TRANSFORMATION INDUCED BY SIMIAN VIRUS 40 IN HUMAN RENAL CELL CULTURES,* II. CELL-VIRUS RELATIONSHIPS

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Earlier reports[1,2] have described cytolytic and proliferative changes occurring in human renal cell cultures chronically infected with SV40, as well as eventual cell transformation as indicated by the emergence of epithelioid forms with abnormal growth characteristics. Experiments to be described in the present paper were undertaken to elucidate cell-virus relationships during the course of these changes.

Materials and Methods.—SV40 virus: SV40 strain VA 45–54 GMK 6/9/61[3] was obtained from M. R. Hilleman of the Merck Institute for Therapeutic Research.

Other viruses: The following were employed: Poliovirus type 2, Stock Lansing strain; Poliovirus type 2, Roca-Garcia, Moyer, Cox strain;[4] Sindbis virus, Stock strain obtained originally from R. M. Taylor; Measles virus, Edmonston strain, 30th human kidney cell passage; Coxsackie A-9, primary isolate in tissue culture; Adenovirus 2, primary isolate in tissue culture; Vaccinia, Stock strain originally obtained from Massachusetts Antitoxin Laboratory; *Herpes Simplex,* Rodanus strain—originally isolated in this laboratory.

SV40 antisera: Heat-inactivated rabbit antiserum to SV40 strain VA 45-54 GMK 1 with a titer of 1:625 versus 1,000 TCD_{50} of that strain in grivet monkey kidney cultures was supplied by M. R. Hilleman. Guinea pig antiserum to SV40 with a complement-fixing antibody titer of 1:1,240 versus strain VA 45-54 was obtained from Microbiological Associates, Inc.[5]

Grivet monkey kidney (GMK) cultures: Primary monolayer cultures of this tissue in 12 × 150 mm tubes were at first kindly provided by Dr. Hilleman and subsequently obtained from Microbiological Associates, Inc. Maintenance medium consisted of 45% Hanks' balanced salt solution, 45% bovine amniotic fluid, 5% inactivated horse serum and 5% beef embryonic extract. Just prior to inoculation with SV40 the medium was withdrawn and fresh maintenance medium added. The medium was changed every seven days thereafter. All media contained 50 units of penicillin, 50 μg of streptomycin and 1 μg of amphotericin B (Fungizone) per ml.

Cultures of human kidney tissue: These were prepared and maintained as previously described.[2]

Infectivity determinations: Infectivity titrations of SV40 were performed in GMK cultures. Three cultures were used for each 10-fold dilution of fluid to be tested. Cultures were examined weekly for characteristic CPE[3] during the six weeks following inoculation. Infectivity titrations of other viruses were performed in cultures of human fetal (HF) kidney and E cells (see below). Procedures were the same as with SV40 except that the observation period was four weeks and the maintenance medium was that employed for GMK cultures. All titers were calculated by the formula of Reed and Muench.

Interferon determinations: These were performed according to the technique

described by Gresser,[6] employing cultures of primary human amnion cells and Sindbis virus as test agent.

Immunofluorescence: (a) *Technique:* The indirect Coons technique was used. Monolayers of human fetal kidney cells on coverslips were washed with cold phosphate buffer (pH 7.2), fixed in cold acetone for ten min, dried, and rinsed briefly in cold phosphate buffer. They were then overlaid with rabbit anti-SV40 serum and a small amount of fresh guinea pig complement and incubated at 37°C for 30 min. The preparations were then drained, washed for 10 min in cold phosphate buffer, overlaid with fluorescein-labeled goat anti-rabbit globulin serum and incubated for 10 min at 37°C. They were again drained and washed and after mounting in glycerin-saline (9:1) were examined with a Zeiss fluorescence microscope and photographed with a Polaroid camera attachment. (b) *Tests for specificity:* SV40-infected HF kidney and rhesus monkey cell cultures exhibited nuclear fluorescence, which was abolished by dilution of the specific anti-SV40 serum. These changes were not seen in uninfected cultures nor in cultures where other antisera were substituted for anti-SV40 serum (i.e., normal rabbit sera or rabbit antisera prepared against diphtheria toxin or polyoma virus).

Experimental.—Terminology: As previously,[2] the various cell forms will be denoted as follows:

F cells (predominantly fibroblast-like) = the population of cells in uninfected controls.

F_i cells (predominantly fibroblast-like) = fibroblast-like and other apparently untransformed cells in infected cultures.

E or epithelioid cells = apparently transformed cells.

Morphologic changes induced by SV40 virus: (a) *In unstained preparations:* Early proliferative and destructive changes induced by SV40 in F_i cells have been described elsewhere.[1] In subcultures of E cells (in which SV40 virus could still be demonstrated by inoculation of grivet monkey kidney cells), there were occasional rounded pyknotic cells, especially in multilayered areas, but no extensive cell destruction ensued comparable to that observed in primary F_i cells.

(b) *In stained preparations:* In subcultures of E cells stained with hematoxylin and eosin, nuclear changes attributable to the virus and similar to those occurring in primary F_i cells[1] and patas monkey kidney cells[7] were noted. Thus, occasional nuclei were swollen with clumping of chromatin; others were vacuolated; and more rarely, still others were encountered in which eosinophilic inclusions were present. As with F_i cells, these changes were more frequent in heaped-up areas. Even after 10 or more serial passages, the nuclear changes were seen in stained E cell cultures. Eosinophilic cytoplasmic masses were occasionally present in primary F_i cells.[1] These masses were also evident in primary and subcultured E cells (Fig. 1) but often were larger and were found in a higher proportion of cells. In some areas, cytoplasmic inclusions were noted in over one half of the cells. Since inclusions of this sort were also rarely encountered in F cells, their specificity and significance remain at present uncertain. It is possible, however, that their greater frequency and larger dimensions in E cells may represent an additional effect of viral infection.

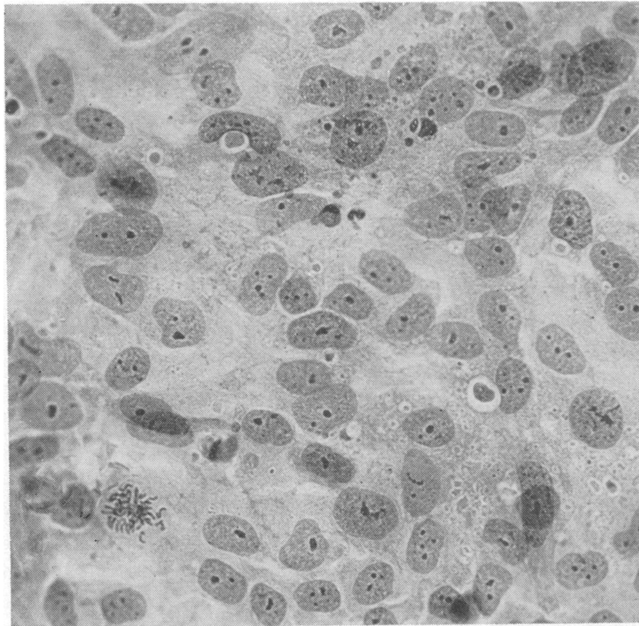

FIG. 1.—E cells in primary culture (from experiment 1, Table 1) on 114th day after SV40 inoculation, stained with hematoxylin and eosin. Magnification 375×. Large eosinophilic cytoplasmic inclusions and random cellular pattern are evident.

(c) *Immunofluorescence:* F_i cells grown on coverslips were examined by the indirect Coons technique at intervals of 1, 2, 3, 4, 8, 15, 24, and 42 days after inoculation of SV40 ($10^{5.5}$ GMK $TCID_{50}$). Beginning on the second day, specific nuclear fluorescence was noted in isolated cells. Antigen was first demonstrated in nuclei of normal size by a diffuse granular staining, which was sometimes associated with small irregular nonstaining areas resembling vacuoles. Other nuclei showed more intense fluorescence that obscured nuclear details. Later, it was also apparent in swollen nuclei, some of which exhibited larger irregular nonstaining areas. In the latter specific fluorescence often seemed to be most intense at the margins of the "vacuoles" (Fig. 2). Intensity of fluorescence tended to diminish with increasing nuclear size.

The proportion of cells showing nuclear fluorescence increased from the second to the fourth day and then appeared to remain constant at about 1 per cent of the population until after the 24th day. During this period fluorescing cells tended to occur in randomly distributed foci, each containing 3–12 loosely grouped fluorescing nuclei. Between the 24th and 42nd day (starting about day 30), destructive and proliferative CPE became increasingly pronounced, so that by the 42nd day much of the cell population was multilayered with necrosis most conspicuous in the upper strata. When a coverslip taken at the 42nd day was examined for fluorescence, many more nuclei fluoresced than previously, especially those in heaped-up areas, but their proportion was impossible to estimate because of extensive multilayering.

Subcultures of E cells representing the 3rd, 7th, and 10th passages were later examined in the same way. Only about 0.01 to 0.1 per cent of the nuclei exhibited

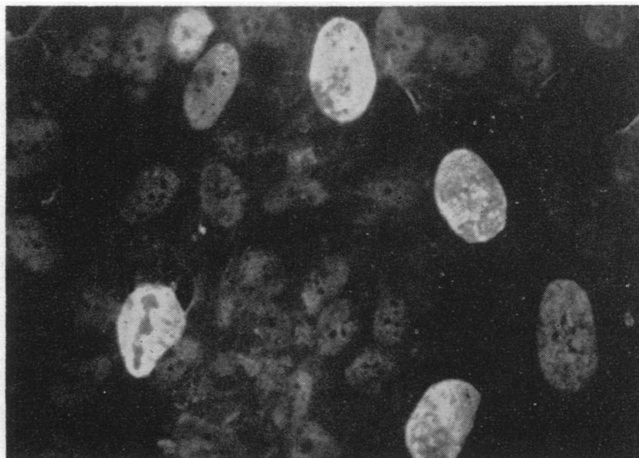

FIG. 2.—Immunofluorescence in E cells in third subculture. Magnification 200×. A group of swollen fluorescing nuclei are shown. Note variation in intensity of staining and presence of nonstaining "vacuole" in one nucleus. Photograph is slightly over-exposed to demonstrate outlines of nonstaining cells in background.

specific fluorescence when cultures were in the monolayer-forming stages. Differences in nuclear fluorescence associated with variation in size were observed as in F_i cells. Fluorescing E cells were also apt to occur in randomly distributed foci. When subcultures were examined after multilayering had occurred, a much higher proportion of nuclei (predominantly those in the thicker heaped-up areas) appeared to fluoresce. However, as in the F_i cells, at 42 days their proportion was impossible to estimate with precision both because of multilayering and also because of the presence of a semiopaque film of scummy consistency which overlay the cells and was diffusely fluorescent, especially where multilayering was most pronounced.

Infectivity titers: (a) *Comparative titers of SV40 virus in E and F_i cell cultures.* Supernatant fluids harvested from primary F_i cells at various intervals after inoculation with SV40 were assayed for infectivity in GMK cultures (Table 1). The concentration of virus remained approximately constant over a period ranging from 25 to 114 days. Emergence of apparently transformed cells in these cultures was not associated with significant change in output of virus. That presence of E cells did not affect viral titer was accounted for by the results of assays of fluids from a subculture consisting largely, if not exclusively, of E cells. In this experiment titers lower by 3 to 4 logs than those previously obtained in fluids from F_i cell cultures were recorded (Table 1). (b) *Comparative titration of SV40 virus in grivet monkey kidney (GMK) cultures and in human fetal kidney (F cell) cultures.* GMK cultures are widely employed as a sensitive assay for the presence of SV40 virus. It was therefore of interest to determine the relative sensitivity of GMK cells and F cells to the cytopathic effect of SV40. To this end, the virus which had previously been passaged three times in HF kidney cultures was titrated in parallel in GMK and HF kidney cultures taking CPE as criterion for the endpoint. The results are given in Table 2. It is seen that CPE appeared earlier and progressed more rapidly in GMK cells, making determination of the endpoint possible after 36 days. In contrast, the endpoint was not attained in HF kidney cells until

TABLE 1

INFECTIVITY TITERS OF SV40 IN FLUIDS FROM E AND F CELL CULTURES AT VARIOUS INTERVALS AFTER INOCULATION

Experiment No.	Tissue	Day fluid collected*	Titer†	Transformation‡
1	HF kid	25	6.5	0
		114	5.5	+
		114	5.8	0
3	HF kid	41	5.5	0
		81	5.5	+
5	Newborn	41	6.8	0
	kidney	89	5.8	+
6	3 month infant kid	41	6.8	0
		89	5.8	+
8	E cells§	35	2.3	+

* = After addition of virus.
† = Expressed as reciprocal log dilution of the endpoint per 0.1 ml fluid. All titrations performed in GMK cultures.
‡ = Transformed cells evident at time fluid collected.
§ = E cells derived from experiment 1 in third subculture.

TABLE 2

COMPARISON OF CYTOPATHOGENIC AND TRANSFORMING CAPACITY OF SV40 IN GMK AND HF KIDNEY CELL CULTURES*

Days after inoculation	Endpoint CPE (titer †)		Highest dilution of virus giving transformation‡	
	GMK	HF Kid	GMK	HF Kid
15	1.8	<0	<0	<0
18	3.8	<0	<0	<0
25	5.3	0.5	<0	<0
36	5.8	2.5	<0	<0
51	5.8	3.3	<0	<0
59	5.8	3.3	nd[x]	nd[x]
82	5.8	3.3	<0	3§

* The same SV40 pool was titered in both GMK and HF kidney cultures, and the cells were observed at intervals for CPE and transformation.
† Expressed as reciprocal log dilution of the CPE endpoint on that day as calculated by the formula of Reed and Muench.
‡ Reciprocal log of highest dilution of virus suspension giving transformation.
§ In more detail, the proportion of cultures with transformation among 3 inoculated with each dilution: 10^0, 1/3; 10^{-1}, 3/3; 10^{-2}, 1/3; 10^{-3}, 2/3.
nd[x] Cultures not examined for transformation on this day.

51 days and was lower by 2.5 logs. These differences suggest that GMK cells are considerably more sensitive than human renal cells to cytopathic effects of SV40.

In the foregoing experiment E cell transformation was not observed in GMK cells nor in HF kidney cells during the first 51 days of observation. It occurred, however, after 82 days in the latter system with all dilutions of virus that produced CPE (Table 2). These observations indicate that concentrations of virus sufficient to cause degenerative changes in HF kidney cells are equally capable of inducing transformation.

Effects of other viruses: Parallel titrations of various viruses were carried out in tube cultures of primary F cells and subcultured E cells. Using CPE endpoint as a criterion, the two systems proved to be equally susceptible to each of the viruses tested (Table 3). However, by certain other criteria the tissues differed in their response to the RNA viruses (with the exception of the RMC agent) but not to the DNA viruses. Both types of virus destroyed F cell cultures rapidly and completely. However, CPE induced by RNA viruses as compared with DNA viruses appeared much later in E cell cultures, progressed more slowly, and differed distinctly in appearance and extent as indicated in Table 3. In general, destructive changes in

TABLE 3

INFECTIVITY AND CYTOPATHIC EFFECTS OF VIRUSES OTHER THAN SV40 IN E* AND F† CELLS

Virus	Infectivity Titer‡ E cells	Infectivity Titer‡ F cells	Maximal CPE§ E cells	Maximal CPE§ F cells	Days after Inoculation Maximal CPE Attained** E cells	Days after Inoculation Maximal CPE Attained** F cells
Vaccinia	6.3	5.8	4+	4+	1	2
Adeno Type 2	3.5	3.5	4+	4+	7	6
Herpes Simplex	4.5	4.5	4+	4+	1	1
Polio Type 2 (Lans.)	3.3	3.5	1+	4+	7	3
Polio Type 2 (RMC)	<0.0	<0.0	0	0	—	—
Coxsackie A 9	4.5	4.5	4+	4+	12	5
Measles	2.5	2.5	4+	4+	19	12
Sindbis	4.3	4.8	2+	4+	5	1

* 5th subculture of E cells derived from experiment 1 (Table 1).
† Primary HF kidney cells.
‡ Reciprocal log of highest dilution of virus suspension giving CPE: volume inoculum = 0.1 ml.
§ 0 = no apparent CPE; 1+ = 25 per cent cells affected; ...; 4+ = 100 per cent cells affected.
** In cultures receiving undiluted virus suspension.

E cells were less characteristic of the viral species, consisting of rounding and fragmentation radiating from centers of affected cells. Of the RNA viruses, only Coxsackie A-9 and measles[8] agents destroyed the entire population of E cells within the period of observation (four weeks). It seemed possible that these modifications of cytopathogenicity might depend upon the presence of interferon developing as a result of infection with SV40 virus. Fluids from E cultures, therefore, were tested in primary human amnion cell cultures for interferon using Sindbis virus as challenging agent; none was demonstrated.

Subsequent experiments employing an independently derived line of E cells have confirmed the above-noted differences in susceptibility to RNA viruses, so that the differences presumably do not depend on the selection of one particular line of E cells.

Interferon: The chronic nature of the SV40 infection in HF kidney cultures prompted attempts to demonstrate interferon in this system. Nutrient medium removed from SV40-infected cultures (F_i cells) at 10-day intervals was tested for the presence of interferon by challenge with Sindbis virus in primary human amnion cultures. No protection was demonstrated even when undiluted fluids were employed. These negative findings do not preclude the presence of interferon in low titer which might be demonstrated by a more sensitive assay or by concentrating the fluids before assay.[9]

Continuing association of SV40 with E cells: As noted previously, fluorescent antibody studies of E cells revealed SV40 antigen in a small proportion of the population even after 10 or more subcultures. Therefore, an attempt was made, which proved unsuccessful, to determine whether virus-free E cells could be obtained and propagated in subculture with retention of characteristic properties. E cells (derived from the fifth passage: see experiment 2, Table 1 of previous paper) were subcultured daily for eight days in the presence of 10 per cent hyperimmune guinea pig anti-SV40 serum. Cells from the last subculture were then washed six times (to remove antiserum). They were thereafter subcultured at weekly intervals for six weeks in the absence of antiserum. During these subcultures the characteristics of E cells were retained. Tests for viral antigen employing the immunofluorescence technique were carried out on materials from the 1st, 3rd, and 5th cell passages after withdrawal of antiserum. All were negative. Examination of preparations stained with hematoxylin and eosin likewise revealed no cytopathic changes

attributable to effect of the virus. However, GMK cultures inoculated with fluid and cells from the fifth cell passage exhibited characteristic vacuolization and cytolyis as well as specific immunofluorescence. Further attempts to free E cells of virus are in progress.

Discussion.—Mechanism of proliferation: Only a small proportion of cell nuclei in SV40-inoculated cultures fluoresce at a time when cell proliferation is 2–5 × that in control cultures and when large amounts of virus are present in the medium. This indicates that most of the cells which have proliferated do not exhibit specific fluorescence although heavily exposed to infection. The factors that are responsible for this failure of a large proportion of cells to fluoresce remain obscure. To determine them eventually is clearly of much significance since in this way the manner in which the virus induces cell proliferation might become clearer.

One may consider two possible explanations of the failure of many proliferating cells to demonstrate evidence of infection. First, the nonfluorescing cells may have escaped infection. If so, the observed increase in proliferation might depend upon stimulating factors produced by infected cells and transferred via the medium. Second, virus may enter all or nearly all cells but induce fluorescence in only a small portion. Because of the presence of high concentrations of extracellular virus, this possibility seems more likely. In cells that exhibit fluorescence, virus may multiply without restraint to reach levels incompatible with cell survival, whereas in nonfluorescing cells viral multiplication may be restricted to the point where it is not demonstrable by the fluorescent technique. Under the latter conditions, cell division may be stimulated. As factors limiting viral multiplication in many of the cells, one can visualize (*a*) spontaneous variations in susceptibility, (*b*) development of interferon or other viral inhibitors, (*c*) rates of cellular division equivalent to or exceeding that of viral increase, and (*d*) failure of virus to replicate: the entering virus itself induces an inheritable alteration in the growth regulatory mechanism of the cell. So far, with the test methods employed, interferon has not been detected in the infected cell systems. It may, however, prove to be present in low concentrations. Absence of viral increase with primary induction of an inheritable alteration of the growth-regulating mechanisms seems unlikely since attempts to cultivate F_i cells in series were unsuccessful.[2] The other two possible factors have not been as yet investigated.

Mechanism of transformation: In a previous paper,[2] it was suggested that E cells arise as a result of an inheritable alteration induced by SV40 rather than by selection of pre-existing cells with the characteristics of E cells. The experiments on relative susceptibility of E and F cells to viruses other than SV40 support this view. In particular, E cells proved resistant to certain RNA viruses which completely destroyed cultures of F cells. If pre-existing E cells had been present among the F cells, a few at least should have survived.

Evidence presented in the previous paper[2] suggests that the site of the primary alteration induced by SV40 in transformed cells is intranuclear. The similar properties of lines of E cells derived from tissues of different individuals further suggest that this alteration is essentially the same in each line. Thus, E cells from different experiments proved to be similar in their morphology, growth characteristics and chromosomal aberrations.[2] In this communication, altered response to RNA viruses of transformed cells derived from different experiments is reported.

Whether or not the factors responsible for the essential features of the transformed cells are established during the stage of increased proliferation remains undetermined. Studies of the chromosomal complement of F_i cells are in progress. Preliminary observations[10] have revealed no abnormalities comparable to those found in E cells. If supported by additional investigation, the normal karyotype of F_i cells would suggest that heritable changes in the nucleus occur coincidently with transformation.

Relative susceptibility of E and F cells to SV40: Although not conclusive, there is indication that E cells are less susceptible than F cells to SV40. Comparative fluorescent antibody studies in which superinfection was induced indicated that a smaller proportion of E cells exhibited specific fluorescence. Furthermore, it was shown that viral replication appeared to be significantly less in cultures of E cells. CPE induced by the virus and consistently present in F_i cultures was less marked and more variable in E cell cultures.

Relative susceptibility of E and F cells to viruses other than SV40: With several DNA viruses, cytopathic effects were indistinguishable in cultures of E and F cells. In contrast, changes induced by RNA viruses in E cell cultures were atypical, delayed, and often incomplete, although the CPE endpoint was the same in both E and F cells. Speculation regarding the mechanism underlying this difference is hazardous in the absence of comparative data on viral synthesis in the two systems. However, future analyses may reveal abnormalities of RNA metabolism in E cells which limit the multiplication of RNA viruses and modify their cytopathic effects.

Summary.—Cell-virus relationships have been studied during the course of the cytolytic, proliferative, and transforming changes that occur in primary human renal cell cultures infected with SV40 virus. In such cultures, viral infectivity titers had attained maxima by the 25th to the 41st post-inoculation day and thereafter remained constant during many weeks (9–16). The cytopathogenic and cell-transforming capacities of the virus were found to be approximately equivalent. Interferon was not demonstrated in these chronically infected cell systems. As compared to cells in uninfected cultures, transformed cells in subcultures exhibited increased resistance to injury by SV40 and an altered cytopathic response to infection with several RNA viruses.

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