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Abstract

In the present experiment, it has been noted that clonizing epithelial-like cells of the intestine 407 were more susceptible to SV-40 virus than normal fibroblasts in primary human cell cultures. In the early stage of the infection the cell growth was enhanced by the inoculation of DNA virus but many cells died, showing lysis characterized by CPE, clumping of chromatin and formation of inclusion bodies. On the other hand, the cells surviving infection have given rise to virus-free long term cultures and cellular responses to the virus characterized by cell proliferation which is classified in four phases. (Phase. I: infection and cell alteration. Phase. II: crisis. Phase. III: fibro-reticulum cell formation. Phase. IV: recovery and proliferation). The most remarkable morphological characteristic was fibroblastic cell alteration from epithelial cells at 5 weeks of virus inoculation. By this study an interesting generalization of human epithelial-like cells can be made about the differentiation of the transformed cells in relation to SV-40 virus and it has been shown that an established human cell line is still susceptible to the reverting action of the SV-40 virus.

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CELL TRANSFORMATION OF ESTABLISHED HUMAN CELL LINE INDUCED BY SV-40 DNA

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The simian vacuolating virus (SV-40) is oncogenic for the newborn hamster^{1,2} and induces transformation of human tissues^{3,4,5} maintained *in vitro*. Conclusive evidence that virus could grow in human cells was lacking until SHEIN and ENDERS^{3,4} (1962, 1963) demonstrated primary cultures of human renal tissues to support the growth of virus. SHEIN and ENDERS^{3,4} described that cell transformation is characterized by epithelioid growth pattern, greatly accelerated growth rate and chromosomal aberrations. KOPROWSKI, PONTÉN and JENSEN (1963⁶) reported that SV-40 virus infected primary human diploid fibroblasts and noted morphologically epithelioid changes in the cells accompanied by distinct chromosomal difference. EDDY⁶ (1962) has demonstrated continued multiplication of SV-40 in continuous lines in HeLa. ROBSON⁷ *et al* (1962) also described that primary cultured cells of the adult human thyroid tissue are susceptible to SV-40 virus and the virus passaged through these cells has oncogenic capacity in newborn hamsters.

In an earlier report⁸ the oncogenicity of DNA of SV-40 virus *in vivo* was presented. SV-40 DNA induced fibrosarcoma in newborn hamsters and intact virus developed from viral DNA in a host cell could induce not only extensive fibrosarcoma but gland-cell carcinoma in the intestine of newborn hamsters at the early stage of infection. The availability of the viral agents infectious for primary cultured human cells and endowed with carcinogenic property for hamster tissue suggested initiation of the present study in which the behavior of established human cell lines infected by the viral agent was investigated and an assay for cellular responses of human cells to virus developed from infectious DNA was studied to obtain more information of relationship between oncogenesis in mammalian and that in human cells.

MATERIALS AND METHODS

Preparation of SV-40 and viral antigen developed from DNA⁸: The original SV-40 virus (10^{8.5}TCID 50/0.1 ml) was obtained through the courtesy

of Dr. Yohn of Roswell Park Memorial Institute, Buffalo, N.Y. U.S.A. Monolayers of BS-C-1 cultured in test tubes (average cell number 5×10^4 /tube) were used for propagation of SV-40. The cell monolayers of 200 test tubes were used for DNA extraction. DNA was prepared from the suspension by the use of cold phenol⁸. As shown in the previous report⁸ the viral antigen developed from DNA can induce subcutaneous sarcoma at the site of inoculation several months later and intestinal carcinoma in newborn hamsters at the early stage of infection. For the present experiment the viral antigen developed from DNA had the fourth passage through BS-C-1 and the specific CPE in the BS-C-1 cells was observed.

The permanent human clone cell line (Intestine 407): Intestine 407 was isolated by HENLE and DEINHARDT⁹ in 1955 from the jejunum and ileum of a two-month human embryo. The original cells were fibroblasts and histiocytes: however, by the fourth passage epithelial-like cells were observed (Fig. 6). The intestine 407 had a broad viral spectrum but was not as sensitive to most viruses as HeLa. The cell has modal chromosome number 76; range 56~153. Tumorigenicity of the cell forms intraperitoneal tumor in irradiated and cortisonized rats with 1×10^4 cells. The line is now available at the Microbiological Associates, Inc., U.S.A. and cells for the experiment were supplied by the Cell Service Department of the Roswell Park Memorial Institute.

EXPERIMENTALS

Infection of cultured cells: Thirty T type flask (Pyrex. T-30. 1×10^6 /ml cell number) cultures and twelve bottle cultures (16 ounce bottle, each bottle inoculated with 3×10^6 cells) were used on transformation studies of the intestine 407 when the clonizing cells were fully developed (Fig. 6). In cases of T type flask cultures each culture received five ml of diluted virus (3.0 ml of 199 Difco medium supplemented with 2 per cent calf serum: 2.0 ml of undiluted virus suspension: $10^{8.8}$ TCID 50/0.1 ml) and twenty ml of diluted virus (12 ml of 199 medium containing 2 per cent calf serum supplemented with 8.0 ml undiluted virus suspension: $10^{8.8}$ TCID 50/0.1 ml) were added to each bottle (16 ounce bottle) cultures. The cell cultures were inoculated at 37°C in a regular incubator for 24 hours. Twenty-four hours later the cells were replaced by $1 \times$ Eagle medium supplemented with 10 per cent fetal calf serum and 100 units penicillin. Thereafter this medium was used as the maintenance medium for all experiments and as the growth medium for cell lines. Cultures were held in stationary position at 37°C and medium fluids were changed every day or every other day as indicated by acid production. Samples of fluids from infected cultures were pooled at weekly intervals and stored at -70°C until virus testing. Tests for SV-40 virus were carried out in tube cultures of BS-C-1. Supernatant of medium

were assayed for virus titration. The first subculture was prepared by 0.2 per cent trypsinization (3 or 5 minutes) 10 days after virus inoculation and the cells were propagated in flasks (T type 30) and bottles (8 or 16 ounce bottle) by trypsinization at four-day intervals. Samples (0.5 ml in volume) were reserved for the estimation of cell numbers by enumerating stained nuclei¹⁰, and for the determination of cell viability by tests for eosin permeability¹¹. Other cells were cultured in 16×150 mm tubes for growth rate studies or on 12×40 mm coverslips in Leighton tubes (Bellco) for detailed cytological examination. The coverslip cultures were fixed in Zenker formalin, stained with hematoxylin eosin. Control cultured cells received no virus and they were maintained in the same manner as the experimental groups.

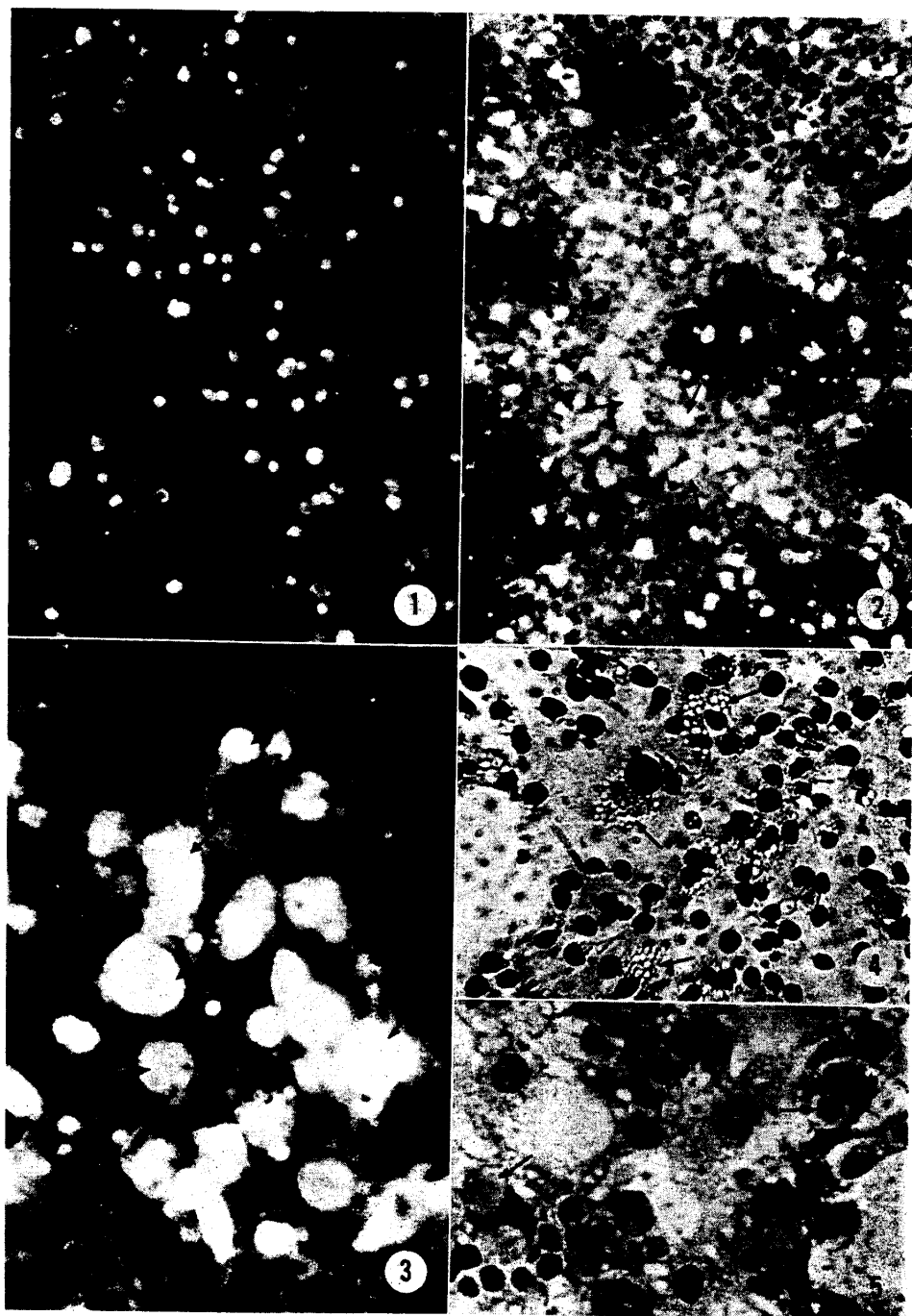
Primary human embryonic cell cultures: For the comparison with established human cell lines the human embryonic intestines removed from two-month human embryo and two-month human whole embryo obtained by the courtesy of Dr. Niwayama and Dr. Levine of the tumor procurement of Health Research (Roswell Park Memorial Institute) were minced. All cultures were grown in MacCoy 5 A supplemented with 20 per cent fetal calf serum and maintained in 1×Eagle's medium supplemented with 10 per cent fetal calf serum. The monolayer culture was inoculated with the identically diluted virus as the intestine 407 cultured in T type flask.

Immunofluorescence: Cells grown on coverslips were examined by the indirect Coon's technique. Cells were air-dried, fixed with acetone for five minutes and air-dried again. The coverslips were then stained for forty minutes at 37°C with fluorescein isothiocyanate labeled globulin derived from the serum of rabbits immunized against SV-40 virus⁸. Observations were made with the American Optical Fluorescent Microscope using dark field illumination.

RESULTS

The intestine 407 are mainly composed of epithelial-like cells which can be passed, clonized serially and resemble HeLa cells morphologically (Fig. 6). The epithelial-like cells are dissociated or attached together loosely with rough cell membranes.

In infected primary cultured human cells, about one per cent of cells exhibited very faint specific nuclear fluorescence when stained 10 days after viral inoculation. On the other hand, the human intestine 407 showed high susceptibility to virus and about 20 per cent of cells exhibited specific nuclear fluorescence after 24 hours of viral inoculation (Fig. 1) and about 80 per cent of cells were infected at 7 days after the inoculation (Figs. 2 and 3). Exposure of these cells to SV-40 virus did not cause immediate morphological changes but

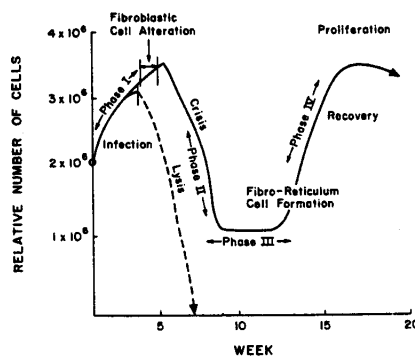


it enhanced growth potentials of the cell lines for 1 or 2 weeks of SV-40 inoculation (Phase 1 in the diagram) provided the culture is maintained without attempting to transfer the cells to new culture. However, following the first cell transfer, cells with vacuolated cytoplasm appeared either singly or in groups on the tenth day after the inoculation. As shown in the diagram a culture of human virus shows a marked cytotoxic effect within five weeks of the infection, and it was characterized by vacuolating cells (Fig. 4), clumping of chromatin, and formation of inclusion bodies (Fig. 5). With the fluorescent staining, it was possible to observe various changes in the cells about 2 or 5 weeks after the infection of a clone. The infected cells of a clone exhibited specific nuclear fluorescence as mentioned above and antigen was released from the nucleus into the cytoplasm through the nuclear membrane and cytoplasmic inclusions were observed in some cells of clones within 4 weeks after SV-40 inoculation (Fig. 4). Although many of the cells die (lysis), many survive (proliferation), and the establishment of such combination state of lysis and proliferation revealed that not all the cells in culture respond equally to infection with virus. Morphological analysis of these phenomena developed in the epithelial-like cells indicates that in a clone there are cells with different susceptibility to virus.

In 5 weeks cell alteration from epithelial-like cells to fibroblastic cells occurred but it was different from cellular patterns of human diploid cells in that the central portion of many such clones of fibroblastic cells was consisted of a thick area of many cells and the cells had lost cell contact-inhibition (Figs. 7 and 9).

In the center of the heaped-up cells superficial necrosis and sloughing of cells

Diagrammatic representation of cell alteration of human epithelial-like cell (Intestine 407) infected with SV-40.



Figs. 1-3 Fluorescent Staining of Human Established Clonizing Cells Infected with Virus Developed from SV-40 DNA.

Fig. 1 About 20 per cent cells infected with virus show nuclear antigen at 24 post-inoculation hours of virus.

Fig. 2 Massive nuclear antigens are seen in the clonizing cells at 7 post-inoculation days.

Fig. 3 High magnification of infected cells at 7 post-inoculation days.

Figs. 4 and 5 Lytic Changes of the Infected Epithelial-like Cells.

Fig. 4 Note cytoplasmic vacuolation, resembling CPE of BS-C-1 infected with SV-40. $\times 100$, Wright-Giemsa staining.

Fig. 5 Cytoplasmic inclusion bodies (arrows). Six weeks of post-inoculation of virus. Wright-Giemsa staining.



Figs. 6~9 Proliferation and Transformation of Human Clonizing Epithelial-like Cells Infected with Virus Developed from SV-40 DNA.

Eig. 6 Uninfected cultured cell of human epithelial-like cells (Intestine. 407) $\times 100$, H-E staining.

Fig. 7 Fibroblastic cell alteration.

Note cells heaped up in the center of clones and losing of contact-inhibition. Phase. I. (5 weeks, 5 passages) $\times 100$, H-E staining.

occurred. They have since been successfully subcultured at intervals of 3 days. In addition, they appear to be readily damaged by extended trypsin treatment, and by adverse conditions of cultivation, e. g., alkaline or acid medium, infrequent medium changes.

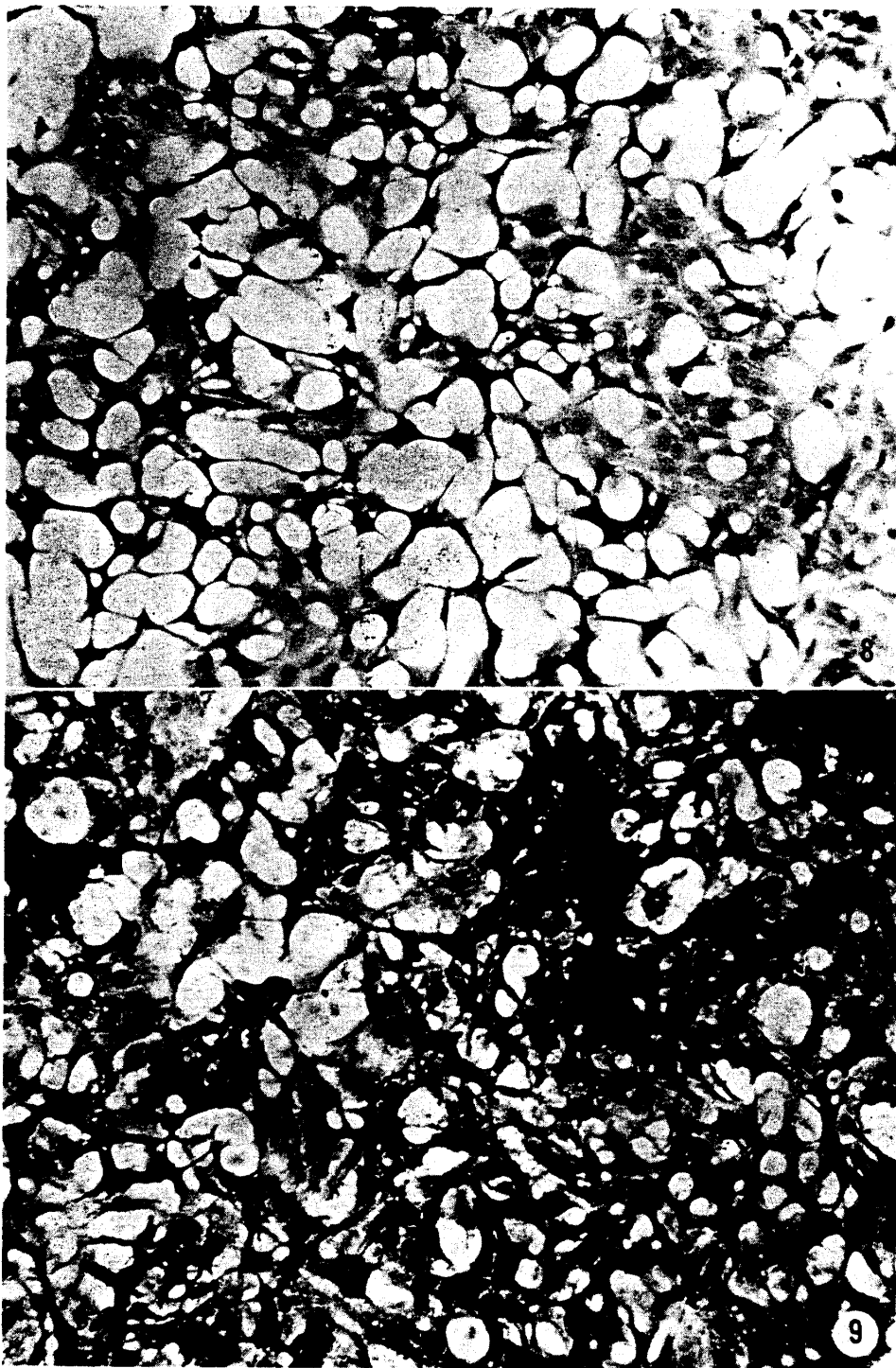
The observations of the dynamics of this cellular response indicate that SV-40 is capable of converting human epithelial-like cell lines to fibroblastic cell line morphologically.

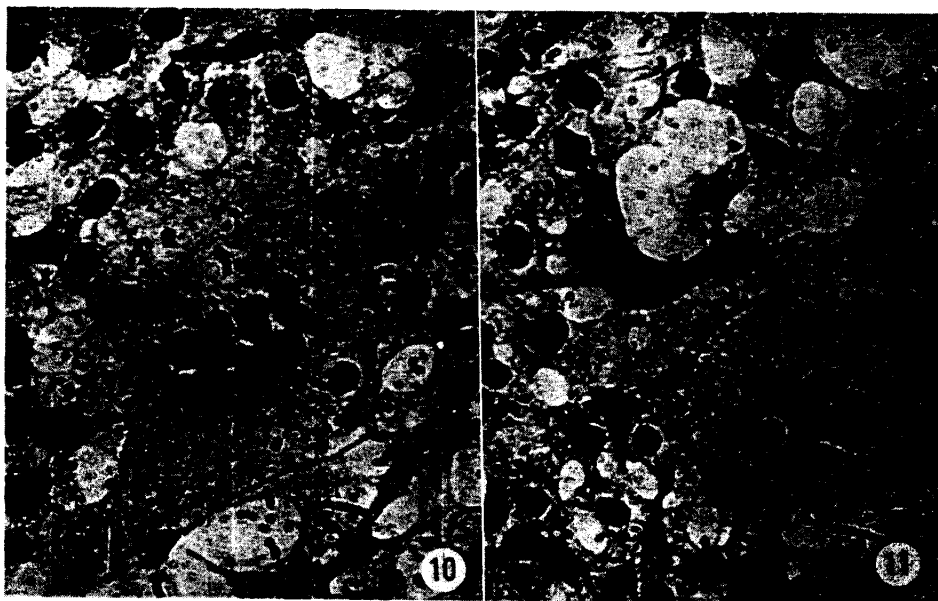
As the other proliferative cellular response, a selective phenomenon of cells was observed in the clone and epithelial-like cells were gradually pressed to the edge of the clone by proliferating fibroblastic cells 5 weeks after viral inoculation. Although some epithelial-like cells degenerated themselves as the sequence of metabolic disturbances, the cells infected by virus showed the fibroblastic cell patterns in some portion of the clones, but had no proliferative capacity in further cultures. In the phase of morphological fibroblastic cells altered from epithelial-like cells the rate of cell death was increased for about 5 weeks (Phase II, crisis) but this rate of the cell death gradually declined after several weeks of growth. At the end of stage of crisis virus becomes unnoticeable in many clone cells but only a small proportion of the clones is still infected by the virus. These findings show that virus production is maintained by a population phenomenon called carrier state as DULBECCO^{13,14} described concerning cell transformation by polyoma virus. The stage of crisis also is connected with resistance of the cells to the virus and the transformation of the clonizing cells by the virus may also be connected with resistance. After about 20 weeks of growth finally no viral antigen was discovered in proliferating clone cells by fluorescent staining. At the stage deviated from crisis to recovery the extensive reticulum cellular patterns were predominant in the fibroblastic altered cells (Phase III: fibro-reticulum cell formation, Fig. 8). These reticulum cellular patterns reconstructed from fibroblastic cells were accompanied with giant cells which have chromosomal abnormalities after 10 or 15 weeks of growth (Figs. 10 and 11). The reticulum-formed cells associated with fibroblastic cells show a marked proliferation after the stage of crisis (Phase IV) and phenomenon of contact-inhibition was lost in clonizing cells (Fig. 9). The proliferative phase has continued *in vitro* now for about 6 months, through 40 passages.

DISCUSSION

The cell transformation which ensues infection with virus has been followed in a more direct way by studying the transformation of established human cells *in vitro*.

Concerning the cell transformation *in vitro* by SV-40 virus the morpholo-





Figs. 10 and 11

Giant cells accompanied by chromosomal abnormalities infected with virus (arrows) at the Phase III (13 passages, 13 weeks). H-E staining.

gical transformation of cells is characterized by epithelial-like cells transformed from fibroblast in the primary organ cultures of human^{8, 4, 5, 15, 16} as well as hamster, rabbit, mouse and porcine^{17, 18, 19, 20}.

More recently it has been noted by BLACK and ROWE^{17, 18} *et al* that clones derived from transformed cells of hamster kidney tissue *in vitro* were consisted of epithelial differentiation into tubular structures, as well as sarcomatous cells when the clones were transplanted to hamster, and it revealed that SV-40 virus induced sarcomatous and cancerous differentiation *in vitro*. In addition, successive transformations of an established cell line 3T3 by polyoma virus and SV-40 has been reported by TODARO and GREEN^{21, 22}. It has been shown that a cell line transformed by one oncogenic virus is still susceptible to the transforming action of the second virus.

In an earlier study⁸ it has been recognized that SV-40 virus developed from DNA in a host cell was infective for fibroblasts and could induce not only extensive fibrosarcoma but gland-cell carcinoma in the intestine of newborn hamsters

Fig. 8 Proliferative reticulum cell formation associated with fibroblastic cells at the stage deviated from crisis (Phases II-IV). $\times 100$, H-E staining.

Fig. 9 Proliferation of fibroblastic cells transformed from epithelial-like cells. Note adhesiveness of each clone and altered cells lost contact-inhibition. Phase IV, 20 weeks, 28 passages. $\times 100$, H-E staining.

at the early stage of infection. In the present report, an analysis of cellular responses of established cell lines and epithelial-like cell altered from fibroblasts of the human embryonic intestine, was conducted in order to study the susceptibility of human cells to SV-40 virus developed from DNA. As mentioned above, it has been noted that clonizing epithelial-like cells of the intestine 407 were more susceptible (80 per cent) to SV-40 virus (Figs. 1, 2, and 3) than normal diploid fibroblasts (1 per cent) in primary human cell cultures. This difference in the susceptibility to SV-40 virus may depend on alteration of the cell membrane by acquisition of inheritable properties different from original cells.

As shown in the diagram, in the early stage of the infection the cell growth was enhanced by inoculation of DNA virus (2 weeks) but many cells died, and lysis, characterized by cytopathic effects, clumping of chromatin and formation of inclusion bodies, was observed in many clonizing cells (Figs. 4 and 5).

In contrast, the cells surviving infection give rise to virus-free long term cultures under certain conditions and cellular response to the virus was characterized by the proliferation which is classified in four phases as shown in the diagram.

The most remarkable morphological characteristic was fibroblastic cell alteration from epithelial clonizing cells (5 passages) at 5 weeks of virus inoculation and this phase was characterized by fusiform shape resembling the original cell cultures (Fig. 7), more rapid cell multiplication, necessitating repeated subcultivations (3-day intervals), and by susceptibility to trypsin. The reticulum-formed cells connected with fibroblastic cells (Phase IV: Fig. 8) proliferated markedly and heaped up in the stage of recovery as shown in the diagram and continuous proliferating cells combined with fibroblastic formed-cell (Fig. 9) were maintained through the recovery stage when the antigen could no longer be detected in any clonizing cells. In Phase IV of rapid proliferation of the altered cells, there was less difference between the number of subcultures obtained with a transformed cell line and with its control. The life potential of the control culture was long. Although the proliferation Phase IV has been continuing for about 6 months after infection, through 40 passages, the ability of transformed cells to multiply seems to be limited in the established cell lines under various conditions, such as susceptibility to trypsin, alkaline or acid change of fluids and the amount of cellular debris increase.

As MOYER (1964)¹⁶ observed in the transformed cell from the established human diploid cell strains, it is possible that cell destruction and cessation of growth in the late passage of the transformed cells and the lobation of cell nuclei and appearance of pyknotic micronuclei are caused by mitotic disturbances resulting from virus infection.

These do not seem to be bacterial or fungus infection, and no pleuropneu-

monia-like organisms, could be demonstrated in any cultures.

By this study an interesting generalization of human epithelial-like cells can be made about the state of differentiation of the transformed cells in relation to SV-40 virus. The cells which arise *in vitro* after transformation by SV-40 virus are star-shaped and the appearance of fibroblastic cells is similar in shape to the cells cultured from the subcutaneous fibrosarcoma that has been developed in the newborn hamsters by inoculation of SV-40 DNA.

The altered growth potential and morphological alteration of the epithelial-like cell lines of intestine 407 may be attributable to alterations of the cell nuclear apparatus and the mechanism for biosynthesis resulting from the SV-40 infection (Figs. 10 and 11). Continuation of the virus-cell interaction might cause many abnormalities so that mitosis eventually becomes impossible in the established human cell lines.

CONCLUSION

In the present experiment, it has been noted that clonizing epithelial-like cells of the intestine 407 were more susceptible to SV-40 virus than normal fibroblasts in primary human cell cultures.

In the early stage of the infection the cell growth was enhanced by the inoculation of DNA virus but many cells died, showing lysis characterized by CPE, clumping of chromatin and formation of inclusion bodies.

On the other hand, the cells surviving infection have given rise to virus-free long term cultures and cellular responses to the virus characterized by cell proliferation which is classified in four phases. (Phase. I : infection and cell alteration. Phase. II : crisis. Phase. III : fibro-reticulum cell formation. Phase. IV : recovery and proliferation). The most remarkable morphological characteristic was fibroblastic cell alteration from epithelial cells at 5 weeks of virus inoculation. By this study an interesting generalization of human epithelial-like cells can be made about the differentiation of the transformed cells in relation to SV-40 virus and it has been shown that an established human cell line is still susceptible to the reverting action of the SV-40 virus.

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