*STIMULATION OF DNA SYNTHESIS AND COMPLEMENT-FIXING ANTIGEN PRODUCTION BY SV40 IN HUMAN DIPLOID CELL CULTURES: EVIDENCE FOR "ABORTIVE" INFECTION**

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Morphological transformation of human cells infected with SV40 during early passages after explantation usually does not become apparent for many weeks, probably because only a small fraction of the culture is infected,[1, 2] and the residual cell population continues to multiply at a normal rate. Thus, it is difficult in this system to quantitate the virus-induced alterations which precede the onset of morphological transformation.

Human diploid cells at the end of their *in vitro* lifetime, however, provide an excellent system for such studies, since the fraction of cells synthesizing DNA is reduced to a minimum,[3] the number of cells infectible by SV40 is considerably increased,[4] and the cultures transformed rapidly.[5, 6]

The data obtained from the study of SV40 infection in such a host-cell system revealed that there is, besides a lytic infection, an "abortive" [6a] infection and that cellular division is stimulated in those cells which carry the abortive infection.[6b]

Materials and Methods.—Virus: The SV40 strain Rh 911 grown in African green monkey kidney cells (GMK) was obtained from Dr. A. J. Girardi. This pool titered $10^{7.5}$ $TCID_{50}$/ml in primary GMK cells.

Cells: Human diploid cells (WI-38) were used at a high passage level (between passages 50 and 53), when mitotic activity is greatly reduced. The cells were planted on small coverslips (9 × 22 mm) in Petri dishes and incubated in a 5% CO_2 atmosphere. The culture medium was Earle's balanced salt solution with a 2× concentration of amino acids and vitamins in Eagle's basal medium, supplemented with 10% calf serum (Flow Laboratories, Rockville, Md.).

The experiments were started approximately 2 weeks after planting of the cells, when the coverslips were fully sheeted and when mitotic activity was virtually absent. At this time the number of cells per coverslip was 8×10^4.

Experimental procedure: Coverslip cultures were washed with phosphate buffered salt solution (PBS) and were then infected with an equivalent of 350 $TCID_{50}$/cell. After an adsorption period of 2 hr the cultures were washed with PBS and culture medium was added which was supplemented with 10% calf anti-SV40 serum (Flow Laboratories, Rockville, Md.). The antiserum titered 1:640 vs. 100 $TCID_{50}$ (heterologous titer). Tritiated thymidine was added to the culture fluid at an initial concentration of 0.02 μc Tdr-H^3/cc (spec. act. 6 c/mmole). Noninfected control cultures were treated in the same manner as the infected cultures, except that during the adsorption period culture medium was added instead of virus suspension. Every second day, half of the medium was replaced by fresh medium containing antiserum and the same concentration of the labeled precursors. Throughout the period covered by the experiments to be described, the cultures were not trypsinized and subcultivated.

Immunofluorescent staining: The coverslip cultures were washed twice in PBS, fixed in acetone at −20°C for 10 min, and stained either with fluorescein-isothyocyanate (FITC) coupled with baboon anti-SV40 serum (Flow Laboratories, Rockville, Md.), or with hamster SV40 tumor antiserum, FITC-labeled (Flow Laboratories, Rockville, Md.), followed by a second incubation with FITC-labeled goat antihamster γ-globulin in order to reinforce the staining. Appropriate controls to exclude a nonspecific staining with this technique were made. The proportion of cells being positive for viral (VP) or complement-fixing antigen (ICFA)[7] was determined by counting at least 800 cells per sample.

Autoradiography: The coverslip cultures were fixed in alcohol-acetic acid (6:1; v/v) for 1 min

and transferred to 80% ethanol. Kodak NTB3 emulsion was used for the autoradiography. The developed autoradiographs were stained with hemalum. The proportion of labeled nuclei was calculated on the basis of 800 nuclei.

Determination of the mitotic index: An average number of 1800 cells was examined for the presence of mitotic figures. Meta-, ana-, and telophases were counted.

Results.—(1) *Distribution of virus-induced antigens in the cell population:* ICFA-containing cells appeared in the cultures as early as 15–18 hr after infection. The fluorescence, however, was faint and weak, and no clear identification of positive cells could be made. On the second day postinfection, even though a high virus multiplicity was used, only 20 per cent of the cells were positive for ICFA; the proportion of such cells increased rapidly, comprising nearly 60 per cent of the population by the 11th day (Fig. 1). The majority of mitotic cells showed strong immunofluorescence for ICFA, indicating that cells positive for this viral-induced antigen were able to undergo multiplication.

Viral (VP) antigen appeared later, between the first and second day postinfection. Sequential appearance of ICFA and VP has also been observed in GMK cells.[7, 8] On the second day, the number of cells positive for VP was about 10%—half the number of cells that were ICFA-positive. The proportion of VP-positive cells increased slowly up to the 7th day and then declined. In cultures that have been maintained for longer periods of time and then split, VP-positive cells became extremely rare. No mitoses with VP were observed.

Since the cultures were maintained under a high concentration of antiviral serum, the slight increase with time of VP-positive cells may be interpreted as due to asynchrony and delay of viral protein synthesis. Further infection via direct cell-to-cell transfer of virus particles cannot be excluded, even though only rarely were groups of neighboring cells positive for VP observed.

It was reported recently that BS-C-1 cells which exhibit VP fluorescence after infection with SV40 contain ICFA as well.[9] It is probable, therefore, that the total number of ICFA-containing cells in the human cell system includes the fraction of cells which are positive for VP. Hence, on the second day postinfection 10 per cent of the total cell population consists of cells which synthesize ICFA but which do *not* synthesize VP. This particular type of cell then increases rapidly, comprising about 45 per cent of the population by day 11 (Fig. 1).

(2) *Stimulation of DNA synthesis by SV40 infection:* The method of continuous labeling was chosen rather than pulse-labeling, since all cells which were undergoing DNA replication and mitotic division prior to fixation could be detected, and no variables, such as possible synchrony or parasynchrony of cell multiplication, could interfere with the interpretation of the results.

The WI-38 cells have a minimal level of DNA synthesis at the end of their *in vitro* lifetime and when forming a confluent monolayer. However, even when completely stationary cultures are supplemented with fresh medium, as was necessary in these experiments, some cells are stimulated to DNA synthesis. A similar observation has been reported by Todaro and Green with stationary cultures of 3T3 cells.[10] Thus an accumulation of Tdr-H^3-labeled cells was observed in the control cultures during the experimental period, which, however, did not exceed 30 per cent by the 7th day.

At day 1 the percentage of labeled cells in the infected culture did not differ from

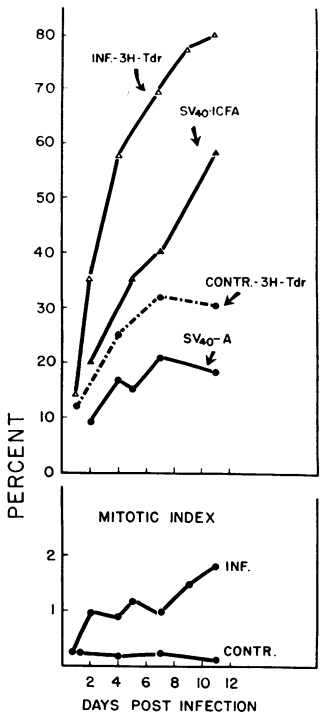

FIG. 1.—SV40-induced alterations in human diploid cell cultures. Stationary WI-38 cells at a high passage level were infected with 350 $TCID_{50}$/cell and maintained after the adsorption period under continuous presence of 10% SV40-antiserum and 0.02 μc Tdr-H^3/ml. △——△, Tdr-H^3-labeled nuclei in infected cultures. ●. .—. . ●, Tdr-H^3-labeled nuclei in controls. ▲——▲, Nuclei containing SV40-induced complement-fixing antigen (ICFA). ●——●, Nuclei containing SV40 viral antigen (VP). *Lower:* mitotic index in infected and control cultures.

that of the control, but by the second day a drastic increase occurred in the infected cultures (Fig. 1). Accumulation of labeled cells continued throughout the experiment, so that by the 11th day 80 per cent of the cells were labeled. Thus at any time the total number of labeled cells exceed considerably the number of ICFA-positive cells (Fig. 1). The rate of accumulation of labeled cells in both infected and control cultures showed a decline after the 4th–5th day rather than an increase, as would be expected on the basis of the steady level of the mitotic index. Such a decline may be explained by the fact that cells which had undergone several divisions in the presence of Tdr-H^3 had accumulated a lethal amount of the isotope.[11]

A burst of mitotic activity was observed between the first and second day postinfection concomitant with the increase of labeled cells. The mitotic index remained persistently high throughout the period of the experiment, far above that of the control cultures.

All mitoses even in the early samples of infected and noninfected cells were labeled, indicating that the cells in the confluent monolayer prior to the exposure of Tdr-H^3 must have been arrested in the G1-period. This finding suggests that the activation of DNA synthesis by SV40 is the result of an interaction with cells in the resting G1 or possibly early S-phase and not with cells in the G2-premitotic period or in mitosis.

(3) *Effect of different input multiplicities on the appearance of ICFA and DNA synthesis in the cultures:* If activation of DNA synthesis is a phenomenon regulated by the virus, the proportion of labeled cells should depend upon the multiplicity of infection. That this, indeed, is the case is illustrated in Table 1. Cultures were infected with a multiplicity of 350, 70, or 35 $TCID_{50}$/cell and were examined at the 2nd and 6th day. The percentage of ICFA-positive and Tdr-H^3-labeled cells was in linear relationship with the multiplicity of infectious units used. If the percentage of labeled cells in the controls is subtracted from the percentage of labeled cells in the infected cultures, the resulting proportion of labeled cells is closely related to that of the ICFA-positive cells.

Discussion.—Increase of cellular DNA and of enzymes related to DNA synthesis has been reported for polyoma virus under conditions in which a lytic interaction occurs in a large part of the cell population.[12–14] It has not been possible so far to define whether the increase in DNA synthesis is an unbalanced process which eventually may lead in all cases to cell death or whether the activation of cellu-

TABLE 1

EFFECT OF DIFFERENT INPUT MULTIPLICITIES ON DNA SYNTHESIS AND ICFA PRODUCTION

Days postinfection		Multiplicity of Infection* 350	70	35
2	ICFA-positive cells†	22	2.5	1
	Tdr-H^3-labeled nuclei‡	20	4	1
6	ICFA-positive cells	37	12	4
	Tdr-H^3-labeled nuclei	31	7	1

Two days postinfection 20% of the controls were labeled; six days postinfection 25% of the controls were labeled.
* $TCID_{50}$/cell.
† Per cent ICFA-positive cells.
‡ Per cent of labeled nuclei in the infected culture above the per cent of labeled nuclei present in controls at the same time.

lar DNA synthesis is compatible with cell survival and multiplication. In the experiments reported here, it is demonstrated that activation of DNA synthesis in a portion of resting cells after infection with SV40 results in a viable and progressively growing cell population.

The different categories of cells existing at any time after infection with SV40 in a culture in which only a fraction of the population is infected, may be indicated in the following scheme:

(*a*) Positive for ICFA and for VP → only ICFA synthesis; → ICFA and VP synthesis

(*b*) Tdr-H^3-labeled → (I) noninfected; → infected → (II) viral DNA; → (III) cellular DNA

(*c*) Nonlabeled

Among the cells in which specific viral products can be detected by immunofluorescence (*a*), the initial ICFA-positive fraction is clearly higher than the fraction exhibiting VP fluorescence. The same phenomenon has been reported in SV40-infected BS-C-1 cells.[9] The VP-positive cells presumably die due to a lytic virus-host cell interaction. There is a progressive disappearance of these cells as the cultures are carried, and, in addition, VP-positive mitotic cells have never been observed.[1, 2, 9] On the other hand, the portion of cells which continues to increase contains ICFA but is not capable of VP production to a detectable amount.

In contrast to this system it usually takes several weeks for the ICFA-positive population to prevail[15] when actively dividing cultures of human diploid cells are infected at an early passage level.

Unlike the stationary 3T3-cell system, which required one division in order to express the SV40-induced loss of contact inhibition,[10] DNA synthesis and mitotic activity are initiated directly in stationary WI-38 cultures infected at high passage levels when a large fraction of the cells have lost the capacity to divide.

Even though we cannot distinguish by the autoradiographic technique between cellular and viral DNA, we have to consider three different groups of cells in the infected cultures which contribute to the population that shows incorporation of Tdr-H^3: (*b*, I) There are noninfected cells undergoing divisions, presumably at the

same level as in the control cultures. This interpretation is supported by the data in Table 1, which indicate that the background of dividing cells present in the controls seems to contribute to the amount of labeled cells in infected cultures. The observation of occasional mitotic figures which are negative for ICFA fluorescence among the more numerous ICFA-positive mitotic cells in infected cultures provides additional evidence for this conclusion.

Another group consists of VP-producing cells, in which virus maturation occurs (*b*,II). It is unclear whether, in these cells, in addition to replication of viral DNA, synthesis of cellular DNA occurs. In fact, an initial depression of host-cell DNA synthesis has been reported in mouse embryo fibroblasts infected with polyoma[16] and in SV40-infected GMK cells in which VP synthesis takes place.[17]

Finally, there remains a group of Tdr-H^3-labeled cells (*b*,III) which contain ICFA but which do not exhibit VP fluorescence. Thus, the rapidly increasing pool of DNA-synthesizing cells in infected cultures apparently is composed of three different fractions of cells. When the fraction of VP-positive cells and the number of labeled cells which are present in the controls are subtracted from the total number of labeled cells in the infected culture, one obtains values which correspond closely to the fraction of ICFA-positive but VP-negative cells (*b*,III). Because of this correlation and since the majority of mitotic cells shows positive ICFA fluorescence, it can be concluded that at least some cells in which the SV40 genome has induced the synthesis of a new specific product are stimulated to division.

The last category (*c*) is constituted of cells in which no Tdr-H^3 is incorporated. Presumably, these are cells in which no (efficient) infection took place and which eventually disappear from the cultures due to lack of multiplication.

Although it seems likely that only an initially stimulated fraction of "target" cells contributes to the continuously dividing population, we cannot rule out, at present, an additional recruiting of cells, which might occur later during the time of infection. Morphological alterations concerning the growth pattern and the cellular shape can be recognized in such cultures approximately 2 weeks postinfection.[5, 6] Whether all cells which are initially stimulated to division will finally reach the stage of morphological transformation is not clear, since there may be a continuous elimination of cells because of extensive chromosomal damage.[18]

SV40-transformed cells of various species are eventually 100 per cent positive for ICFA even when no infectious virus can be recovered.[19] This information and the data presented here lend strong support to the hypothesis that the transforming cells must originate from the pool of cells in which only part of the information present in the complete viral genome is expressed, since they contain only ICFA but are not capable of producing VP in any detectable amount. In these cells, synthesis of cellular DNA occurs which is compatible with their survival. Whether this "abortive" infection is produced by defective particles[19] of an inhomogeneous virus pool, or whether the host cells are unable to support the proper transcription of the complete viral genome, or whether both possibilities have to be taken into consideration is an open question which will be the subject of further investigations.

Summary.—Stationary human diploid cultures at the end of their lifetime *in vitro* were infected with SV40. By a combination of autoradiography for H^3-thymidine incorporation and of immunofluorescence for the complement-fixing (ICFA) and

viral (VP) antigens, it was determined that a fraction of this cell population is positive for ICFA and not for VP.

Stimulation of DNA synthesis and mitosis takes place between the first and second day postinfection. The cell population, in which cellular DNA synthesis is activated by SV40 infection and which starts to divide, consists of abortively infected ICFA-producing cells.

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[6a] "Abortive" infection is intended here as a condition in which a specific product (ICFA) induced by the incoming virus is shown by immunofluorescence in cells in which no viral antigen can be demonstrated by immunofluorescence. Thus "abortive" infection is ultimately as correct as the sensitivity of the detecting method.

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