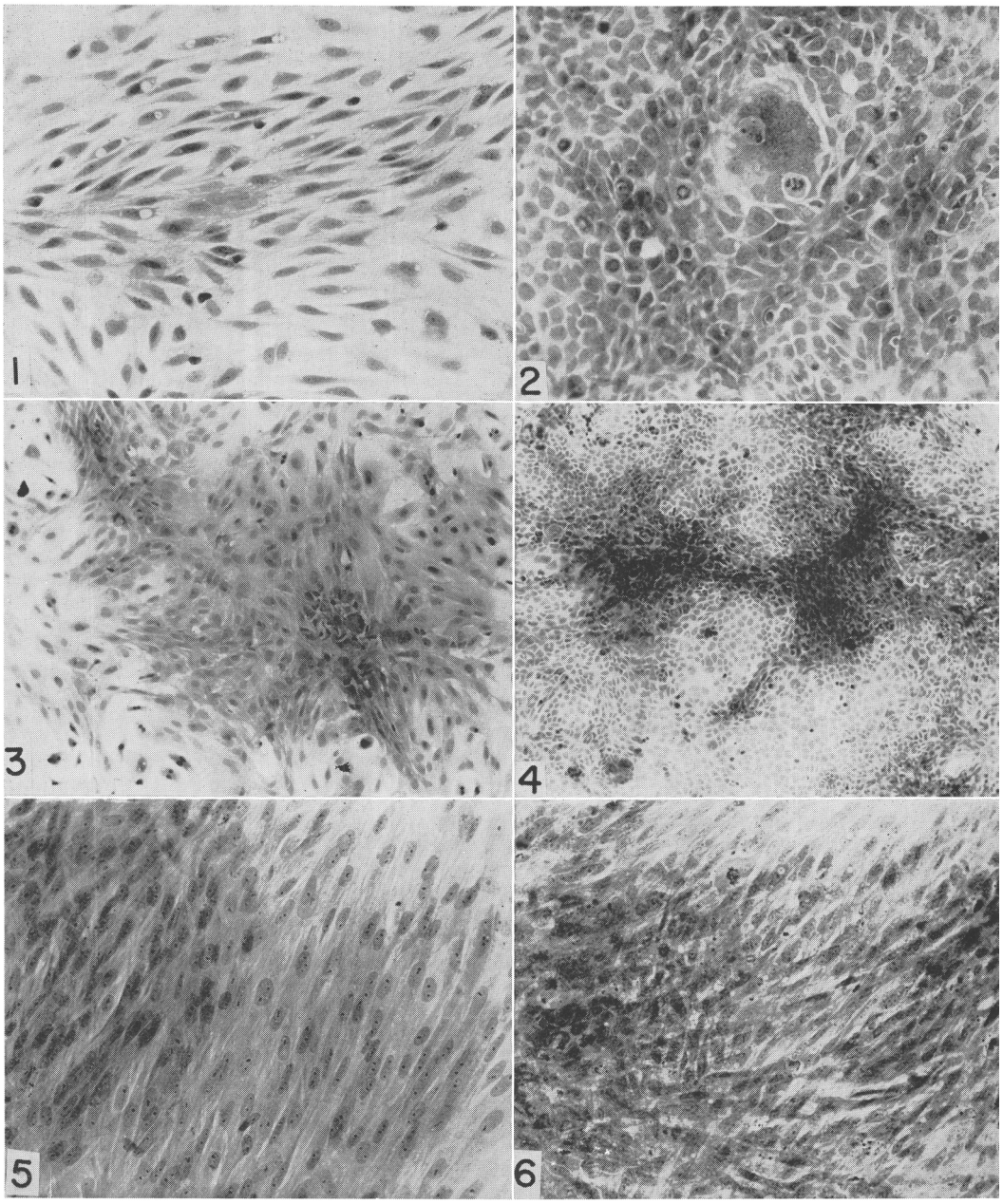

FIG. 1. (1) Normal rabbit kidney monolayer culture in 2nd passage. H & E stain. 150×. (2) TRK-1 cells in 7th passage. Note epithelioid morphology and multinucleated giant cell containing cells in mitosis. H & E stain. 150×. (3) Normal Swiss mouse kidney monolayer culture in 2nd passage. H & E stain. 63×. (4) TSMK-1 cells in 2nd passage. Note epithelioid morphology. H & E stain. 63×. (5) Normal porcine kidney monolayer culture in 2nd passage. H & E stain. 150×. (6) TPK-1 cells in 3rd passage. Note random growth pattern and frequent mitotic figures. H & E stain. 150×.

into the subcutaneous tissue of the back of eleven 3-day-old rabbits, and 2×10^6 cells similarly inoculated into 2 weanling rabbits; intracutaneous injection of 1×10^6 cells was also made in each of the latter animals at a different site on the back. After 5 months, no tumors have appeared in the 7 surviving rabbits inoculated in the newborn period, or in the 2 weanlings.

Mouse kidney cultures. Three experiments, using mouse kidney monolayers derived from 2 strains of mice, were carried out. A total of 23 cultures were inoculated with SV-40; those prepared from NIH strain Swiss mice received $10^{4.8}$ $TCID_{50}$, while those which derived from C3H mice were inoculated with $10^{5.3}$ $TCID_{50}$. Since the patterns of response were similar they will be discussed together.

In inoculated cultures, scattered multinucleated giant cells appeared within 6 to 10 days. By the 14th day, foci of new growth had appeared in several cultures. The morphology of the proliferating cells was difficult to characterize, being of a triangular, "epithelioid" shape for the most part; spindle-shaped cells and multinucleated giant cells were also present in the new growth. As in the rabbit kidney cultures, piling up of cells and rapid overgrowth of surrounding tissue occurred, so that thickened plaques were observed macroscopically by the 22nd day. At the 33rd day, 18 of the surviving 19 inoculated cultures showed these changes. Over the next 20 days, this unrestrained growth continued, forming a dense mass of tissue up to 1 mm thick. At this time, tearing and retraction occurred in several cultures with subsequent degeneration. In some cultures, regrowth of tissue succeeded the tearing and retraction. Some cultures, surviving as long as 206 days after inoculation, have repeated this pattern several times.

The 17 control cultures showed no evidence of focal proliferation. In all control tubes, tearing and retraction of the monolayer commenced by the 16th day, and most of the tissue had degenerated by the 33rd day. Some regrowth of fibroblasts occurred in several cultures from the 29th to 55th day but no piling up was observed. All control cultures had degenerated by the 65th day.

Cell lines of "transformed" mouse kidney cells. Two cultures, one each from Swiss and C3H mice, and showing the changes described above, were trypsinized at 44 and 55 days, respectively, and cell lines established; these lines, designated TSMK-1 and TCMK-1, are currently in the 22nd and 21st passages, respectively. Both lines are composed predominantly of epithelioid cells, but spindle-shaped cells and multinucleated giant cells are present as well (Fig. 1). As in the TRK-1 line, cells from both these lines have pleomorphic nuclei, increase in the nucleus-cytoplasmic ratio, and a high mitotic index. The cells tend to overgrow one another, forming thickened multi-layered foci as in the primary cultures.

Too few cells remained in control cultures for passage. However, fresh monolayer kidney cultures, prepared from NIH Swiss mice, were serially passed at 1-2 week intervals; this line could not be maintained beyond the 6th passage because of non-specific deterioration.

Third passage TSMK-1 cells were inoculated subcutaneously into 14 newborn ($1\text{-}2 \times 10^5$ cells) and 6 weanling ($1\text{-}2 \times 10^6$ cells) NIH Swiss mice; approximately the same number of 3rd passage TCMK-1 cells were inoculated into 8 newborn and 8 weanling C3H/Mai mice. All Swiss mice survived, and none had tumors after 4 months' observation; neither were any takes apparent after 4 months in 6 surviving C3H mice inoculated when newborn, nor in the 8 C3H mice challenged as weanlings.

Porcine kidney cultures. Five porcine kidney cultures, each inoculated with $10^{5.3}$ $TCID_{50}$ of SV-40, resembled uninoculated control cultures for the first 39 days; the cell type was predominantly fibroblastic. By the 47th day, inoculated cultures appeared to be more heavily populated with cells than control cultures. At 51 days, foci of proliferation with piling up of the cells were observed in 3 of the 5 infected cultures. Within the proliferating foci, cells of both epithelioid and fibroblastic morphology were present. During the following 20 days the process continued with the production of thickened, nodular areas, visible macroscop-

ically, and with more rapid acidification of the medium. All the inoculated cultures contained proliferative foci by the 85th day. In general, the pattern of proliferation, piling up, and retraction was similar to that observed with rabbit and mouse kidney cultures, but the changes occurred more slowly and were not as extensive. Two cultures observed for 185 days continued to show this pattern.

The 5 control cultures remained well populated and contained areas of fibroblastic outgrowth from nodal areas through the 63rd-72nd day. However, these areas did not persist and soon degenerated, as did the other parts of the monolayer; by the 85th-104th day only a few scattered small islands of cells remained.

Cell line of "transformed" porcine kidney cells. On the 104th day after inoculation an altered culture was trypsinized and a cell line established; the line (TPK-1) is currently in the 9th passage. Too few cells remained in control cultures for passage. Fresh porcine kidney cultures were trypsinized but the cells could be maintained for only 3 serial passages.

As observed with the other continuous lines described above, the TPK-1 line contains predominantly "epithelioid" cells which grow randomly and pile up to form thick nodular growth centers (See Fig. 1). Marked nuclear pleomorphism with an increased nucleus-cytoplasm ratio is also present in these cells. No efforts to transplant these cells have been made.

Recovery of virus. Table I summarizes the virus isolation results in the various experiments. In rabbit and porcine cultures, SV-40 was recovered from the culture fluid throughout the period of maintenance of the primary cultures. Virus was recovered in moderate titers from the Swiss and C3H mouse cultures through the 44th and 55th days, respectively. Supernatant fluids from the C3H cultures were devoid of demonstrable infectivity at 4 to 5 months, at which time the entire cell population consisted of transformed cells.

After passage of cells and establishment of cell lines, virus was no longer readily detectable, either in culture fluids or cell suspensions, in the rabbit and mouse cultures. The swine cultures produced larger amounts of virus in the primary cultures, and the cell line contained virus in moderate titer.

Discussion. The proliferative changes in rabbit and mouse cultures described here resembled closely the transformation of hamster and human kidney cultures by SV-40 (3-9). The focal nature of the proliferation, predominance of epithelioid cells, multinucleated giant cells, nuclear abnormalities, abnormal growth pattern, ability to propagate in prolonged serial passage, and increased rate of acid production are common to all four systems, and would appear to warrant use of the term "transformation" for the changes reported here. The changes in pig kidney cultures were essentially the same, differing only in the lack of giant cells.

The similarity of these changes to hamster cell transformation, plus the absence of such changes in uninoculated controls, leaves little doubt that the alterations were induced by SV-40.

In contrast to transformed hamster cultures, the rabbit and mouse cells did not induce tumors on transplantation into homologous hosts. However, the attempts thus far can only be considered as preliminary. The rabbits and Swiss mice used for transplantation were not inbred, or conditioned by corticosteroids or X-irradiation; it is unlikely that histoincompatibility played a role in the failure of transplantation of the transformed C3H mouse cells in isologous mice.

The results of virus recovery attempts in the rabbit and mouse cell cultures were also very similar to the hamster cell system(13). Virus was continually present in most of the original cultures, but declined in titer and was not detectable in fluids from mouse kidney cultures maintained for 4 to 5 months or in the fluids or cells of the passaged cell lines. It may well be that infectivity could have been detected by overlaying the cells on AGMK cultures(2,14), a procedure necessary for detection of virus in SV-40 transformed hamster cells(13,15). Despite the low virus titers in the rabbit and mouse cultures, there is little doubt that the virus propa-

gated. The virus was readily recovered after the theoretical dilution of the inoculum by fluid changes had surpassed 10^{-18} in the case of the mouse cells, and 10^{-37} in the rabbit cultures. The virus growth pattern in the swine cultures resembled that seen with transformed human kidney cells, with moderate titers being recovered continually(9, 16).

Although the passage lines of transformed rabbit and mouse cells did not contain extractable virus, we have shown elsewhere(17) that they, as well as the transformed pig kidney cell line, elaborate the "tumor specific" SV-40 CF antigen found in transformed and tumor cells of hamster origin(18). It thus appears probable that the transformed rabbit and mouse cells contain at least a portion of the SV-40 viral information. The presence of this antigen provides further evidence for

TABLE I. Virus Recovery from Rabbit, Mouse and Porcine Kidney Tissue Cultures.

			Test for virus AGMK		
Species	Passage No.	Days in culture	Material tested	Incubation period* (days)	Titer (log_{10})
Rabbit (Exp 1)	0	→ 56	Supernatant	20, 20	
		88	"	20, —	
		109	"	24, 28, —	.0†
	1	115	"	trace‡	
	2	118	"	—	
	4	141	"	—	
	4	141	Cells§	—	
	12	236	"	—	
Rabbit (Exp 2)	0	9	Supernatant	7, 8	
		31	"	9, 9	
		→ 57			
		58	"	9, 16	
		85	"	16, 21	
		107	"	16, 21	
		129	"	20, —	.0
Mouse (Swiss)	0	→ 22			
		25	Supernatant	13, 16	
		32	"	20, 20	
		38	"	16, 16	
		44	"	16, 16	1.5
	2	55	"	—	
	2	55	Cells	—	
	16	132	"	—	
Mouse (C3H)	0	→ 14			
		36	Supernatant	13, 13	
		49	"	16, 16	
		55	"	16, 16	1.5
		149	"	—	
		169	"	—	
	2	65	"	—	
		65	Cells	—	
	4	69	"	—	
Pig	0	15	Supernatant	8, 9	
		36	"	6, 6	
		→ 51			
		57	"	6, 6	
		78	"	6, 6	
		92	"	6, 6	4.0
		148	"	6, 6	>3.5
	2	113	"	6, 6	2.8
	2	113	Cells	5, 5	3.5

→ Indicates day transformation first noted.

* Length of time prior to appearance of SV-40 cytopathogenic effect in each AGMK culture.

† Expressed as log_{10} $TCID_{50}$/0.1 ml culture fluid or disrupted cell suspensions.

‡ Virus recovered on blind passage of the pooled fluids.

§ See *Materials and methods* section.

the specificity of the transformations described here.

The findings presented here indicate that SV-40 has a remarkably broad host range for tissue culture cells, both in its capacity to multiply, and to a lesser extent, to induce cell transformation. The only reported failure of SV-40 to multiply in tissue cultures is the report of Gerber(19), with rabbit kidney cell cultures; however, our data indicate that under somewhat different conditions the virus does propagate in these cells. In experiments carried out identically with those described here, we have found that fetal bovine kidney cell cultures also showed the same pattern of virus growth as the rabbit and mouse cultures, with liberation of virus for at least 99 days, but without evidence of cellular transformation (unpublished data).

In the past, transformation by viruses *in vitro* has been correlated with oncogenicity for the same species *in vivo*. Since SV-40 has not yet been found to produce tumors in rabbits and mice(10,11), our findings suggest that there may not necessarily be complete correlation. If this be true, the transformation of human tissue cultures by SV-40 (7-9) would have less dire implications.

Summary. A proliferative response and cellular changes occurred in primary rabbit, mouse, and porcine kidney cultures infected with SV-40 which resembled the transformations induced by this virus in hamster and human tissue cultures. Continuous cell lines of the altered cells from the 3 species were established; preliminary attempts to transplant these cells to homologous or isologous species have failed. SV-40 proliferated in all 3 species cultures but was not present as extractable infectious virus in the passage lines of rabbit and mouse cells.

The authors wish to acknowledge the excellent technical assistance rendered by Mr. Richard Maloof.

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Received September 9, 1963. P.S.E.B.M., 1963, v114.