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[9] Heath, T. L., in *Euclid's Elements* (New York, 1956), vol. 2, Book IX, Proposition 14.

[10] *Added in proof:* Recently the author has established the infinitude of pure non-Euclidean models. In addition, he has established the recursive unsolvability of the decision problem as to pureness of an arbitrary model of *FTA*.

AN ANALYSIS OF SV40-INDUCED TRANSFORMATION OF HAMSTER KIDNEY TISSUE IN VITRO, I. GENERAL CHARACTERISTICS

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Several reports have described transformation of newborn and adult Syrian hamster kidney and hamster embryo tissue cultures by the simian vacuolating virus SV40.[1-4] This report describes the effect of various factors on the rate of transformation of hamster kidney cell cultures, the tumors induced by transplantation of transformed cells, and the pattern of virus recovery from the transformed cultures.

Materials and Methods.—Virus: Strain 777 of SV40 was obtained from Dr. Paul Gerber, and virus stocks were prepared as previously described.[5] The pools used in the majority of these experiments were derived from the 3rd, 4th, and 5th Cercopithecus kidney (African green monkey, AGMK) passages and had infectivity titers of $10^{6.5}$, $10^{4.8}$, and $10^{5.3}$, $TCID_{50}$/0.1 ml, respectively, as determined in AGMK cultures. Two pools of strain A426 were obtained from Dr. Bernice Eddy. One of these (A426-AGMK-5, HeLa-1) was derived from HeLa cell cultures chronically infected with SV40.[6] Prior to use, this pool was extracted with chloroform (see below). Infectivity titrations and viral isolation techniques have been described previously.[5]

Hamster kidney cultures: Primary cultures of trypsin-dispersed kidney cells of weanling (WHK) and 15–16-day embryonic (EHK) Syrian hamsters were obtained from Microbiological Associates, Inc. Cultures grown in Eagle's basal medium (BME) with 5% calf serum were maintained with one of the following media: (A) BME with 5% heated (56° 30 min) horse serum; (B) BME with 5% heated calf serum; (C) BME with 5% unheated agammaglobulinic newborn calf serum;[7] or (D) NCTC 109 with 10% unheated agammaglobulinic calf serum. All media contained 100 μ penicillin and 100 μg streptomycin per ml. Media were changed twice weekly, and tubes were kept in a stationary position at 36°C. Cultures were examined 2–3 times each week and were held for at least 57 days. Culture fluids were saved each week and stored at −60° until tested for virus.

Tests for virus: Four methods were used to test for virus in the hamster tissue cultures. (*i*) Supernatant fluids were tested by inoculation of 0.1 ml of the undiluted culture fluid into 2 or 3 AGMK culture tubes. These cultures were observed 30–40 days, then frozen and thawed 2 or 3 times, and the cell extracts passed to fresh AGMK cultures. (*ii*) Disrupted cells were tested as follows. Cultures were trypsinized, and the cells suspended in medium D (see above) to give $3–4 \times 10^6$ cells/ml. The cells were disrupted by either freeze-thawing 2–3 times, sonicating for 2 min, or exposing to 10% chloroform for 10 min at 0°C, followed by centrifugation at 1,000 rpm for 10 min. These extracts were then tested in AGMK cultures as described above. (*iii*) Viable cultures were tested by a modification of the overlay method described by Eddy *et al.*[8] and Gerber and Kirschstein.[9] Transformed cells were trypsinized and suspended in medium D; dilutions of the cell suspension were immediately inoculated into 3 or 4 tube cultures of AGMK. A 24-hr period of attachment was allowed before the medium was changed. These dual cultures were

observed for 4 weeks for development of the characteristic SV40 CPE in the AGMK portion. (*iv*) Tests for virus in tumors produced by cell transplants provided an additional method for determining the capacity of transformed cells to produce infectious virus (see next section).

Transplantation of tissue culture cells: Cells were removed from the glass by trypsinization or scraping with a rubber policeman, and suspended in medium B. Approximate cell counts were made, and 0.5–1.0 ml of suspension inoculated into the subcutaneous tissue of the back of 3–4-week-old noninbred Syrian hamsters. The techniques used for assessment of size, preparation of tumor extracts, tests for virus, and histological examination have been described.[5]

Results.—General description of transformation of hamster kidney cultures: The over-all pattern of transformation, observed throughout the present work, closely resembled that described in our initial experiment.[2] After a variable length of time, depending on various factors discussed below, several focal areas containing multinucleated giant cells appear within the infected cell sheet. Cellular proliferation occurs 10–20 days later in some of the areas populated by the polykaryocytes. Frequently, only one focus of growth occurs in any single culture, but as many as 4 have been observed. The new growth is composed chiefly of polygonal to triangular-shaped epithelioid cells, but spindled fibroblasts are also evident in the outgrowth. Many giant cells are present within the new growth. The proliferating tissue overgrows the adjacent cells and forms a multilayered, thickened patch of tissue which is visible macroscopically (Fig. 1). Concomitant with the new growth, the cultures begin to acidify more rapidly. During the next several months the transformed cells may obliterate the entire cell sheet; necrosis and retraction often occur in the central, heaped-up areas, followed by regrowth of transformed cells. Tubes held as long as 153 days showed repeated cycles of growth, retraction, and regrowth. Of the more than 100 uninfected control cultures, none showed comparable cell growth, and all degenerated "nonspecifically" in 40–60 days.

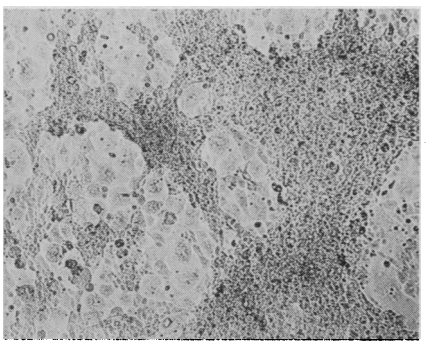

FIG. 1.—Transformed WHK culture, 53 days after inoculation and 35 days after the appearance of transformed cells. Unstained. Note multinucleated giant cells and piling up of cells. × 45.

Factors affecting rapidity of transformation: Table 1 summarizes the results of various tests of SV40 in hamster kidney monolayer cultures, and permits some evaluation of the relative influence of several experimental variables on the reproducibility and rapidity of transformation. In this table, each experiment number refers to a different lot of kidney cultures.

The composition of the medium was of much importance. With Medium A, results were variable, and in expt. 6–12 only a small proportion of cultures showed transformation. Cultures maintained in Medium B occasionally transformed relatively early, but only 3 of 17 cultures transformed. In the media (C and D) containing unheated agammaglobulinic calf serum, 38 (95 per cent) of 40 cultures receiving unheated, undiluted virus preparations showed transformation; expt. 11–14 indicated that the response occurred more rapidly in Medium D than C.

With the optimal medium, there was no marked variation in response between different batches of cultures infected under comparable conditions, as shown by

TABLE 1

SUMMARY OF EXPERIMENTS, SHOWING TIME OF TRANSFORMATION IN CULTURES INFECTED UNDER VARIOUS CONDITIONS

Virus pool	Expt. no.	Tissue	$TCID_{50}$ inoculated per tube (log_{10})	Medium*	Day of appearance of giant cells	Frequency of transformation, no. tubes pos./ no. tubes inoc.	Day transformation first observed in positive tubes	Length of observation of negative tubes (days)
777 (AG3)†	4–18	WHK	6.5	A	18–20	2/2	31, 34	
	4–18	"	4.5	"	?	2/2	41, 45	
	6–12	"	6.5	"	20–41	3/12	49, 52, 59	66
777(AG4)	9–7	WHK	4.8	B	—	0/8	—	80
	9–26	"	"	"	26	1/3	34	75
	10–18	"	"	"	21	1/3	21	57
	10–23	"	"	"	16	1/3	21	60
	11–14	"	"	C	13–16	5/5	26, 26, 37, 51, 55	
	11–14	"	"	D	5–6	5/5	16, 20, 20, 20, 20	
	11–28	"	Chloroform extracted	"	6	4/4	22, 22, 22, 28	
	11–28	"	Heated 60° 30′	"	—	0/4	—	60
777 (AG5)	1–5	WHK	5.3	D	5–6	4/4	17, 20, 24, 24	
	1–5	"	5.3 + SV40 antiserum	"	—	0/4	—	60
	2–4	EHK	5.3	"	6–10	4/6	15, 15, 15, 29	36 and 87
	2–4	"	Heated 56° 30′	"	10	2/3	42, 42	60
	2–28	WHK	5.3	"	8–11	3/3	23, 23, 23	
	2–28	EHK	"	"	8	3/3	23, 23, 36	
A-426 (AG5, HI)	2–28	WHK	7.0	D	8	3/3	23, 33, 33	
	2–28	EHK	"	"	5–8	3/3	15, 23, 23	
A-426(AG5)	4–30	WHK	7.5	D	11–16	4/4	16, 21, 21, 24,	
	4–30	"	5.5	"	20	2/4	31, 55	65
	4–30	"	3.5	"	—	0/4	—	65

* See *Materials and Methods* for meaning of symbols.
† Figures in parentheses give passage history; AG3 = 3rd passage in Cercopithecus kidney cultures; H1 = 1st passage in HeLa cell cultures.

comparison of expts. 11–14 Medium D with 11–28, 1–5 with 2–28 WHK, and 2–4 with 2–28 EHK (Table 1).

Since SV40 is primarily tumorigenic in the newborn period,[10] comparisons were made of the rapidity of transformation in cells derived from embryos as compared with cells from weanling hamsters (expt. 2–28, Table 1). With both SV40 strains, there was no marked difference between the rapidity of transformation in EHK and WHK, but the EHK cultures tended to respond somewhat more quickly.

The effect of virus dosage is seen in expt. 4–30 in Table 1. The undiluted virus material of strain A-426 ($10^{7.5}$ $TCID_{50}$ inoculum) produced relatively rapid transformation, but a 10^{-2} dilution thereof produced transformation in only two of four cultures, and with a longer latent period. No transformation occurred with the 10^{-4} dilution of this pool. With strain 777, used in doses of $10^{4.8}$–$10^{5.3}$ $TCID_{50}$ per culture, new growth appeared as quickly as with $10^{7.0}$ $TCID_{50}$ of strain A-426, as shown most clearly in expt. 2–28. These findings suggest that various SV40 strains differ in ability to transform or, less likely, that an additional factor in undiluted culture fluids is necessary for rapid transformation.

Specificity of SV40 transformation: In order to determine the specificity of both the giant cell and transformation responses, and to attempt to rid the pools of any potential contaminating simian agents, several procedures were carried out upon the virus pools. In contrast to most viruses, SV40 is relatively stable to heat at 56°C for 30 min; in addition, it is resistant to treatment with chloroform. The two agents (SV5 and the SA_1 virus[11]) which occur in AGMK cultures and produce syncytial giant cells are inactivated by these procedures. Heating SV40 virus at 56°C for 30 min did not prevent giant cell formation or transformation, but did delay the effects (Table 1, expt. 2–4); the data of Sweet and Hilleman[12] indicate that this heat exposure would reduce infectivity titer by about 6-fold. Heating at 60°C for 30 min destroyed ability to transform (Table 1, expt. 11–28). A pool treated with chloroform produced giant cells and transformation at the same time as in cultures infected with untreated virus (Table 1, expts. 11–14 Medium D and 11–28).

Further evidence of the specificity of the effect was provided by a neutralization test with hyperimmune anti-SV40 rabbit serum. WHK cultures were inoculated with 0.2 ml of mixtures containing $10^{5.3}$ $TCID_{50}$ of SV40 and 2 per cent immune or normal rabbit serum. The immune serum had a neutralizing antibody titer of at least 1:2560. The 4 cultures which received neutralized virus also contained 0.5 per cent rabbit antiserum in the maintenance medium, and were maintained continuously in antibody-containing medium. Transformation did not occur in these cultures during a 60-day period of observation, but occurred in all 4 control cultures within 17–24 days (Table 1, expt. 1–5).

Continuous cell line of transformed cells: As described previously,[2] a transformed culture in expt. 4–18 was trypsinized 69 days after inoculation, and a cell line established. This line, referred to as THK-1, is currently in the 73rd passage. The cell line is highly pleomorphic, consisting predominantly of triangular to polygonal-shaped "epithelioid" cells, with many spindle-shaped cells as well. The nuclei are large and pleomorphic with an increased number of nucleoli. The cells grow rapidly in a disorderly fashion and pile up on each other with the formation of thickened, nodular, growth centers which are macroscopically visible.

TABLE 2

TESTS FOR VIRUS IN HAMSTER KIDNEY CULTURES BEFORE AND AFTER TRANSFORMATION

Original Culture			Cell Passages (Tests for Virus)								
Days after inoculation	Test of supernatant* Results	Day CPE first observed	Passage no.	Days after orig. inoc.	Test of supernatant Results	Day CPE first observed	Disrupted cells	Overlay method (no. cells) $10^{5.2}$	$10^{4.2}$	$10^{3.2}$	Tumor extracts (no. tumors positive/ no. tumors tested)
3	+	6									
6	+	6									
9	+	7									
13	+	7–8									
20	+	7									
27	+	7									
37†	+	7									
48	+	9									
58	+	9–11									
69	+	11‡									
			1	75	+	18, Neg.					
			1	77	+	18, 21					
79	+	11	2	81	+	25, Neg.					
			3	85	+	18, Neg.					1/3
92	+	11–18	4	92	Neg.						
			5	96	"						0/1
			8	104							
			9	108	Neg.						
			10	114	"						
			11	120	"						
			15	134							2/3
			16	142							1/1
			18	153	Neg.						
			24	204	"		+§				
			35	279	"			+	+	−	
			51	364	"		Neg.				
			59	384	"		Neg.				
			66	401	"		Neg.	+	+	+	

* Pooled fluids from 2 culture tubes.
† Transformation noted 31 and 34 days after inoculation.
‡ $10^{2.2}$ $TCID_{50}$/0.1 ml culture fluid.
§ $10^{2.3}$ $TCID_{50}$/0.1 ml, representing extract of 3–4 × 10^5 cells.

Giant cells with 30–40 aggregated nuclei in the central portions are present within the cell sheet. These characteristics noted in the earliest passages[2] have been maintained through the present passage level.

Detailed cytogenetic studies of this continuous cell line have been reported elsewhere.[13] Marked, progressive chromosomal changes consisting of both numerical and morphological alterations were described. No stem line was detectable after 18 passages.

Transplantation to weanling hamsters was done with THK-1 cells at the 3rd, 8th, 15th, and 16th passage levels. The inoculating dose was generally 10^6 cells; the smallest inoculum was 10^5 cells. Tumors developed in all animals, being first evident at 3–4 weeks, and attaining a diameter of 1 cm by the 6th–7th week. There was no difference in growth characteristics between tumors induced with cells of different passage levels. Virus was isolated from extracts of 4 of 8 tumors; these virus-positive tumors arose from cells from the 3rd, 15th, and 16th passages (see Table 2). As judged from the latent period of CPE in AGMK cultures, about 10–100 $TCID_{50}$ of virus/0.1 ml tumor suspension were present. No tumors arose over a 4-month period of observation when 10^6 normal WHK cells were inoculated subcutaneously into hamsters of comparable age.

The tumors induced with THK-1 cells were round to ovoid, lobular, and yellow to gray in color. They remained fairly well localized and were easily dissected out despite their large size, which often attained 5–7 cm diameter. The central portions frequently were composed of a sharply circumscribed core of necrosis. Grossly, there was no evidence of metastases.

Microscopically, 3 of 8 tumors were purely sarcomatous, having highly cellular areas of undifferentiated cells and other areas resembling fibrosarcoma. The remaining 5 tumors had, in addition to sarcomatous cells, areas of undifferentiated carcinoma and frank adenocarcinoma (Fig. 2). All tumors had many multi-

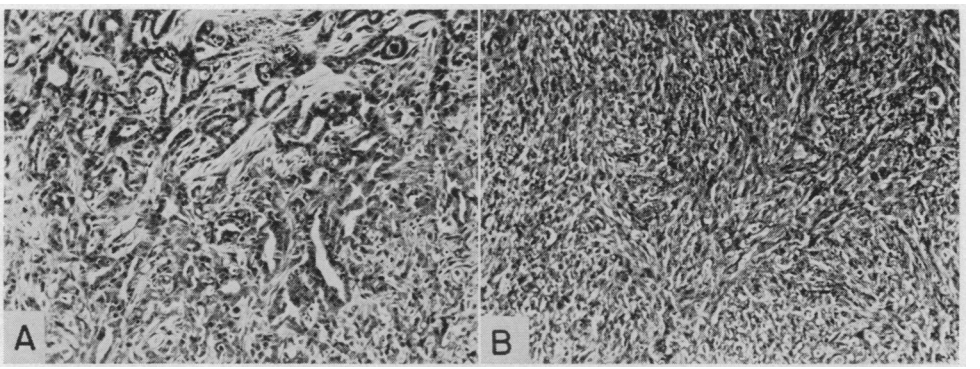

FIG. 2.—(*A*) Mixed tumor arising from transplantation of THK-1 cells in the third passage, showing area of epithelial differentiation into tubular structures. H&E stain ×71.5. (*B*) Fibrosarcoma arising from transplantation of THK-1 cells in the third passage. H&E stain ×71.5.

nucleated giant cells. In one animal, serial biopsies from the same tumor showed both epithelial and sarcomatous elements on one biopsy, and only sarcoma cells on a later biopsy. The tumors frequently showed microscopic invasion of blood vessels and adjacent muscle, but no metastases were observed in microscopic sections of representative portions of most of the viscera of 4 animals.

Presence of virus in infected cultures: Virus was readily recovered from culture fluids of the WHK and EHK primary cultures for at least 2–3 months. The amount of virus tended to decline somewhat as the proportion of transformed cells increased. Inoculated cultures which did not undergo transformation also elaborated virus for prolonged periods.

Table 2 summarizes the attempts to recover virus from the cultures in the original transformation expt. 4–18[2] and from the continuous cell line THK-1 derived therefrom. The progressive decline in virus titer in the original culture tubes after transformation began is evident. Virus was also recovered from culture fluids of the first 3 cell passages, but in distinctly lesser amount than from primary cultures which had not been trypsinized. Supernatant fluids from the continuous cell line were thereafter consistently negative. Virus was recovered from the disrupted cell pack at the 24th passage, but not at the 51st or 59th passages. That virus is continuously present in this cell line in some form is also shown by the recovery of virus by the overlay method and from the transplant-induced tumors. Thus, the carriage of virus in subinfective form or amount in the transformed cell line resembled closely that described for SV40 tumors induced *in vivo*.[9, 14, 15]

Discussion.—From the experiments described above, certain conclusions can be made concerning the variables affecting transformation of hamster kidney tissue cultures by SV40. The composition of the medium was an important factor affecting both the reproducibility and the latent period before transformed cells appeared. The best medium of those tested consisted of NCTC 109 with 10 per cent uninactivated agammaglobulinic calf serum; it seems likely that other media could result in even more rapid transformation. The differences between sera with regard to their effect on rapidity of transformation was not attributable to virus inhibitors in the sera (unpublished data). For a given SV40 strain, dosage of virus was also a major factor; however, the two strains of SV40 employed differed markedly in ability to transform when used in comparable dosage. The age of the hamsters from which the kidney tissue was obtained did not markedly affect the latent period.

No efforts have as yet been made to determine the efficiency of transformation. The proportion of cells transformed is probably extremely small, since only 1–4 foci of new growth appear in cultures containing about 10^5 cells. This low frequency of transformation may reflect differences in physiological state of cells, as thought to be the reason for the low transformation frequency with polyoma virus,[16] or susceptibility of only one cell type of the several present in primary kidney cultures.

Since both epithelioid and fibroblastic elements were present in the outgrowth of transformed cells and in many of the tumors derived therefrom, it was originally assumed that two cell types were transformed. However, evidence will be presented in the subsequent report[17] indicating that the mixed tumors were not derived from mixtures of transformed cell types.

Mixed tumors arising from transplantation of SV40 transformed newborn hamster kidney cells were also described by Rabson and Kirschstein;[1] however, the tumors obtained from transformed newborn hamster kidney cells by Shein and Enders were described as adenocarcinomas,[3] and some of our tumors were purely sarcomatous.

The proliferation of SV40 in hamster kidney cultures is in marked contrast to the lack of virus proliferation in the pretumor period following inoculation of SV40 into suckling hamsters.[5, 9] After subcultivation of transformed cultures, the amount of recoverable virus declined progressively, suggesting that the infectivity was derived primarily from the nontransformed cells, which cannot be maintained in serial transfer. After the third passage of the THK-1 cell line, no virus was detected at any time in the supernatant culture fluids. In late passages, no virus was demonstrable in cell extracts, but could be detected by growing the cells on AGMK cultures. Thus, in this respect the cell-virus relationship in the transformed cell line closely resembled that in primary SV40-induced hamster tumors.[9, 14, 15] The fact that SV40 can be recovered consistently from passage lines of transformed cells only by the overlay method[8, 9] may explain the negative findings of Shein *et al.*,[3] and Ashkenazi and Melnick.[4]

The chronic production of virus in the primary cultures may be one of the reasons for the occasionally long latent period before transformation, in that cells are being continually exposed to virus. On the other hand, the long latent periods may be similar to the delayed appearance of transformation by polyoma virus, where it

seems clear that the input virus alone provided the stimulus.[18] The close association of foci of giant cells with sites of subsequent transformation may also be related to this chronic infectious process; if the giant cells are sites of maximal virus growth, the neighboring cells may be most affected. In this connection, Shein *et al.*[3] noted that nuclei of the early giant cells consistently showed immune fluorescence with anti-SV40 rabbit serum.

Summary.—When hamster kidney monolayers are exposed to the simian vacuolating virus—SV40, abnormal proliferation of tissue occurs after a variable latent period. The length of the latent period is dependent on the type of medium, virus strain, and dosage of virus used. The transformed cells produce mixed tumors (carcinosarcomas) when transplanted to hamsters. SV40 proliferated continually in the primary cultures and became progressively more difficult to detect in transformed cultures; in late passages of transformed cells, virus could only be recovered when these were planted directly on Cercopithecus kidney monolayers.

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*REACTIONS OF NORMAL AND TUMOR CELL SURFACES TO ENZYMES, I. WHEAT-GERM LIPASE AND ASSOCIATED MUCOPOLYSACCHARIDES**,†

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Within the last few years many scientists have focused their attention on the nature of the cell membrane. It is well established that cellular adhesions and interactions are largely dependent on the surface properties of cells. With the observation that neoplastic cells differ from normal cells in the nature of these