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*TRANSFORMATION INDUCED BY SIMIAN VIRUS 40 IN HUMAN RENAL CELL CULTURES, I. MORPHOLOGY AND GROWTH CHARACTERISTICS**

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At least three tumor viruses have now been shown to induce *in vitro* transformations in susceptible cells. Rous sarcoma and avian myeloblastosis viruses induce epithelioid transformation in cultures of chick embryo fibroblasts,[1–4] and SE polyoma virus brings about fibroblastic transformation in cultures of mouse, rat, and hamster tissues.[5, 6] No analogous transformation has been previously demonstrated in primate cells. As will be shown in this communication, simian virus 40 (SV40), which is oncogenic in suckling hamsters,[7, 8] can induce a reproducible epithelioid transformation in primary cultures of human renal cells. Previously, cellular changes suggesting transformation in this system were briefly described.[9]

MATERIALS AND METHODS.—*SV40 virus:* SV40 strain VA 45-54 GMK 6/9/61[10] was obtained from Dr. M. R. Hilleman of the Merck Insitute for Therapeutic Research.

Cultures of human kidney tissues: Primary monolayer cultures of trypsinized cells from kidney tissues of fetuses of 2 to 3 months' gestation and from newborn and three-month-old infants were prepared in 12 × 150 mm tubes. Cover-slip cultures in Leighton tubes were similarly established.

During the first 2–4 days, cultures were incubated at 37°C in a stationary position and thereafter in a roller wheel with the exception of Leighton tubes. Cultures were used in experiments usually 4 to 8 weeks after preparation, when they consisted predominantly of fibroblast-like cells.

Growth medium for primary human cultures consisted of 90% Eagle's basal medium, modified as described by Berg *et al.*[11] and 10% fetal bovine serum (Colorado Serum Company). To each culture 1 ml of medium was added and replaced every 2–4 days. Immediately before addition of SV40 virus, medium 199 containing 2% chicken serum was substituted. One to 3 days later the latter was replaced by the original medium which again was changed every 2–4 days. Cultures were incubated at 37°C. Subcultures were made either with trypsinized suspensions of cells from primary cultures or by removing portions of the cell growth with a capillary pipette. The medium, consisting of 80% Puck's medium, 4% NCTC 109, 8% fetal bovine serum, 8% inactivated horse serum, and 0.4% lactalbumin hydrolysate, was changed every 2–4 days.

EXPERIMENTAL.—*Introductory:* In our earlier report,[9] growth-stimulating together with destructive effects of SV40 infection on renal cells from human fetuses, newborn and three-month-old infants were described. A further, more preliminary, observation suggested the occurrence of transformation analogous to that de-

scribed by Vogt and Dulbecco in hamster cells infected with polyoma virus:[5] Thus, in one infected culture after about 60 days, groups of rapidly proliferating epithelial-like cells appeared which were easily distinguishable amid the predominantly fibroblastic population by increased transparency, accelerated growth rate and tendency to form in multilayered nodules composed predominantly of viable cells.[9]

On more prolonged incubation of infected renal cultures included in this earlier study, similar changes suggesting transformation were seen in a large proportion. Accordingly, experiments here described were undertaken to compare systematically these apparently transformed cells with other cell forms present in both infected and uninfected systems. The results will be considered under two main headings: (1) those relating to differences in cellular morphological and growth characteristics (presented in this paper); (2) those concerned with differences in cell-virus relationships (to be presented in a second paper). Reference to the various cell forms mentioned above will be as follows: F cells (predominantly fibroblast-like) = the population of cells in uninfected control cultures; F_i cells (predominantly fibroblast-like) = fibroblast-like and other apparently untransformed cells in infected cultures; E or epithelioid cells = apparently transformed cells.

Cell morphology: Both in primary cultures and in subcultures, F cells appeared as slender fibroblasts with elongated oval nuclei containing usually from one to three nucleoli. Variations in nuclear dimensions and over-all size were slight (Fig. 1).

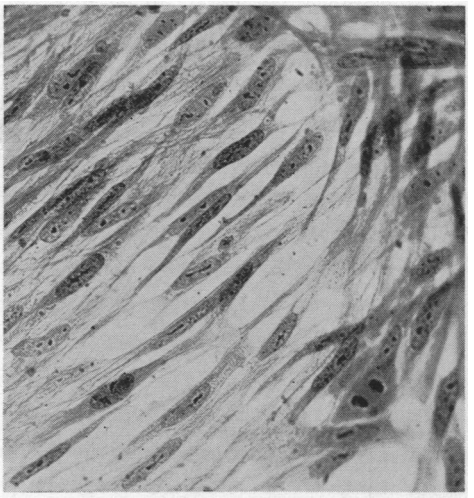

FIG. 1.—F cells in (uninoculated control culture. Stained areas (H. and E.) at magnification of 375×. Nuclei vary little in size and shape and contain 1–3 nucleoli. No large cytoplasmic inclusions evident. Overgrowth of a few cells is apparent at right of the monolayer.

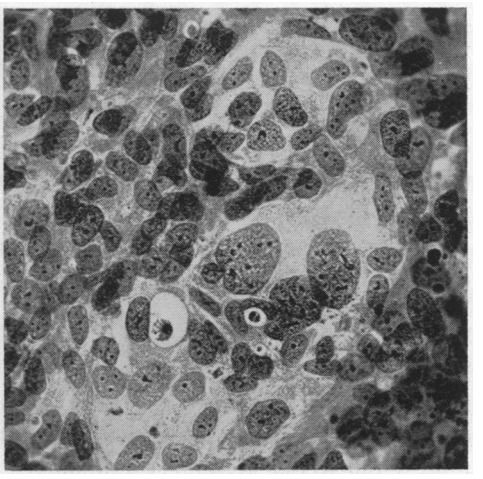

FIG. 2.—E cells in primary culture (from same exp. as Fig. 1 on 114th day after SV40 inoculation. H. and E. stained area at magnification of 375×. Notice variation in nuclear size and shape and in nucleolar number. Random cellular pattern and multilayering are evident.

F_i cells not obviously damaged by viral action exhibited in general a similar appearance but in heaped up areas tended to be somewhat broader in cross section, occasionally even oval in outline. Mitotic figures were more frequent in primary cultures of F_i than of F cells.[9] In contrast to F and F_i cells, E cells presented a thin almost transparent polygonal aspect. Nuclei varied widely in size and shape,

ranging from circular to slightly oval and containing from one to six or more nucleoli (Fig. 2). As compared with F cells, mitotic figures were by inspection more numerous in primary as well as subcultured E cells. While primary cultures of E and F_i cells showed no apparent differences in this respect, dividing cells were more frequent in subcultures of E cells than in either primary or subcultured F_i cells.

Chromosomes in E and F cells: In collaboration with G. Yerganian and R. Kato of the Children's Cancer Research Foundation, chromosomal analysis at the metaphase in E, F, and F_i cells is in progress and will be reported in detail elsewhere. Preliminary findings with E and F cells are summarized here. Studies of chromosome complements of F cells have revealed in nearly all 46 disomically paired chromosomes (the normal complement). E cells in contrast have exhibited in the majority either aneupoloidy or quasi-diploidy, i.e., either the total number of chromosomes was altered (aneuploidy), usually because certain types lacked one member of a chromosome pair (monosomy); or the total number of chromosomes remained unaltered in the presence of such abnormalities (quasi-diploidy) because this monosomy was "balanced" by trisomy (an extra member) for other types. In most E cells from three independent transformations so far examined there was monosomy involving chromosomes of Group 21–22 (Denver Classification). More rarely, monosmy involving chromosomes other than 21–22 was also seen, both in cells abnormal in respect to Group 21–22 and in others.

In addition to aneuploidy or quasi-diploidy, over half the metaphase figures in E cell cultures exhibited a grouping of certain chromosomes which either was not seen or was rarely present in F cells. This grouping consisted of an association by their satellited processes of two or more satellited chromosomes (i.e., numbers 13–15, 21, 22).

Patterns of cell growth: F cells tended to remain in a monolayer composed largely of whorls of fibroblasts aligned side by side. With prolonged incubation growth of cells over the monolayer occurred to a limited extent (Fig. 1). This regular growth pattern has been considered characteristic of normal fibroblasts mutually inhibited by contact[12] as contrasted to the multiplication and mobility of sarcoma cells, which have been found to be relatively independent of such inhibition.[13] F_i cells in a monolayer presented essentially the same arrangement. However, in the areas of multilayering[9] these cells lay at random and were more rounded.

In primary cultures E cells were first seen usually at the periphery of multilayered foci of F_i cells. Initially E cells formed a monolayer in which more centrally located elements were markedly polygonal in outline while cells at the advancing edge were more elongated. Later the monolayer became multilayered through overgrowth of cells. Foci where thickening was most pronounced presented whitish opaque masses to the naked eye (Fig. 3). Although progressively extending, such thickened areas were not observed to replace completely the surrounding growth of F_i cells. As they heaped up, E cells retained in general their epithelioid character but were more rounded and thickened as contrasted with their thin, almost transparent configuration when growing as a monolayer. At all stages E cells exhibited a completely random pattern which strongly contrasted with the regular arrangement of F cells and of F_i cells when in a monolayer (Fig. 2). In subcultures E cells exhibited essentially the same characteristics except growth was more rapid and extension unrestricted. Heaping-up in E cell cultures was increased by incubation in a roller

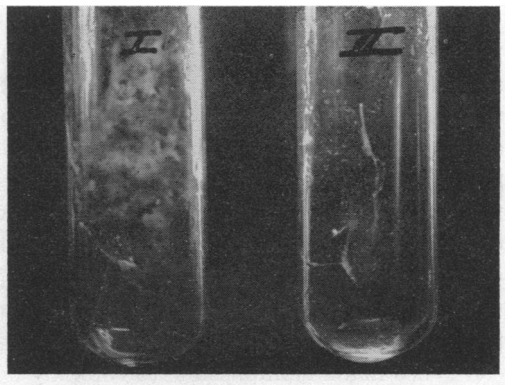

FIG. 3.—Primary HF kidney cultures 112 days after SV40 inoculation showing extensive opaque cell growth in inoculated culture.
I, Culture inoculated with SV40.
II, Control uninoculated culture.

wheel and was exaggerated in certain instances by retraction of cells from the glass. The epitheloid appearance was most pronounced when Eagle's medium was used; it was less striking in Puck's medium, in which more elongated forms were observed. In two infected cultures an apparent transformation to a more elongated cell form was observed. These cells were propagated serially and are now under study.

Cell multiplication: In primary cultures it was evident by inspection[9] that multiplication of F_i cells subsequent to the 30th day after addition of SV40 virus exceeded that of F cells. This difference in growth capacity was confirmed in experiments to determine the relative number of cells in primary F and in F_i cell cultures at various intervals following SV40 inoculation (Table 1). After periods ranging from 61 to 114 days, cell counts of trypsinized cell suspensions from single cultures were in each experiment found to be 2 to 5 times higher with F_i cells (Table 1). This differential was maintained irrespective of the presence or absence of E cells in F_i cultures. These findings indicate that increase in cell replication in SV40-infected primary cultures does not depend upon the emergence of E cells.

TABLE 1

INDEPENDENCE OF PROLIFERATIVE EFFECT AND TRANSFORMATION

Experiment	Tissue	Days after inoculation when trypsinized	SV40 inoculated (I) or control (C)	* Cell count $\times 10^{-5}$	Transformation	
					Prior to trypsinization	After trypsinization
1	HF kid†	114	I	7.2	0	0
			C	2.1	0	0
2	HF kid	83	I	7.5	+	+
			I	5.7	0	+
			C	3.0	0	0
			C	1.7	0	0
3	HF kid	81	I	6.6	+	+
			I	7.3	+	+
			C	1.5	0	0
			C	1.2	0	0
4	HF kid	61	I	3.5	+	+
			I	5.3	0	0
			C	1.6	0	0
5	Newborn kid	92	I	7.5	+	+
			C	2.1	0	0
6	Infant kid (3 mo.)	92	I	8.1	+	+
			C	1.8	0	0

* Each figure represents the average of four hemocytometer counts performed on a trypsinized cell suspension from one primary culture.
† Human fetal kidney.

In subcultures of F cells, made usually 90 days or more after establishment of the primary culture, growth was slow and scanty. The cells appeared more elongated and mitotic figures were rarely seen. When the cells from a single primary culture were used to establish two subcultures, monolayers were not complete until 14–21 days. In over 10 experiments of this type cell multiplication in successive subcultures was increasingly diminished, so that after three or four passages further attempts to subculture these cells failed. Although in early subcultures confluent monolayers were attained somewhat earlier with F_i than with F cells, in three experiments serial passages also failed after 4–5 subcultures.[14]

Although numerous mitotic figures were seen in foci of E cells emerging in primary cultures, the multilayering of such cells and their interdigitation with other cell types made it impossible to compare accurately their rate of multiplication relative to primary F and F_i cells. Behavior of E cells in subculture, however, was easily observed and was found to contrast with the other forms in two essential respects. First, multiplication of E cells was more rapid. Thus when these cells from a single culture were distributed into three subcultures confluent monolayers were formed within 2–3 days; when 10 subcultures were prepared from a single culture monolayers were established within 5–7 days. Secondly, E cells were readily maintained in serial subculture. Thus, during the passage of 8 different lines of E cells through 10 to 20 subcultures, diminution of growth was not encountered.

Capacity to multiply from small numbers: The relative autonomy of E cells was also shown in attempts to propagate the various cell forms from small inocula. Trypsinized suspensions from primary and subcultured E, F_i, and F cells were diluted. Approximately 10 to 100 cells were introduced into each of several 50 mm Petri dishes which were then maintained in a CO_2 incubator. After 24 hours the positions of single cells as well as small aggregates of cells was determined by inspection. With each of the cell forms the numbers adherent were roughly comparable.

Upon subsequent incubation, F and F_i cells became progressively elongated and granular and assumed a jagged outline. Isolated cells or cell groups remained intact for several weeks. However, in a total of 17 experiments, each of which included 5–10 preparations, multiplication failed or was minimal, since no more than 10 cells were ever observed in any locus. After 4–5 weeks all cells disintegrated.

E cells, in contrast, usually assumed their characteristic polygonal shape and presented a normal smooth appearance. Within 14 days many of the cells initially observed had multiplied to form macroscopic colonies. In three experiments, employing a total of 36 Petri dishes, colonies of E cells developed in all but two dishes. Subculture of such colonies was subsequently carried out. Cells from single colonies of E cells were removed by suction with a capillary pipette under microscopic observation, suspended in 3 ml of medium and transferred to other dishes. Again within 14 days grossly visible colonies appeared. This procedure was twice repeated serially. In the last passage cells were inoculated into rubber-stoppered glass bottles. Subsequently colonies also developed in these bottles. Although the organization and cytologic characteristics of E cells within the colonies differed somewhat from bottle to bottle, the morphology of all colonies within any given bottle was the same. This finding suggested a common progenitor cell for all the

colonies within a given bottle. Certain E cell colonies derived in this manner have now been carried for over 3 months in culture.

Influence of F_i cells on growth of E cells: As stated earlier, E cells after their emergence rarely completely overgrew the fibroblasts in primary F_i cultures. Failure to overgrow persisted even after 120–140 days. In contrast to this limited growth in primary culture, E cells subcultured in the apparent absence of F_i cells rapidly extended throughout the available growth area. This rapid extension took place not only under the stimulus of frequent trypsinization but also when explants of E cells were made by means of a capillary pipette from primary cultures and used to initiate plasma clot cultures. These findings suggest that apparently untransformed F_i cells may in some way limit the growth of the transformed elements.

Reproducibility of transformation: Seven experiments were undertaken to determine the frequency of epithelioid transformation in cultures of cells from human fetal kidney tissues of different individuals and from kidney tissues of newborn as well as three-month-old infants (Table 2). In each experiment transformation was seen in at least half of the infected cultures but at no time in uninfected controls. Among a total of 38 inoculated cultures transformation occurred in 30 (80%); among a total of 28 uninoculated control cultures, transformation was not recognized.

TABLE 2

REPRODUCIBILITY OF TRANSFORMATION

Experiment	Tissue	Days observation after SV40	Occurrence of Transformation* Inoculated†	Occurrence of Transformation* Controls
1	HF kid	114	1/2	0/2
2	HF kid	83	2/3	0/3
3	HF kid	77	3/3	0/3
4	HF kid	69	5/6	0/6
5	Newborn kid	92‡	1/2	0/2
6	Infant kid (3 mo.)	92‡	1/2	0/2
7	HF kid	87	17/20	0/10
Totals			30/38	0/28

* $\frac{\text{Number cultures showing transformation.}}{\text{Total cultures in experiment}}$

† All inoculated cultures exhibited destructive and proliferative CPE.

‡ One inoculated tube without apparent transformation was fixed for H & E staining on 42nd day after inoculation.

DISCUSSION.—*Origin of E cells:* While it is conceivable that E cells were present initially in small numbers and subsequently multiplied selectively during the course of infection, this explanation of their origin seems improbable. During the course of examining several hundred uninoculated cultures of varying ages, groups of cells exhibiting the morphology or growth characteristics of E cells were not seen. In view of the demonstrated capacity of E cells to multiply extensively from small inocula, such elements if originally present should have multiplied sufficiently during the course of observation to be recognizable. If it is urged that failure of E cells originally present in small numbers to multiply in primary cultures depended upon inhibition by F_i cells, it should have been possible, at least occasionally, to demonstrate their presence by subculture. Many attempts to do so were unsuccessful. Nevertheless, final exclusion of the initial presence of E cells must await development of clones of F cells and induction in these of E cell transformation following inoculation of SV40.

There is more reason to believe that SV40 induces an inheritable cellular metamorphosis. This concept is supported by the following data indicating that both viral multiplication and cell transformation are associated with changes in the nucleus. Electronmicroscopic studies with SV40 in simian cell systems suggest that the virus multiplies almost entirely within the nucleus.[15] Fluorescent antibody studies of infected human cells (to be described in a second paper) together with those on infected simian cells[16] indicate that viral antigen is present only in the nucleus. We have observed that E cells exhibit increased numbers of nucleoli and consistent chromosomal aberrations involving predominantly the satellited chromosomes. The latter are thought to be nucleolar organizers. In simian renal cells infected with SV40 the earliest morphological and chemical changes (increased RNA content, vacuolation and presence of viral particles) also occur in the nucleolus.[15]

Although it thus seems probable that E cells represent an inheritable alteration induced by the virus, the cell type of origin cannot at present be defined. The original cultures in which transformation occurred consisted predominantly of fibroblast-like elements. However, the possibility remains that many of these may have been epithelial cells which under the conditions of cultivation tended to assume a spindle-like configuration. Moreover, a variable although much smaller number of epithelial-like cells were constantly present before and after inoculation of SV40. It is also impossible to exclude the capillary endothelium as a source of target cells; but this appears less likely, since transformation has not been observed thus far in limited studies of cells from other organs.

Significance of chromosomal aberrations in E cells: The preliminary results indicating high frequency of monosomy involving chromosomes of Group 21–22 are of interest, especially since abnormalities are seen in these chromosomes in both chronic myelogenous human leukemia and in human mongolism. A large proportion of patients with this form of leukemia exhibit in their leukocytes a distal deletion of one of the chromosome pairs 21 or 22[17, 18] and nonfamilial mongolism exhibits trisomy for 21–22.[19] The less common familial mongolism also features a "trisomic" relationship of 21 or 22 in the form of a translocation usually between chromosomes 14 or 15 and 21, but in some cases between 21 and 22.[20, 21] Mongoloid children are also reported to show an increased incidence of leukemia of all types.[22, 23] Aneuploidy and quasi-diploidy, which were observed in E cells, have previously been described in certain neoplasms of man and other species.[24, 25]

With regard to the frequent association of satellited chromosomes seen in E cells as compared with F cells, it should be pointed out that a comparably high incidence has recently been reported in peripheral leukocytes drawn from presumably normal individuals.[26] The significance, therefore, of the increased association of satellited chromosomes in E cells is uncertain. However, since the satellited chromosomes are thought to be involved in nucleolar formation,[27, 28] and since infection with SV40 is associated with early nucleolar abnormalities,[15] the difference may prove useful for the understanding of the mechanisms of viral transformation.

Neoplastic characteristics of the transformed cells: Accelerated growth, abnormal growth pattern, and chromosomal aberrations exhibited by E cells are characteristics commonly associated with rapidly growing tumors and with "continuous" lines of cells in culture. In considering the significance of these properties, however, it should be emphasized that such characteristics arising *in vitro* do not constitute

reliable criteria for malignancy. There is no evidence that all continuous cell lines are malignant or even neoplastic when reinoculated into the host species of derivation.[29] On the other hand, cells transformed *in vitro* by SE polyoma virus have been shown capable of tumor induction in appropriate animal hosts.[5, 30] Although the reinoculation criterion of oncogenic potential cannot readily be applied in the case of transformed cells of human origin, nevertheless evidence concerning the neoplastic capacity of such cells when implanted in experimental animals may be suggestive. Accordingly, experiments are in progress to determine whether E cells proliferate in the cheek pouch of the Syrian hamster, an animal which has been frequently employed in studies of this sort.

Growth of E cells in the presence of F_i Cells: It was recently reported that multiplication of a "spontaneously" transformed line of amnion cells (FL) is inhibited by the presence of untransformed (normal) amnion cells.[31] The apparent restriction of the extension of E cells in primary cultures of F_i cells may afford another example of the same phenomenon. Under these conditions E cells neither overgrow F_i cells nor always extend to fill adjacent empty areas. Factors responsible for this restriction remain obscure; but the frequent failure of E cells to occupy empty spaces suggests that only the presence of F_i cells, not contact with them, is essential.

Significance of the findings: These findings provide an *in vitro* system for studying the mechanisms of viral transformations of human cells. By its application it should be possible to follow the evolution of the immunological, chromosomal, and biochemical alterations that may arise in the course of the transformation.

SUMMARY. Induction by simian virus 40 of a reproducible epithelioid transformation in primary cultures of human renal tissues is described. The transformation is characterized by an abnormal growth pattern, a greatly accelerated growth rate and chromosomal aberrations.

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*REMOVAL OF SIALIC ACID FROM THE CELL COAT IN TUMOR CELLS AND VASCULAR ENDOTHELLUM, AND ITS EFFECTS ON METASTASIS**

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The fate of circulating neoplastic cells depends primarily on their adhesiveness with respect to the vascular endothelium. If they adhere, metastases may be formed.

It is very likely that the capacity of circulating tumor cells to adhere to vascular endothelia must be greatly influenced by the sticky properties of both surfaces which make contact during the process of metastatic tumor formation, although this has not yet been fully established by *in vivo* experiments. A possible variation of the stickiness in the vascular lining has already been suggested by Zeidman,[1] who has observed that mesenteric capillaries in cortisonized rabbits trapped more tumor cells than similar capillaries in normal controls. But changes in the coat of tumor cells, as shown by the Hale staining technique,[2] may be equally important in modifying their stickiness. In our experience (unpublished), the ability of intravenously injected mouse ascitic cells to produce lung tumors seems to be related to the existence of a good mucopolysaccharide coating. However, other properties of this coat, still to be determined, may also play an important role.

In view of the fact that the Hale positive component of the coat in mouse ascitic tumor cells, a sialomucin in nature,[3] as well as the Hale positive coat of the vascular lining (see below), may be removed by the receptor-destroying enzyme (RDE), experiments were performed to determine whether the enzymatic treatment of these two types of cells may affect the frequency of metastases. For this purpose, a