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Studies on the transformation of human fetal cell cultures by simian virus 40

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Abstract

A human fetal fibroblast strain, belonging to a group resistant to SV40 transformation, was transformed by SV40 through a multiple inoculation procedure. Two independently transformed cells were described in comparison with each other. The proportions of cells with the nuclei possessing V antigen were 2.9% at the 5th passage in one strain and 1.1 % at the 4th passage in another, and they declined gradually as frequent passages were repeated. The percentages of the transformed cells with V antigen-positive nuclei were, in both strains, quantitatively compatible with those of the cells with the nuclei full of virus particles in crystalline arrays, which were demonstrated by immunofluorescent studies and electron microscopy.

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STUDIES ON THE TRANSFORMATION OF HUMAN FETAL CELL CULTURES BY SIMIAN VIRUS 40

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Morphological transformation of human cells by simian virus (SV40) has been reported in a variety of tissue-cultured cells. Among these are cells derived from newborn kidney (1), skin and buccal mucosa (2), adult thyroid (3), embryonic lung (4—6), foreskin (7), amnion (8, 9), astrocyte (10), parathyroid adenoma (11), and liver (12, 13). In these studies, characteristic properties of human cell transformation by SV40 have been investigated which, as a rule, distinguish human transformed cells from those of the other species transformed by SV40; (a) the proliferative stage of transformation results in a "crisis" period occurring at a varied time after the initiation of transformation. Characteristics of the "crisis" are a remarkable decline in cell proliferation, increased frequency of abnormal cell division and the eventual decrease in cell number (11, 14, 15). (b) the yield of SV40 virus detectable in the supernatant fluids of transformed cell cultures, ranging in titer from 10^2 to $10^{7.1}$ TCID₅₀ (3, 7, 12, 16, 17, 18), and (c) a variety of proportions (0.01-75 %) of cells with the nuclei possessing V antigen (3, 7, 12, 16, 17, 18).

In addition, a few recent reports have described the transformation of human cells by DNA isolated from SV40 (19—21).

This report describes two independent transformation experiments and some of the characteristic features of a "resistant" fibroblast strain (22) transformed by SV40 through a multiple inoculation procedure. The proportions of cells with the nuclei containing V antigen gradually declined after the onset of transformation, and the percentages were, in both transformed cells, quantitatively compatible with those of cells with the nuclei full of virus particles, which were demonstrated by immunofluorescent studies and electron microscopy.

MATERIALS AND METHODS

SV40 virus: The stock virus of SV40 (Strain 777), originally obtained from

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Dr. KUMAO TOYOSHIMA of Virus Laboratory, Osaka Public Health Institute, was propagated in our laboratory in Vero cells (an established cell line from African green monkey kidney). The infectivity titer of the stock virus was 10^6 TCID₅₀/0.1ml.

Media: Nutrient media for cell cultures consisted of Eagle's minimum essential medium supplemented with 20 % bovine serum for normal cell cultures and 10 % for SV40-inoculated cultures. All media contained 100 units of penicillin and 100 μ g of streptomycin per ml.

Cell cultures and transformation experiments: A human male fetus of an estimated three months' gestation was obtained from a clinically normal adult female. After washing in phosphate buffered saline (PBS), the fetus was minced, trypsinized with 0.02 % trypsin and seeded into flat-bottomed glass culture bottles. The culture media were renewed every other day. Fully-sheeted cultures were digested with 0.02 % trypsin and subcultured at 1:2 split ratio in the control cultures. Culture bottles to be inoculated by dispersed cells were previously conditioned by approximately 1 % of sodium bicarbonate in PBS. The confluent cultures of the cells were 1.4×10^4 cells/ml at the maximum cell number.

Two series of cell cultures were used in transformation experiments at the first passage, 7 days (Series 1) and at the 6th, 43 days (Series 2), respectively, after the primary culture. The former cultures predominantly consisted of elongated cells accompanied by a small number of epithelioid cells (Fig. 1), while in the latter no growth of epithelioid cells could be recognized (Fig. 2) and the cells were occasionally arranged in interlacing bundles (Fig. 3), showing prominent fibers interwoven with the cells.

The cell cultures were inoculated with 0.2 ml of the stock virus for 2 hours at 37°C. Cell cultures of both series were inoculated with the stock virus three times within 45 and 21 days, respectively (as described in "Results").

Immunofluorescent staining: For staining V antigen, the direct method was employed with fluorescein-isothiocyanate (FITC)-conjugated globulin, prepared from antiserum of a rabbit inoculated with purified SV40 virus.

For staining T antigen, the indirect method of POPE and ROWE (25) was employed with SV40 tumor-bearing hamster serum and with FITC-conjugated rabbit anti-hamster globulin. The stained coverslips were observed with a Nikon fluorescent microscope.

Electron microscopy: Transformed cells of two series at the 6th passage, 24 days after transformation (SVHF-1) and at the 5th passage, 31 days after transformation (SVHF-2), respectively, were detached from the glass surface by scraping with a rubber policeman and centrifuged at low speed for 10 min. The cell pellets

Fig. 1 The cells on the 5th day after a primary culture of a human fetus, starting at the cell density of 2×10^4 cells/ml. Both fibroblasts and epithelioid cells are observed. Giemsa stain. (x 300)

Fig. 2 The control culture at the 12th passage. Elongated cells and prominent collagen fibers interwoven with the cells. No epithelioid cells can be recognized. Giemsa stain. (x 300)

Fig. 3 The control culture at the 12 passage. Fibroblasts are arranged in interlacing bundles. This should not be confused with the irregular multilayer characteristic of transformed cells. Giemsa stain. (x 500)



were fixed with 2.5 % glutaraldehyde and 1 % osmium tetroxide. The thin-sectioned specimens were stained with 2 % uranyl acetate and lead citrate, and examined in a Hitachi HU-11C electron microscope.

Chromosome analysis: Chromosomes of two transformed cells were examined, at the 11th passage, 51 days after transformation (SVHF-1) and at the 4th passage, 30 days after transformation (SVHF-2).

The cell cultures during the phase of active growth were treated for 3 hours with 1 μ g/ml of colchicine in culture media. Then they were washed once with PBS, digested with trypsin and centrifuged at 1,500 rpm for 10 min. The cell pellets were suspended in 2 ml of 1 % sodium citrate and incubated for 20 min. at 37°C. Six drops of the fixative mixture of methanol and glacial acetic acid (1 : 1) were slowly put on the cell suspensions, and 1 ml of the fixative was layered on the specimens, which were kept still for 10 min. Subsequently they were centrifuged, and 2 ml of the fixative was added, mixed well and centrifuged again. Finally, the pellets were resuspended in 0.5 ml of the fixative. One to two drops of the fixed materials were dropped on clean slide glasses covered with 50 % ethanol, and a quick burning was done in a weak flame. All the specimens were stained with 2 % May-Greenwald Giemsa solution for 4 hours.

Infectivity tests: For infectivity titrations of SV40 in the supernatants from 2 transformed cell cultures, nearly confluent Vero cells grown in test tubes were inoculated for 2 hours at 37°C with 0.2 ml of serial dilutions of either supernatants or cell extracts from transformed cells (SVHF-1 & SVHF-2). Extracts were prepared by repeated freezing and thawing of the cell cultures.

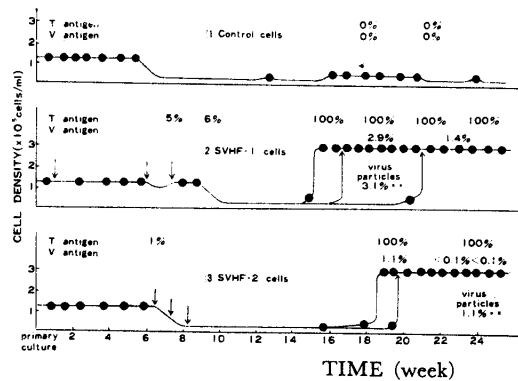
Transplantability of transformed cells: Hamsters weighing from 40 to 50 g at 25 days of age were conditioned with 500 r of X-ray just before transplantation. Transformed cells in a logarithmic growth phase at the 6th passage after transformation were trypsinized, washed once and suspended in 0.1 ml of serum-free culture medium. Normal cells at the 15th passage after the primary culture were also prepared in the same way. The bilateral cheek pouches of each hamster were inoculated with the previously dispersed cell suspensions ranging in cell number from 10^4 to 10^6 for the transformed cells and from 2×10^5 to 10^6 cells for the control cells, according to the procedure described by FOLEY *et al.* (23). Subsequently the hamsters were checked for tumors every 2 to 3 days.

RESULTS

Transformation experiments (Text-Fig. 1): After the first inoculation of the stock virus, cells belonging to Series 1 showed no cytopathic changes but good cell growth, and subcultures could be done once a week for one month. During this period no growth of epithelioid cells was recognized. The second inoculation was performed 37 days after the first inoculation. Forty-eight hours later, approximately 5 % of the nuclei contained T antigen. Then the inoculated cultures showed a considerable degree of cytopathic appearance but it declined within a few days. One week after

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the second, the final virus inoculation was executed. Subsequently, a slight decline in growth rate of the inoculated cells was noted, but the cells could be subcultured with longer intervals. Twelve days after the last inoculation, T antigen was examined. About 6% of the nuclei showed nuclear staining. For the following 6 weeks the cultures revealed lower growth rate and tendency to lose cells.



Text-Fig. 1 The time course of transformation experiments

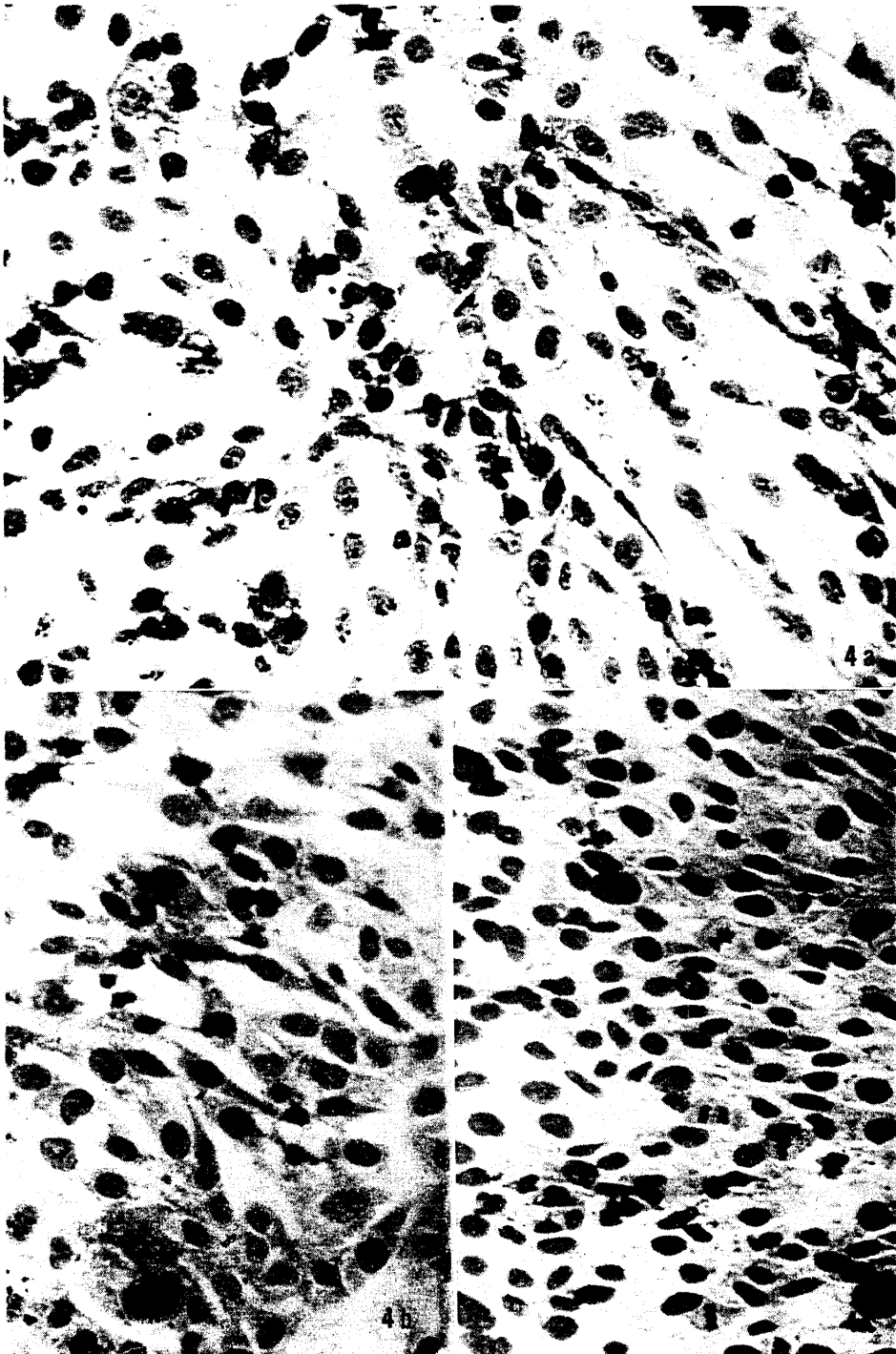
● marks indicate subcultures.

* The frequent passages were intentional for many tests to obtain as many cells as possible.

** Frequency of cells with the nuclei possessing virus particles, observed by electron microscopy.

On the 100th day after the first virus inoculation (56th day after the last inoculation), a focus of rapidly proliferating cells appeared in randomly dispersed fibroblasts, which was easily distinguishable by cluster formation and appearance of numerous mitotic figures. Nine days later, after two more passages, T antigen was examined and detected in all the nuclei. The first transformed cell series was termed SVHF-1. Furthermore, the other two cultures in this series were transformed on the 66th and 90th days after the last virus inoculation.

The cultures in Series 2 were infected by the virus three times with one week intervals. Forty-eight hours after the first virus inoculation, they possessed T antigen in approximately 1% of the nuclei. After three successive virus inoculations, the growth rate of the cells in this series was also lowered, but three subcultures could be done in the following 70 days. On the 71st day after the last virus inoculation, a focus of rapidly proliferating cells appeared just as in Series 1. Eight days later, after two more passages, the proportion of cells with T antigen-positive nuclei was 100%. In addition, another culture in this series was transformed on the



79th day after the third transformation. The transformed cells in Series 2 were termed SVHF-2. No such cell foci were noted in the control cultures. The morphological characteristics of SVHF-1 cells were quite similar to those of SVHF-2 cells.

In the stage of "early transformation" (17) the majority of the cells were epithelioid, and the cell arrangements were abnormal, forming small multilayered piles (Fig. 4). The characteristics of transformed cells were numerous mitoses, pleomorphic nuclei with many nucleoli, and loss of contact inhibition (Fig. 5). The mitotic indices of the transformed cells at the sixth passage after transformation and the control culture cells at the twelfth passage were 3.7 % and only 0.4 %, respectively.

Immunofluorescent studies: One or two passages after transformation were sufficient to exclude the T antigen-negative cells, probably due to the difficulty of the nontransformed fibroblasts in survival after repeated passages. At the 2nd, 5th, 8th and 15th passages of SVHF-1 cells and at the 2nd, 4th and 8th passages of SVHF-2 cells, the cells were tested for the proportion of cells containing T antigen-positive nuclei. The cultures at the 2nd passages of both transformed cells, as described in "Transformation experiments", had no cells with the nuclei unstained by the specific fluorescence. In the subsequent tests all the cells were found to be positive for the T antigen. Cells in the control cultures were all negative for the antigen throughout the experiments. The specific fluorescence was located in the nuclei of all the transformed cells with the sparing of the nucleoli. The intensity and appearance of the fluorescence varied considerably between individual nuclei. Occasionally, the fluorescence was of a reticulated appearance, varying from a fine to a coarse network. In some cells, the nuclei were stained as "doughnut-shaped" bodies consisting of a bright fluorescent ring surrounding a less intensely fluorescent center (Fig. 6).

The transformed cells were examined for the presence of V antigen. Except in occasional cells caught in the process of lysis, V antigen was exclusively located in a small number of the nuclei. In SVHF-1 cells the proportions of cells with V antigen-positive nuclei were 2.9 % at the 5th passage, and 1.4 % at the 12th passage, whereas in SVHF-2 cells 1.1 %

Fig. 4 Cell cultures of "epithelioid" transformed cells.

(a) Pleomorphic nuclei with many nucleoli and small piles are observed, 4th passage after transformation.

(b) Another photograph of the same culture. A giant cell and many nucleoli are evident.

(c) Numerous mitotic cells and irregular nuclei can be seen, 7th passage after transformation. (x 500)



at the 4th passage, and less than 0.1 % at the 6th and 8th passages, respectively (Fig. 7). The cell cultures with the constant, low percentage of V antigen-positive cells (SVHF-2 cells) showed neither abundant yield of infectious virus particles nor characteristic features of the cells during a "crisis" period (8, 11, 14, 15) which most human cells transformed by SV40 were destined to enter at variable intervals after the onset of transformation.

V antigen was not found in cells caught in the process of mitosis. V antigen was negative in the control cultures (Fig. 8).

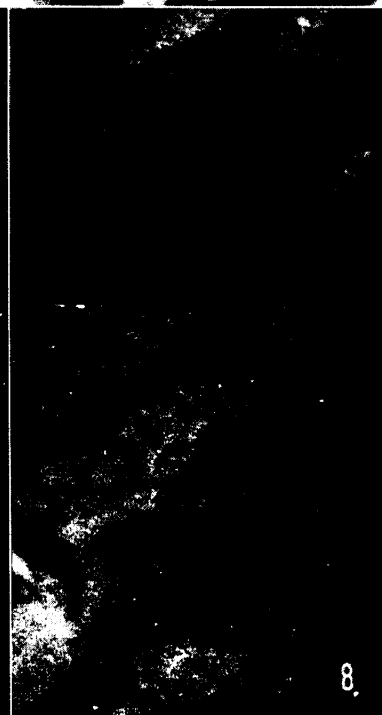
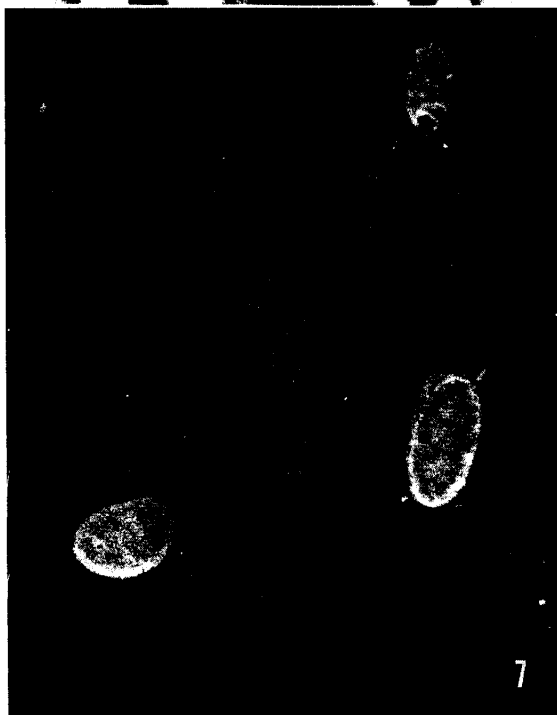
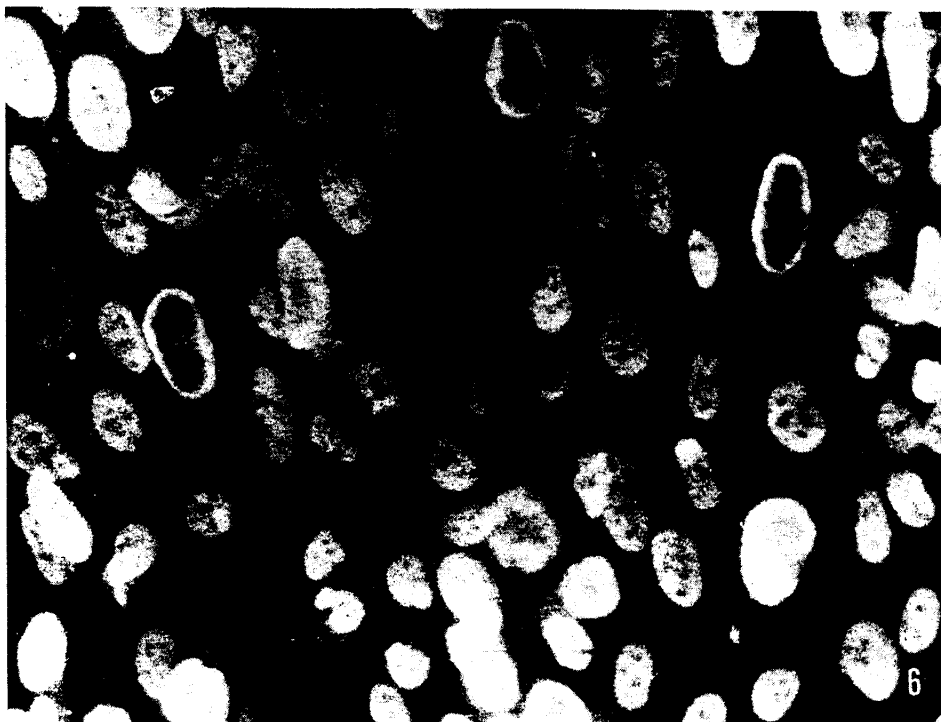
Electron microscopy: SVHF-1 cells at the 6th passage, 24 days after transformation, SVHF-2 cells at the 5th passage, 31 days after transformation, and the control cultures at the 12th passage after the initiation of the culture were examined for the presence of virus particles by electron microscopy.

In the ultrathin sections of SVHF-1 cells, intranuclear virus particles were observed in 3.1 % of the cells (31/1,000 cells). The virus particles in the nuclei were round in shape and uniform in size (40—43 m μ in diameter), and they appeared apt to form crystalline arrangements (Fig. 9). Occasionally, a small number of cells with virus particles both in the nucleus and in the cytoplasm were observed (Fig. 10). These extranuclear viruses were morphologically similar to those observed in the nuclei. This may suggest the leaking of virus particles from the nucleus to the cytoplasm. The cells with the nuclei full of virus particles were apt to appear in randomly distributed foci, which was analogous to the findings of the immunofluorescent studies on V antigen.

In the SVHF-2 cell samples, only one cell with the nucleus containing virus particles in a crystalline array was found in more than one thousand cells observed one by one.

The proportions of virus-possessing cells in SVHF-1 and SVHF-2 cells appeared compatible with the data of the immunofluorescent studies on V antigen that the antigen was positive in 2.9 % of the nuclei of SVHF-1 cells at the 5th passage, and in less than 0.1 % of those of SVHF-2 cells at the 8th passage after transformation. Virus particles were not found in mitotic cells. No cells possessing virus particles were found on the grids in the control cultures.

Fig. 5 Abnormal nuclei and cytopathic changes in transformed cells at the 6th passage after transformation. (a) An inclusion body in a nucleus and vacuoles in the cytoplasm
 (b) An abnormal nucleus, and vacuoles in the cytoplasm
 (c) An abnormal nucleus and a "budding"
 (d) Another abnormal nucleus (x 2,000)



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Infectivity tests: The supernatant of an SVHF-1 cell culture at the 8th passage was inoculated for 2 hours at 37°C on monkey kidney cells just as in stock virus titrations. Typical CPE by SV40 virus appeared on the third day (Fig. 11) and reached into 100 % on the 8th day after inoculation of the supernatant. The infectivity titers of the supernatants of SVHF-1 cells at the 10th passage and SVHF-2 cells at the 6th passage after transformation were 10^4 TCID₅₀/0.2 ml and 10^4 TCID₅₀/0.2 ml, respectively.

Chromosome analysis: As shown in Table 1, most of mitotic cells in two transformed cell series fell into hypoploidy; 72% for SVHF-1 cells

TABLE 1 DISTRIBUTION OF CHROMOSOME COUNTS

Transformed cells	Number of cells with chromosome count indicated												Other chromosome counts	Total metphases examined	Cells with distinctly abnormal chromosomes
	38	39	40	41	42	43	44	45	46	47	48				
SVHF-1	2	3	1	9	7	12	21	11	13	7	3	30, 34, 35, 35, 66, 67, 75, 77, 80, 80	100	26/50	
SVHF-2	4	2	4	3	4	11	20	20	21	4	1	64, 69, 83, 84, 87	100	24/50	

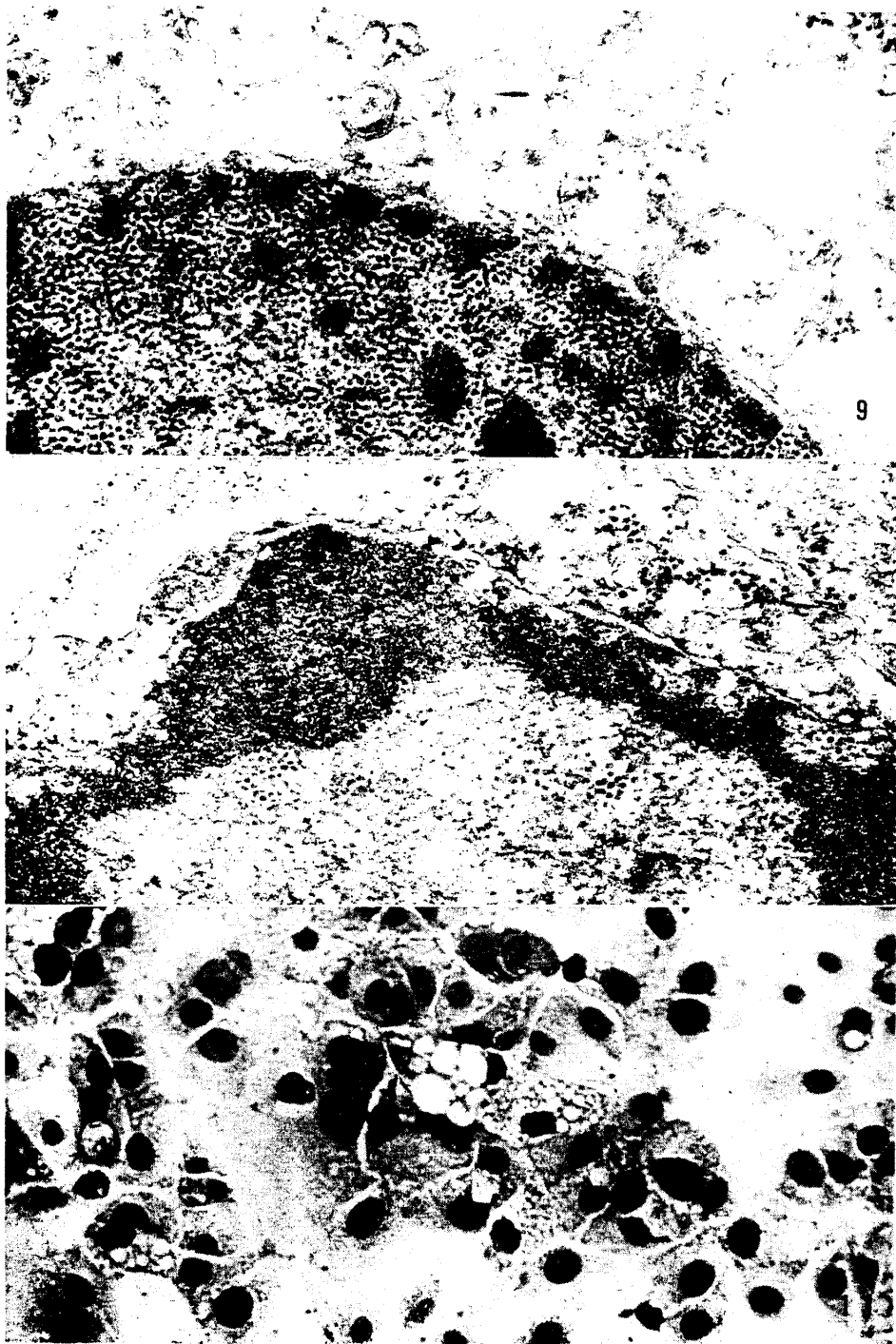
and 79 % for SVHF-2 cells. Cells with distinctly abnormal chromosomes were counted. The results show that about one half of the metaphases were involved in some aberrations. The chromosomal changes were characterized by variable degrees of constrictions and dicentrics, one to several for each abnormal metaphase (Fig. 13). Fig. 12 shows a metaphase of SVHF-1 cells with 41 normal (in appearance) chromosomes and 3 chromosomes with distinct aberrations. Two constrictions and an acentric fragment are evident. The missing chromosomes are one from the 19—20 group and another from the 21—22 group. Any "marker" chromosomes specific for all transformed cells were not recognized.

Transplantability of transformed cells: The ability of the transformed cells to produce tumors in the hamster cheek pouch was investigated. In the cheek pouches inoculated with 2×10^5 and 10^5 cells from transformed cell cultures, three nodules ranging from 2 to 4 mm in diameter were

Fig. 6 Intracellular fluorescence of T antigen in the transformed cells at the 5th passage after transformation. All the nuclei are stained. The mode of the fluorescence varies considerably between individual nuclei. ($\times 1,000$)

Fig. 7 Intracellular fluorescence of V antigen in the transformed cells at the 5th passage after transformation (2.9%). This photograph shows that the cells with the nuclei possessing V antigen are arranged in randomly distributed foci. (1,000)

Fig. 8 No specific fluorescence is observed in the control culture. ($\times 1,000$)



observed from 3 to 6 days after inoculation. Histological examination of one of the three nodules excised on the 6th day after cell inoculation revealed no neoplastic growth of the inoculated cells. The nodule consisted of granulation tissue, necrotic debris and infiltrates of leucocytes. Then the remaining two nodules gradually declined and had faded away by the end of the second week after the initiation of the transplantation experiment.

DISCUSSION

The multiple SV40 inoculation experiments were performed for two reasons ; (a) previous failures in transformation experiments of two independent human fetal cell strains by SV40 through a single virus inoculation procedure after careful maintenance and frequent observations on the infected cells for 281 and 285 days, and (b) the low percentages of the nuclei positive with T antigen, 48 to 72 hours after the first virus inoculation (as low as 1 % or so), followed by the second inoculation after an adequate period, which increased the proportion of T antigen-positive nuclei up to 2 to 10 %. However, the previous failures in transformation might be due to the low titer of SV40 virus (as low as 10^5 TCID₅₀/ml) and resistance of the cells against transformation.

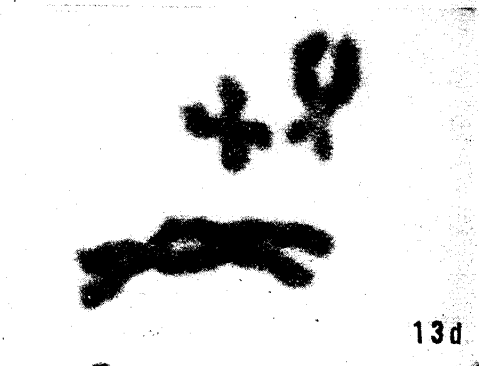
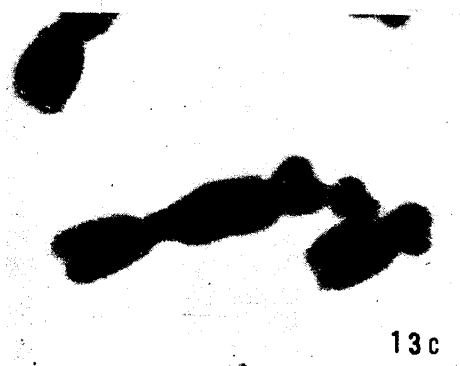
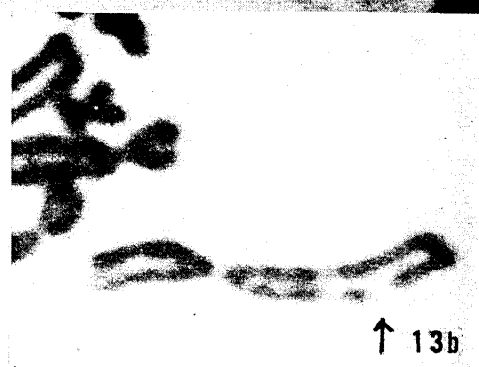
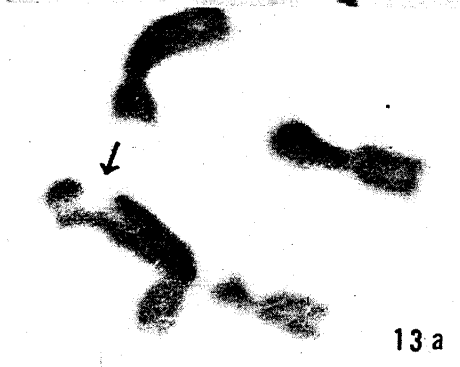
A number of authors have reported the percentages of V antigen positive cells at variable passages and virus titers of the supernatant fluids from the transformed cell cultures (Table 2). In the studies of NOORDAA (12), 70—80 % of the nuclei of a population of transformed cells contained V antigen, and the titers of the supernatant fluids from the transformed cell cultures varied from 10^2 to 10^3 TCID₅₀/ml. One reasonable explanation of the data may be that in most cells, synthesis of viral coat protein occurred without the subsequent production of matured viruses. RABSON *et al.* (3) reported that approximately 5 % of nuclei in "infected" (or transformed) cultures were constantly positive in repeated V antigen detection tests for 10 weeks after the initiation of the transformation.

The data described in this report do not agree with theirs in that both in two independently transformed cells, the proportions of cells with

Fig. 9 Electron micrograph of a SVHF-1 cell with the nucleus full of virus particles (x80,000)

Fig. 10 Electron micrograph of another SVHF-1 cell with virus particles both in the nucleus and in the cytoplasm. This photograph suggests the leaking of virus particles from the nucleus to the cytoplasm. (80,000)

Fig. 11 Prominent cytopathic effects on monkey kidney cells on the 4th day after inoculation of a supernatant from a SVHF-1 cell culture. (x 500)



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TABLE 2 V ANTIGEN AND VIRUS ISOLATION

Author	Cell origin	V antigen (%)	Virus titers of supernatants (TCID ₅₀)
Shein (1962)	fetal kidney	0.01—0.1	2.3/0.1ml (3rd passage)
Rabson (1962)	adult thyroid	appr. 5(a)	10 ^{6.8} —10 ^{7.1} /ml
Moyer (1964)	fetal lung	undone	10 ³ —10 ^{6.5} /0.1ml
Bissett (1966)	skin	less than 1	undone
Petursson (1966)	foreskin	less than 0.1	10 ^{4.9} , 10 ^{5.9}
Noordaa (1968)	liver	appr. 75	10 ² —10 ³ /0.1ml
Khoury (1969)	liver	undone	none(b)

(a) This percentage persisted for more than 3 months, still showing a rapid cell growth pattern.

(b) No infectious viruses were found in the supernatant fluids. Virus induction experiments using cocultivation and cell fusion techniques were all negative.

the nuclei possessing V antigen gradually declined and, in one group of the transformed cells, remained constantly less than 0.1 %, showing neither abundant yield of virus particles nor characteristic features of a "crisis" period. One important thing lies in the fact that their transformed cells had no morphological features of "epithelioid" transformed cells reported by all the others (5, 6, 8, 11, 14, 15).

The infectivity titers of virus particles produced by transformed cell cultures in the supernatant fluids were detected somewhat in proportion to the percentages of cells with the nuclei containing V antigen, except for the two cases of NOORDAA (12) and KHOURY (13). KHOURY made the first report on SV40-transformed cells from which no infectious virus particles were recovered before crisis.

In electron microscopic studies, RABSON (3) demonstrated intranuclear localization of SV40 virus particles in approximately 5 % of the cells, and the percentage of cells with the nuclei filled with virus particles was approximately equal to that of the cells with the nuclei containing V antigen. However, PETURSSON (7) found no intracellular virus particles in his transformed human cells but only clusters of extracellular particles morphologically similar to those observed within the nuclei of SV40-infected (but not yet transformed) cells.

Fig. 12 Metaphase chromosomes of a SVHF-1 cell with 41 normal (in appearance) chromosomes and 3 abnormal chromosomes. Arrows indicate two constrictions and an acentric fragment. The missing 2 chromosomes are one from the 19—20 group and another from 21—22 group. (x 5,000)

Fig. 13 Portions of metaphase chromosomes, showing strong constrictions (a and b) and dicentrics (c and d) (x 10,000)

In the present report, the quantitative data on the ultra-thin sections of two transformed cells ascertained RABSON's observations; namely, intranuclear virus particles were observed in crystalline arrangements and the percentages of cells with the nuclei possessing virus particles (3.1 % for SVHF-1 cells and less than 0.1 % for SVHF-2 cells) appeared compatible with the data of immunofluorescent studies on V antigen that the antigen was positive in 2.9 % for SVHF-1 cells and less than 0.1 % for SVHF-2 cells in the nuclei.

In SVHF-1 cell cultures, V antigen was positive in 2.9 % of the nuclei at the 5th passage, 23 days after transformation and 1.4 % at the 12th passage, 51 days after transformation. Within these 4 weeks, the total cell population increased, on a liberal calculation, up to more than 700 times as large as that at the 5th passage. It is impossible for the cells with the nuclei containing V antigen to keep occupying the half of the previous percentage even after 7 more passages, provided that the cells with the nuclei full of virus particles have no or only very small potentiality to divide because no virus particles were observed in the nuclei of the cells in mitosis. One possibility is that transformed cells are being involved in reinfection by SV40. This idea agrees with the fact that both V antigen-positive cells and SV40 virus-containing cells are frequently observed in "randomly distributed foci". However, a recent report on "superinfection" by SV40 (24) suggested that human cells transformed by SV40 were incapable of producing infectious viruses after exposure to SV40. Another possibility is that some transformed cells initiate SV40 virus production through some unknown activation mechanisms.

SUMMARY

A human fetal fibroblast strain, belonging to a group resistant to SV40 transformation, was transformed by SV40 through a multiple inoculation procedure. Two independently transformed cells were described in comparison with each other.

The proportions of cells with the nuclei possessing V antigen were 2.9 % at the 5th passage in one strain and 1.1 % at the 4th passage in another, and they declined gradually as frequent passages were repeated. The percentages of the transformed cells with V antigen-positive nuclei were, in both strains, quantitatively compatible with those of the cells with the nuclei full of virus particles in crystalline arrays, which were demonstrated by immunofluorescent studies and electron microscopy.

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