Increased Resistance to Herpes Simplex Virus of Hamster and Human Cells Transformed by SV_{40}.* (31289)

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Altered responses of human kidney cells morphologically transformed by SV_{40} to infection with several DNA and RNA viruses were briefly described in an earlier report(1). In these cells particularly after infection with RNA viruses modifications in cytopathic effects were observed as compared with normal primary human kidney cells. However, because of the lack of comparable lines of control cells, these findings were difficult to evaluate. More recently we have had at our disposal lines of SV_{40}-transformed Syrian hamster embryo cells as well as comparable lines of "normal" non-oncogenic control cells(2). The effects of two DNA viruses (vaccinia and herpes simplex) and one RNA virus (Sindbis) were studied in these two systems. We also have investigated comparatively the effect of herpes simplex virus (HSV) on human embryonic skin-muscle cells transformed by SV_{40} and on early lines of "normal" cells derived from the same source.

Materials and methods. Cell cultures: Hamster cells. Lines of SV_{40}-transformed Syrian hamster embryo cells and of control cells were initiated and developed as already described(2). Five lines of transformed cells (D, X, 2Pst, 3D, 3Z) and 5 lines of "normal" control cells (E, I, P, Ks, Ps) all of which were derived from the same suspension of hamster embryo cells, were used.

Culture media have been described previously(3). Cells of all the transformed lines produced the SV_{40} T ("tumor") antigen and were shown to be oncogenic at the 10th passage level in weanling hamsters. None of the control lines replicated T antigen or exhibited oncogenic properties at the 15th, 30th and 50th (2 out of 5 tested only) passage levels (2). In addition to the hamster embryo cell lines, 3 clonal lines of transformed hamster kidney cells (EG, 15J, 6F) previously developed in this laboratory(4) were employed in certain experiments.

Human cells. Lines of transformed human embryonic skin-muscle cells were derived from primary embryonic skin-muscle monolayer cultures inoculated with SV_{40}. Subcultures of uninoculated primary cultures prepared with aliquots of the same cell suspension were used as controls. Cells of transformed skin-muscle cultures carried the SV_{40} T antigen as shown by the indirect fluorescence antibody staining method.

In addition to these embryonic cells an established line of SV_{40}-transformed human kidney cells was tested at its 105th passage level. This line has been described(5).

Viruses. Herpes simplex virus (HSV) of the 4th passage of the Rhodanus strain (isolated in this laboratory) in primary human amnion was used. Previously this agent was passed twice in primary cultures of human skin-muscle and 3 times in primary cultures of chick embryo fibroblasts. Vaccinia virus, originally obtained from the Massachusetts State Antitoxin Laboratory, was grown in the chorioallantoic membrane of the chick embryo. Sindbis virus (Taylor strain) was grown in cultures of primary chick embryo fibroblasts.

Titration of herpes simplex virus. Infectivity of HSV suspensions was determined by the plaque method. Cells of one of the hamster embryo control lines (line I) were grown to monolayers in plastic petri dishes (Falcon, diameter: 35 mm). Serial 10-fold dilutions of

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TABLE I. Progression of CPE of Herpes Simplex Virus in 2 Lines of SV_{40}-Transformed Hamster Embryo Cells and in 2 Lines of Control Cells.

Cell line and passage No.	Days after inoculation 1	2	3	4
X 57 (transf.)	0	0	+	++
2Pst 54 (")	0	±	++	+++
Ks 28 (control)	++	+++	++++	no cells left
E 34 (")	+	++	++++	no cells left

0 = no CPE; ± = occasional focus; + = 25% of the cells affected; ++++ = 100% of the cells affected.

virus suspension were prepared and 0.1 ml aliquots of each were added to each of 3 dishes after removal of the medium. After one hour at 37°C in an atmosphere of 5% CO_2 in air, the cells were covered with 2 ml medium containing 1.5% methylcellulose(6) (Methocel Dow 4000 CSF). After 3 days the cells were fixed with 10% formalin, stained with hematoxylin and the plaques counted.

Interferon. Tests for interferon were done by determining the degree of reduction in plaque formation by vesicular stomatitis virus (VSV) in petri dish cultures of primary hamster embryo cells. The pH of the fluid to be investigated was lowered to 2 by dialysis against McIlvain buffer for 24 hours at 4°C when the reaction was readjusted to 7. Primary hamster embryo cells were incubated for 24 hours with 0.2 ml of this fluid. Then VSV was added and after an adsorption period of 1 hour the cells were covered with 0.9% agar overlay. After 3 days they were stained with neutral red and the plaques counted.

Results. Hamster cells. Experiments with this system were initiated by measuring simultaneously the titers of herpes simplex, vaccinia and Sindbis viruses in both transformed and control hamster embryo cells. No differences in infectivity titer or in characteristics of cytopathic effect (CPE) were found for vaccinia and Sindbis viruses. However, the titer of HSV was lower in cultures of transformed cells than in controls. The factors responsible for this difference in titer of HSV were investigated by comparative studies of CPE, plaque formation, growth rate, adsorption and interferon production in transformed and control cell cultures. Finally, the proportion in the two systems of cells capable of inducing "infectious centers" after exposure to herpes virus was determined.

Cytopathic effect. Monolayers of transformed and control cells in tubes (16 × 150 mm) were inoculated with 100 PFU of HSV. Tubes were fixed and stained, employing the collodion embedding technique. Results are shown in Table I. Focal CPE in control cells started earlier and spread rapidly to involve the whole cell sheet. Intranuclear inclusions were seen on the first day after inoculation. In transformed cells CPE started later, spread slowly and intranuclear inclusions were not seen before the second or third day. CPE in transformed cells remained more localized or "focal" for a longer period as is illustrated in Fig. 1a-1d.

Plaque formation. Monolayers of transformed and control cells in petri dishes were inoculated with 200 PFU of HSV. Five dishes per cell line were used. Plaques were counted 3 days after inoculation; results are given in Table II. The number of plaques in transformed cells was consistently lower than that in controls. The plaques in transformed cells were also smaller than those appearing in control cells. Among the 5 transformed hamster embryo cell lines, differences in sensitivity to HSV infection were noted as indicated by variations in the number of plaques that developed.

Growth rate. Monolayers of transformed and control cells in tubes were inoculated with 200 PFU of HSV. Daily, the cells and fluids from 3 cultures of each line were frozen, pooled and stored at −70°C. Before titration the cells were disrupted by freezing and thawing 3 times. As shown in Fig. 2 and 3 the growth rate of the virus in transformed cells was much lower than in the control cells. In the latter the maximum titer was reached

within 3 days, while in transformed cells at least 5 days were required to reach the same level. The difference was most pronounced during the first 48 hours.

Virus adsorption. To monolayers of transformed and control cells in tubes, 2 ml medium containing 850 PFU/0.1 ml was added and incubated at 37°C. Samples were taken after 15, 30, 60 and 120 minutes and titrated. Percentage of virus adsorbed after each interval was calculated (Fig. 4). In both systems about 80% of the virus was adsorbed after 2 hours.

Infectious centers. Cultures of transformed

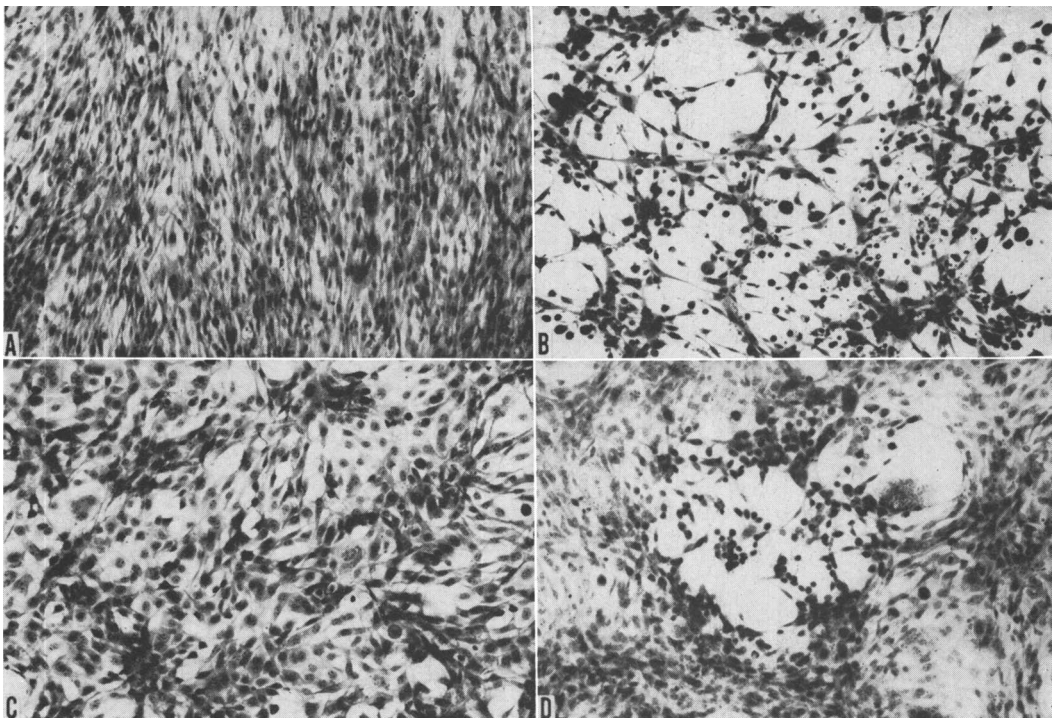
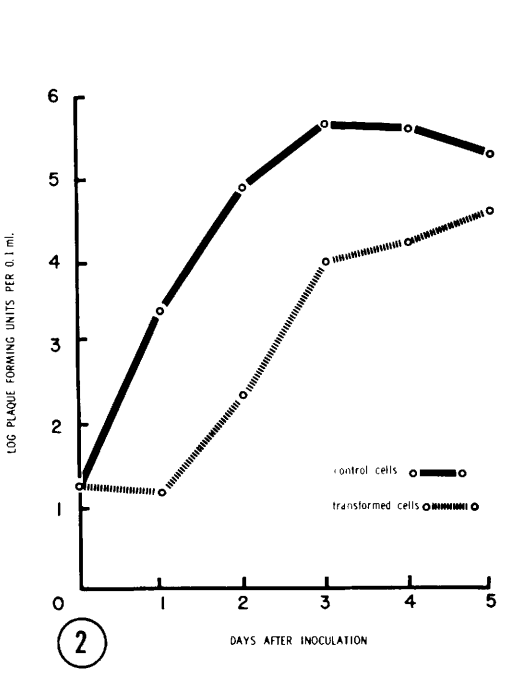


FIG. 1. CPE of herpes simplex virus in control (line E, passage 34) and SV_{40}-transformed (line X, passage 57) hamster embryo cells 3 days after inoculation. a) uninoculated control cells. b) inoculated control cells. c) uninoculated SV_{40}-transformed cells. d) inoculated SV_{40}-transformed cells. Magn. × 336.

TABLE II. Numbers of Plaques Formed by Herpes Simplex Virus in Cultures of SV_{40}-Transformed and Control Hamster Cell Lines.

	Cell line and passage No.		No. of plaques per petri dish					Avg
Controls	E	34	162	167	154	146	145	154
	I	46	120	116	113	101	132	116
	P	36	139	134	126	100	95	138
	Ks	36	154	145	130	147	149	145
SV_{40}-transformed	D	68	59	61	50	62	46	53
	X	69	15	10	6	9	6	9
	2Pst	66	73	77	76	59	71	71
	3D	63	11	13	11	11	6	10
	3Z	63	58	21	38	33	54	50
	EG	152*	3	2	1	1	0	1
	15J	138*	0	0	0	0	0	0
	6F	142*	0	0	0	0	0	0

* Newborn hamster kidney clonal lines; remaining lines are hamster embryo cell lines.



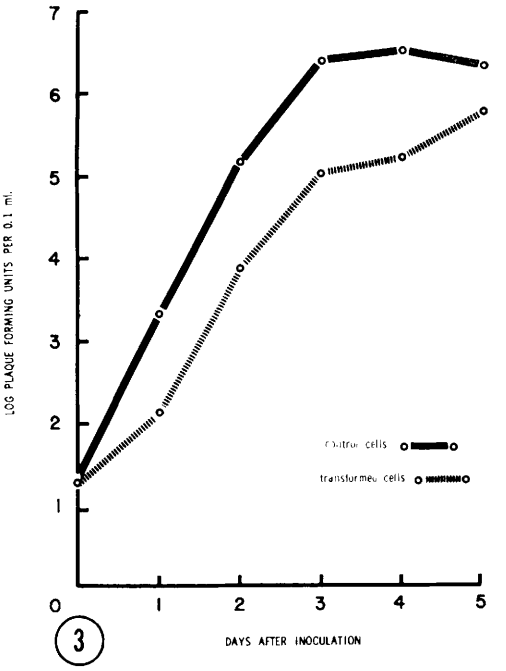


FIG. 2. Multiplication of herpes simplex virus in control (line Ks, passage 28) and SV_{40}-transformed (line X, passage 57) hamster embryo cells.

FIG. 3. Multiplication of herpes simplex virus in control (line E, passage 34) and SV_{40}-transformed (line 2Pst, passage 54) hamster embryo cells.

(line 3D) and control (line Ks) cells, grown to monolayers in tubes, were exposed to 2000 PFU of HSV suspended in 1 ml of neutralized Hanks' BSS. After one hour incubation at 37°C the cells were washed 3 times with neutralized Hanks' BSS. Original fluid and the fluid of the first 2 washings were pooled and titrated; fluid of the third washing was titrated separately. Cells were trypsinized, counted, and resuspended in medium. Ten-fold dilutions of the suspensions were added to monolayers of hamster embryo cells (line I). After an adsorption period of 1½ hours at 37°C in 5% CO_2 in air, medium containing 1.5% carboxy methylcellulose was added. After 3 days plaques were counted and the number of virus-exposed cells required to produce one plaque was calculated. It was found that in control cells one out of every 7 exposed cells and in transformed cells one out of every 200 gave rise to an infective center.

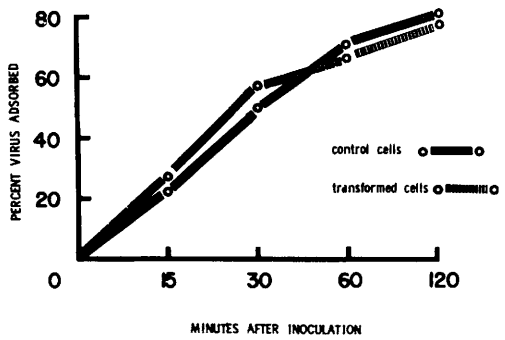


FIG. 4. Rate of adsorption of herpes simplex virus to control (line Ks, passage 32) and SV_{40}-transformed (line X, passage 62) hamster embryo cells.

That this large difference did not depend upon a difference in the capacity of the cells to adsorb virus was shown by the titrational data obtained with the washing fluids. Thus in both control and transformed cells approximately 25% of the virus was adsorbed after one hour as determined by titration of the pooled washings. No virus was detected in 0.1 ml of fluid of the third washing from either control or transformed cells.

Interferon assay. It seemed possible that an enhanced capacity of transformed cells to produce interferon might account for their increased resistance. Accordingly, production of this factor after infection with HSV was

investigated. Control hamster embryo cells (line Ps, passage 41) and transformed hamster embryo cells (line 3D, passage 66) and cells of a clone line (6F, passage 145) of transformed hamster kidney cells were grown to monolayers in 16 oz prescription bottles. Each bottle was inoculated with 300 PFU of HSV. Media were harvested after 3 days and centrifuged for 45 minutes at 30,000 rpm. At the time of harvest of the fluids, CPE in Ps, 3D and 6F cell cultures was recorded respectively as $++++$, $\pm$ and 0. No interferon activity was detected in any of the supernatant fluids by the technique described above.

Results in human cells. Reaction of human skin-muscle cells to HSV infection was tested at 2 different intervals after exposure to SV_{40}. In the first experiment monolayer cultures in tubes were exposed to 200 PFU of HSV three weeks after the SV_{40} had been added. The cultures which were then in the first passage showed no signs of morphological transformation. After 4 days both SV_{40} exposed and control cultures were completely destroyed by HSV. No difference in character or degree of CPE was noted. Three months later an analogous experiment was performed with the cells of 7 SV_{40}-exposed and 5 control lines in the 8th subculture. At that time 5 of 7 exposed cultures had morphologically transformed and were shown to be replicating tumor antigen. These 5 transformed cultures showed a marked resistance to HSV. Thus 4 days after virus was added only rare focal CPE was seen. In contrast, the controls as well as the 2 SV_{40}-exposed cultures which remained untransformed were completely destroyed at this time. Cells of a stable line of SV_{40}-transformed human kidney(5) also proved almost completely resistant to the destructive effect of HSV.

Discussion. A number of factors that might underlie the increased resistance of SV_{40}-transformed cells to HSV infection were considered. The first was a possible difference in adsorption of virus. Diderholm *et al*(7) showed that BHK-21 cells transformed by polyoma virus were insusceptible to certain strains of foot and mouth disease virus. No adsorption of these strains to this line of cells could be demonstrated. In our systems, however, HSV virus was adsorbed by both transformed and control cells to the same degree.

Another possible factor might be an enhanced capacity of the transformed cells to produce interferon, although this seemed *a priori* unlikely, because Sindbis virus known to be highly sensitive to this reagent multiplied as well in transformed as in control cells. Nevertheless the possibility remained that HSV infection of transformed cells stimulated increase in interferon production in cells harboring the SV_{40} viral genome. Thus Glasgow and Habel(8) were able to show that resistance to HSV of polyoma carrier cultures of mouse embryo cells probably was caused by an additive effect of interferon produced by both viruses. However, no interferon was detected after infection with HSV in the fluids from SV_{40}-transformed cells as compared with those from control cells. Since at the time of testing many transformed cells remained intact it is nevertheless possible that they might have contained increased concentrations of interferon had appropriate tests been done.

A third factor may be envisioned as an emergence during the process of transformation of a cell variant with enhanced resistance to HSV infection. The greater resistance of the hamster kidney clone lines may possibly favor this concept, but we have no other evidence to support it.

The fourth, and in our opinion the most likely, factor might be interference with replication of HSV, mediated in some manner by the presence of the SV_{40} genome in the transformed cells. A probable example of such interference has recently been reported by Strohl *et al*(9) who found that cultured cells derived from type 12 adenovirus hamster tumors exhibited a reduced capacity to synthesize infectious type 2 adenovirus. These authors propose that this resistance might depend upon the persisting genetic material of adenovirus 12. In our experiments, of the 2 DNA viruses studied, HSV and vaccinia, only the growth of HSV, an agent known to be synthesized in the nucleus, was retarded in SV_{40}-transformed cells. Vaccinia virus which is synthesized in the cytoplasm replicated equally well in both transformed and control

cells. It is therefore reasonable to suggest that the presence of the SV_{40} genome in the nuclei of the transformed cells interfered with the normal rate of synthesis of HSV.

The synthesis of HSV in proximity to the SV_{40} genome obviously suggests the possibility of hybridization such as occurs between SV_{40} and adenoviruses(10). With this idea in mind, 3 passages of HSV in transformed hamster cells (line X) were made and the virus then added to cultures of control hamster cells (line Ks). After 24 hours, when early herpes virus CPE was evident, tests for the presence of SV_{40} T antigen were made by the indirect immunofluorescence technique. Failure to detect this antigen, although far from conclusive, may suggest that hybridization of the type occurring between SV_{40} and adenovirus does not occur and is consistent with the findings of Rabson and his coworkers(11) who have lately shown that simultaneous infection of green monkey kidney cells with SV_{40} and HSV may take place, although they obtained no evidence of hybridization under these circumstances. However, they propose that phenotypic mixing with a consequent alteration of biological properties of SV_{40} might result from association under these conditions.

The demonstration that SV_{40}-transformed cells are more resistant to HSV infection taken with analogous findings in the case of adenovirus tumor cells(9) may be useful in the search for viruses etiologically associated with tumors of unknown origin. Thus if cells from a neoplasm specifically exhibited enhanced resistance to a certain virus or group of viruses, one might be encouraged to attempt to obtain additional evidence of the presence and nature of the interfering agent.

Summary. Lines of cells derived originally from various hamster and human tissues and transformed *in vitro* by SV_{40} consistently exhibited increased resistance to infection with herpes simplex virus, as compared with control cells not exposed to SV_{40}. Increased resistance did not appear to depend upon differences in cellular capacity to adsorb HSV or to allow penetration into the cell; nor could it be related to enhanced production of interferon. It is suggested that the SV_{40} genome in the nucleus of transformed cells interferes in a manner as yet undefined with the synthesis of HSV which occurs at the same site.

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The "Critical Period" for Production of Humorally Conditioned Necroses.* (31290)

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A number of qualitatively different morbid lesions can be elicited by conjoint treatment with two classes of potentially pathogenic agents: 1. Sensitizers or conditioning factors which create a specific predisposition for cer-

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