

# Properties of Hamster Embryo Fibroblasts Transformed In Vitro After Exposure to Ultraviolet-Irradiated Herpes Simplex Virus Type 2

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An in vitro method which led to the transformation of hamster embryo fibroblasts after exposure to herpes simplex virus type 2 (HSV-2) inactivated with ultraviolet irradiation is described. The transformed cells (333-8-9) produced tumors when inoculated into newborn Syrian hamsters but not when injected into weanling Syrian hamsters of the same LSH inbred strain. However, after one in vivo passage, the 333-8-9 cells became highly oncogenic in weanling hamsters. No infectious virus was recovered from these cells. Herpes simplex virus antigens were detected in the transformed cells by the indirect immunofluorescence technique. Sera from tumor-bearing hamsters contained antibody with highly specific neutralizing activity against HSV-2. These studies indicate the continued involvement of the HSV-2 genome in an oncogenic cell line.

Viruses belonging to herpesvirus groups have been implicated as possible etiological agents in cancer in humans as well as in neoplasms of lower animals (21). Herpes simplex virus type 2 (HSV-2) was first associated with human cervical carcinoma by epidemiological methods (15, 16, 22); however, direct evidence of the role of HSV-2 in cellular transformation has not been obtained. A few tumors have been observed in newborn hamsters after injection of many animals with HSV-2, but virus antigens were not demonstrated in these rare tumors (14). HSV-2 antigens have been observed in cells isolated from patients in the early stages of cervical carcinoma (2). In addition to HSV-2, a second herpesvirus has been shown to be associated with human neoplasms. The Epstein-Barr virus has been observed by electron microscope techniques in transformed cells obtained from Burkitt's lymphoma (6, 7). Visual observations of herpesvirus in the transformed cells have been correlated to the presence of circulating antibodies against the virus in the serum of the human host (8). In addition, recent studies have revealed deoxyribonucleic acid in the Burkitt tumors hybridizable to Epstein-Barr virus deoxyribonucleic acid (25).

Two other herpesviruses are generally considered to be important in the induction of

malignant disease in lower animals. The first is probably the agent which plays an important role in Marek's disease of chickens (4, 17). In addition, it has recently been reported that *Herpesvirus saimiri* induces lymphomas when injected into adult primates (10-12).

With the possible exceptions mentioned above, direct demonstration of the oncogenic potential by herpesviruses has been extremely difficult. Much of this difficulty results from the characteristic cytopathic effect of HSV which destroys the infected cells soon after in vitro infection. Several investigators have reported that ultraviolet (UV) irradiation of oncogenic viruses does not decrease the oncogenic potential of these viruses as rapidly as it decreases the lytic potential (1, 3, 9). The cellular cytopathic effect of these UV-irradiated viruses is greatly reduced or removed long before the oncogenic potential is eliminated. Therefore, UV irradiation was chosen as a method with the potential to demonstrate the possible oncogenic activity of HSV. Transformation experiments described in this report were carried out in cell culture as a result of previous observations that this method is more sensitive for the detection of viral transformation than direct injection of animals with the virus (20). The in vitro and in vivo characterization of

hamster cells transformed after infection with UV-irradiated HSV-2 are described in this report.

### MATERIALS AND METHODS

**Cells.** Syrian hamster embryo fibroblasts (HEF) cells (inbred LSH strain, Lakeview Hamster Colony, New Field, N.J.) were prepared from 13-day-old embryos by a previously described method (5). Briefly, the embryos were treated with 0.25% trypsin for 20 min and then centrifuged to remove the trypsin. The cells were suspended in Eagle's medium containing 10% fetal bovine serum, 10% Tryptose phosphate broth, and 0.075%  $\text{NaHCO}_3$ . The newly prepared HEF suspension then was placed into 8-oz (ca. 240 ml) glass prescription bottles at a cell density of  $5 \times 10^6$  cells per bottle.

Rabbit kidney (RK) cells were prepared from weanling (21- to 28-day-old) rabbit kidneys by a procedure similar to that described for the preparation of HEF. After the trypsinization procedure was complete, the RK cells were suspended in Eagle's medium with 10% fetal bovine serum, 10% Tryptose phosphate broth, and 0.225%  $\text{NaHCO}_3$ . RK cells were grown in 60-mm plastic petri dishes and used for routine HSV assays.

Primary human embryo kidney (HEK) cells were obtained from HEM Research Inc., Rockville, Md. After three tissue culture transfers in medium 199 plus 10% fetal bovine serum, 10% Tryptose phosphate broth, and 0.075%  $\text{NaHCO}_3$ , the cells were seeded into 8-oz bottles and utilized for the preparation of HSV stocks.

**Virus.** HSV-2 was obtained from W. Rawls, Baylor College of Medicine, Houston, Tex., after isolation from a primary genital lesion. The isolate was designated HSV-2-333. When HSV-2-333 was used for HEF transformation experiments, this virus strain had been passed three times in HEK cells. Herpes simplex virus type 1 (HSV-1) was obtained from the same source after isolation from an oral lesion and was designated HSV-1-Patton. This virus isolate was treated identically to HSV-2-333 and was used as the prototype HSV-1 in these experiments. HSV-2-333 and HSV-1-Patton were stored at  $-76^\circ\text{C}$  in a Revco freezer.

**Virus assay.** The assay method for HSV-1 and HSV-2 in RK cells has been previously described (19). Confluent monolayers of RK cells in 60-mm plastic petri dishes were inoculated with the appropriate dilution of either herpesvirus type. After adsorption at room temperature for 1 hr, the infected cell cultures were overlaid with Eagle's medium containing 10% Tryptose phosphate broth, 10% fetal bovine serum, 0.225%  $\text{NaHCO}_3$ , and 1% methylcellulose. Four days after virus infection, a 0.005% solution of neutral red was added to each petri dish 2 hr before plaques were counted.

**Immunofluorescence techniques.** The indirect method of immunofluorescence was used to detect HSV-specific antigens. Cover slips with monolayers of cells containing known or suspected viral antigens were washed three times in warm tris(*t*:hydroxymethyl) aminomethane (Tris) buffer ( $\text{pH}$  7.4) and air-dried. After fixation for 3 min in acetone, HSV-specific antiserum was adsorbed on the cells for 30 min. The

cells then were washed three times in Tris buffer and exposed to anti-hamster gamma globulin conjugated to fluorescein isothiocyanate. The cover slips again were washed three times in Tris buffer, air-dried, and mounted on slides. Specific fluorescence was detected by using a Zeiss microscope with a UV light source.

HSV-specific antibody was prepared by injecting weanling Syrian hamsters with HSV-1 or HSV-2 three times at weekly intervals. Viruses used for the first injection were inactivated by UV irradiation for 30 sec. This prevented death of the hamsters after injection. The second and third injection used nonirradiated herpesvirus. At 3 to 5 weeks after the final injection, the hamsters were bled by heart puncture, and, after separation, the serum was stored at  $-20^\circ\text{C}$ .

**Neutralization of HSV.** A 0.5-ml amount of an appropriate dilution of serum was mixed with  $10^3$  plaque-forming units of HSV-1 or HSV-2. These mixtures were incubated in a  $37^\circ\text{C}$  water bath for 40 min. After incubation, 0.1 ml of the virus-serum mixture was placed on an RK cell monolayer in a 60-mm plastic petri dish. Four plates were used per sample. After virus adsorption for 1 hr, the infected cultures were overlaid with Eagle's medium containing 10% fetal bovine serum, 10% Tryptose phosphate broth, 0.23%  $\text{NaHCO}_3$ , and 0.5% methylcellulose. After 4 days, the cells were stained with neutral red, and plaques were counted.

### RESULTS

**UV inactivation of HSV-1 and HSV-2.** Herpes simplex viruses effectively destroy cells infected *in vitro*. Therefore, a method was necessary which would prevent this rapid cell death, permitting possible HSV-induced transformation to be manifested. UV irradiation was chosen for a variety of reasons, primarily reproducibility of results and rapid reduction of viral cytopathic effect. The UV exposure was calculated to maximize inactivation without destroying transformation potential. A 2-ml amount of the virus was exposed to UV light for various times with constant rotation of the petri dish to insure even exposure. Throughout the times of UV irradiation tested, HSV-2-333 was inactivated at a rate faster than that of its type 1 counterpart (Fig. 1). This difference of inactivation rates is in accord with results reported by other investigators (24).

**Transformation of HEF by UV-irradiated HSV-2-333.** After UV irradiation for 30, 60, 120, 240, and 480 sec, separate samples of HSV-2-333 were adsorbed onto HEF cell monolayers for 1 hr at  $37^\circ\text{C}$ . Approximately  $5 \times 10^6$  HEF cells were exposed to an equivalent of  $5 \times 10^6$  HSV-2-333 plaque-forming particles (assayed before irradiation in RK cells). After virus adsorption, the infected monolayers were trypsinized, divided, and grown in 250-ml plastic tissue culture bottles. Cells infected with HSV-2-333 irradiated for 30 and 60 sec were destroyed with typical

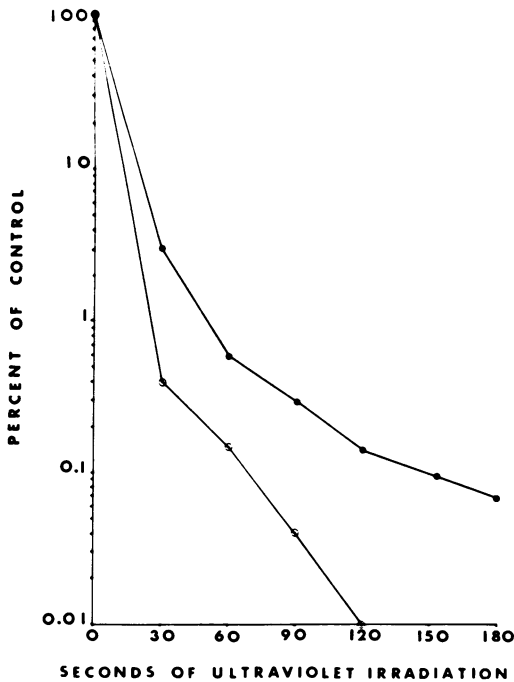


FIG. 1. Effect of UV irradiation on infectivity of HSV-1 Patton (●) and HSV-2-333 (○). The virus was irradiated in 60-mm plastic petri dishes by a General Electric G8T5 UV bulb at a distance of 20 cm.

HSV cytopathic effects. Cells infected by virus irradiated for 120 sec showed evidence of cytopathic effect but were not destroyed by HSV until the cells were passed 30 days after initial virus infection.

The remaining cell cultures, which had been infected with virus exposed to UV for either 240 or 480 sec, showed no HSV cytopathic effects either before or after transfer. In two of the bottles infected by HSV-2-333 irradiated for 480 sec, transformed foci were observed 30 days after infection. Each bottle contained one transformed focus that was distinguished from the cell monolayer by an altered morphology and a definite loss of contact inhibition. Each transformed focus was isolated and transferred into a 35-mm plastic petri dish. The resulting cell lines were designated 333-8-8 and 333-8-9, respectively.

At passage 11, 333-8-8 entered into crisis from which it did not recover, probably because of technical difficulties. However, 333-8-9 entered crisis during cell culture passage 10 and recovered during passage 14. At present, this cell line has been passed 45 times, and the passage histories of both 333-8-8 and 333-8-9 are summarized in Fig. 2. One bottle culture of 333-8-9 was destroyed by typical HSV cytopathic effects during passage 4.

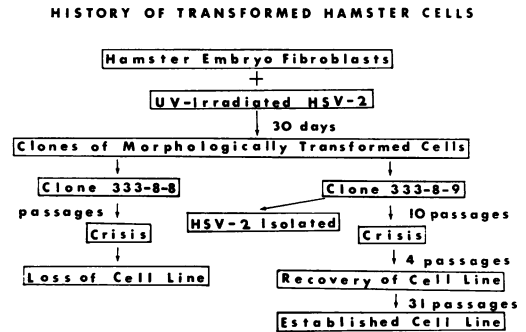


FIG. 2. *In vitro* history of 333-8-9-transformed hamster cells.

The HSV strain isolated was found to be closely related to the parental HSV-2-333 when compared by differential neutralization and immunofluorescence techniques. Further characterization of this virus is under investigation.

The transformed cells were stained with hematoxylin and eosin (Fig. 3) to establish the histological patterns of the culture. The 333-8-9 cell culture contained some cells which appeared generally undifferentiated; others appeared to be fibroblastic. A type of giant cell was distributed throughout the cell culture. Transformed 333-8-9 cells were larger, were stained more densely, and had larger nuclei than the control HEF cells. Over 25 control cultures observed in parallel failed to yield transformed foci, and none survived passage 10.

**Oncogenicity of transformed cells in Syrian hamsters.** To determine the oncogenic potential of the *in vitro* transformed cell line (333-8-9), 4-week-old LSH hamsters were injected subcutaneously with  $10^6$  cells in their 14th passage after viral exposure. These animals were observed for 8 months, and no tumors developed. The oncogenicity of this cell line was further examined by injection into newborn Syrian hamsters. Each newborn LSH strain hamster was injected subcutaneously during the first 24 hr after delivery with approximately  $2 \times 10^6$  viable 333-8-9 cells from passage 21. After 10 weeks, a small (20-mm) solid tumor was observed near the site of inoculation in one hamster. After 16 weeks, a total of 11 tumors had appeared in 11 separate hamsters out of 31 weaned (Table 1). A complete study of the pathology of these tumors is in progress; however, the majority of these tumors are invasive and appear to contain cells with a basically undifferentiated morphology.

***In vitro* characteristics of 333-8-9 cells extracted from a hamster tumor.** The first hamster tumor to develop after injection of 333-8-9 cells was surgically removed. The excised tumor was tryptic

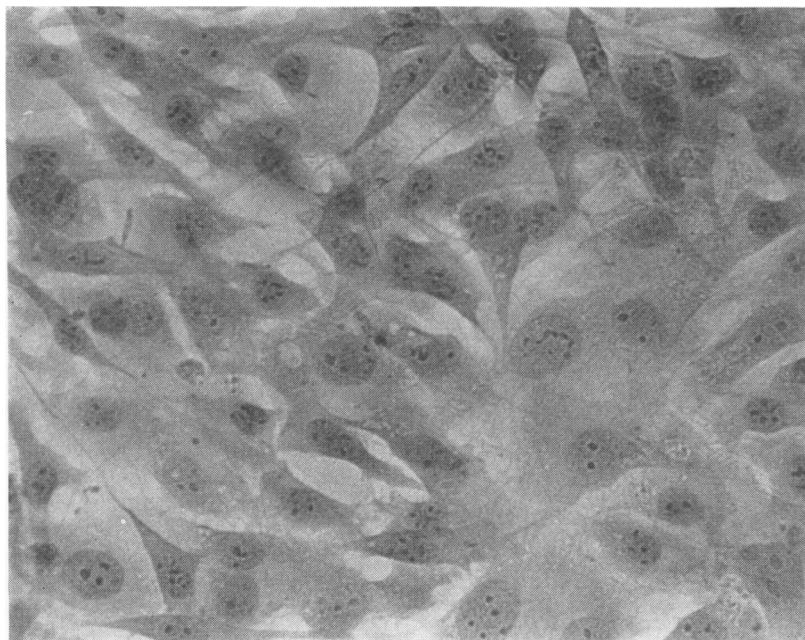


FIG. 3. Photomicrograph of 333-8-9-transformed hamster cells stained with hematoxylin and eosin.  $\times 300$ .

sinized and grown in medium 199 containing 10% fetal bovine serum, 10% Tryptose phosphate broth, and 0.075%  $\text{NaHCO}_3$ . Three morphological types of cells were found to grow in cell culture from the tumors. The predominant cell type was rather undifferentiated but appeared somewhat epithelial. Cells with a fibroblastic morphology were also found. A number of giant cells were observed; many of these contained several nuclei or a single large nucleus often with a bizarre morphology. Approximately 1% of the tumor cells contained cytoplasmic inclusion bodies (Fig. 4). This inclusion body was stained by eosin and was not observed in the nuclei. (Tests kindly carried out by Leonard Hayflick revealed no evidence of mycoplasma in any of the cell cultures described in this report.) Such inclusion bodies have been reported associated with the presence of herpes-type virus particles in Marek's disease (18). The tissue culture-adapted 333-8-9 tumor no. 1 cells were reinjected into weanling Syrian hamsters during passage 3, and induced tumors in 100% of the animals injected with  $10^6$  viable cells. The observed increase in the oncogenic potential of 333-8-9 cells after animal passage may be the result of selection within the host animal for the more oncogenic cells.

**Antigenic characteristics of transformed cells.** The original *in vitro* transformed 333-8-9 cell line and cells extracted from tumor numbers 1 to 5 were examined by indirect immunofluorescence for the presence of HSV-specific antigens (Table

TABLE 1. Tumor induction in LSH hamsters by transformed 333-8-9 cells

Week after injection <sup>a</sup>	No. injected <sup>b</sup>	No. with tumor	Per cent positive
1-9	31	0	0
10	31	1	3
11	31	2	6
12	31	3	10
13	31	5	16
14	31	9	29
15	31	10	32
16-24	31	11	35

<sup>a</sup> Each hamster was injected with  $2 \times 10^6$  viable cells.

<sup>b</sup> Total animals injected represents the number of animals surviving 3 weeks until weaning. No tumors were observed in any of the animals that did not survive the first 3 weeks.

2). All cell lines, whether established from the primary *in vitro* transformation or from the hamster tumors, contained HSV antigens visible as a diffuse fluorescence throughout the cytoplasm of the tumor cell (Fig. 5). The nuclei of 333-8-9 were relatively free of this type of fluorescence, although some more localized nuclear fluorescence was observed. When these tests were carried out by using normal hamster serum or serum specific for simian virus 40 tumor antigen, no specific immunofluorescence was evident. In addition, when HSV-1- or HSV-2-speci-

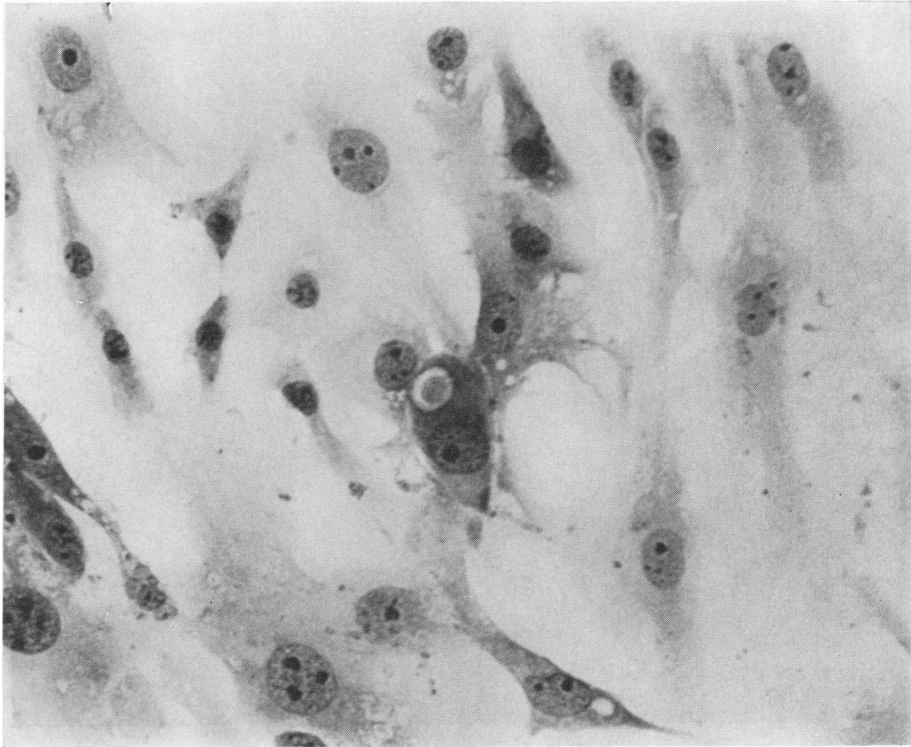


FIG. 4. Photomicrograph of 333-8-9-transformed hamster cells after isolation from tumor no. 1 induced by injection of the cells into a newborn Syrian hamster. The center cell contains a cytoplasmic inclusion body. Stained with hematoxylin and eosin. X300.

fic sera were reacted with normal or PARA (defective simian virus 40)-adenovirus 7-transformed hamster cells, no fluorescence was observed.

**Antibody specificity of sera from tumor-bearing hamsters.** Sera from animals with tumors induced by 333-8-9 cells were collected and examined for the presence of specific HSV antibodies. When indirect immunofluorescence was utilized for this purpose, the sera specifically reacted with RK cells infected by HSV-2-333 (Table 3) or by HSV-1-Patton. This fluorescence was bright but diffuse throughout the infected cell, the type of fluorescence pattern observed when specific sera known to contain HSV antibodies were used. Serum with antibodies to simian virus 40 tumor antigens did not react with HSV-infected cells. Furthermore, sera from 333-8-9 tumor-bearing hamsters did not react with simian virus 40-infected cells.

Sera from hamsters bearing primary 333-8-9-induced tumors were assayed for the presence of HSV-neutralizing antibodies. Sera from five hamsters with tumors induced by 333-8-9 cells neutralized HSV-2 when the sera were diluted 10-fold (Fig. 6). This loss of virus infectivity ranged from 18 to 46% of that observed with un-

TABLE 2. Detection of HSV-2 antigens in 333-8-9 transformed cells

Cell type	Specificity of antisera			
	HSV-1 <sup>a</sup>	HSV-2 <sup>b</sup>	SV 40 (T antigen) <sup>c</sup>	NH <sup>d</sup>
333-8-9	+	+	-	-
333-8-9 T no. 1 <sup>e</sup>	+	+	-	-
333-8-9 T no. 2	+	+	-	-
333-8-9 T no. 3	+	+	-	-
333-8-9 T no. 4	+	+	-	-
333-8-9 T no. 5	+	+	-	-
2-Ct-1b <sup>f</sup>	-	-	+	-
Normal hamster cells	-	-	-	-

<sup>a</sup> Pooled sera from hamsters immunized with HSV-1.

<sup>b</sup> Pooled sera from hamsters immunized with HSV-2.

<sup>c</sup> Pooled sera from hamsters bearing simian virus 40 (SV40)-induced tumors.

<sup>d</sup> Pooled sera from normal weanling hamsters.

<sup>e</sup> Tumors were numbered in the order in which they were removed from the host hamster.

<sup>f</sup> Hamster embryo fibroblasts transformed by the PARA (defective SV40)-adenovirus 7 hybrid.

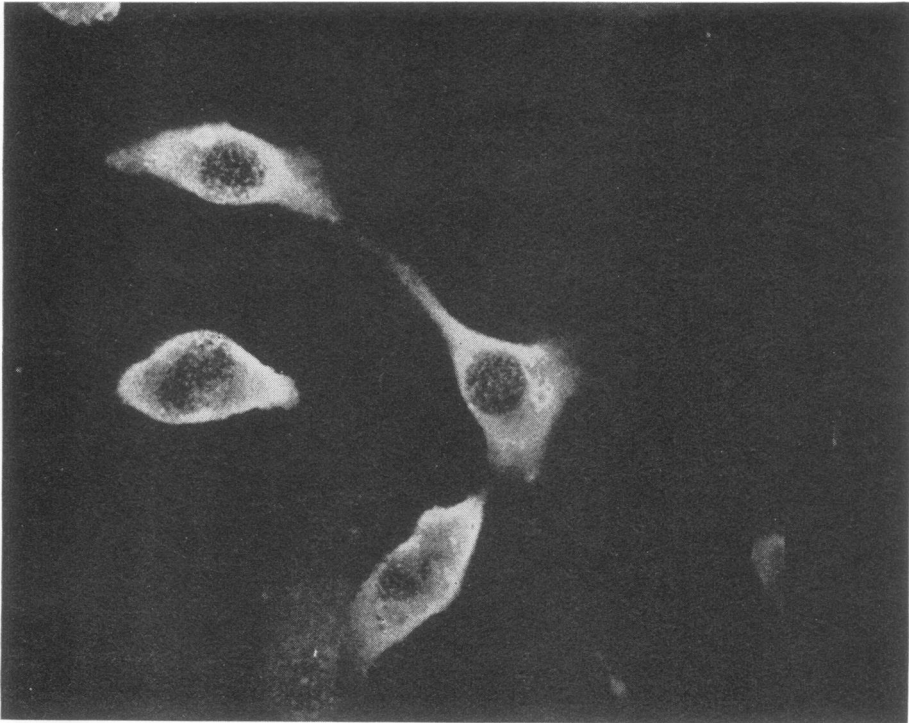


FIG. 5. Immunofluorescence photomicrograph of HSV-2 antigens localized in 333-8-9-transformed hamster cells.  $\times 450$ .

treated HSV-2. Two sera also were tested at a 1:5 dilution with a proportionate increase in virus neutralization.

The HSV-2 specificity of this neutralizing activity of primary 333-8-9 sera was tested by replacing the type 2 virus with type 1 virus. Neutralization of HSV-1 (Fig. 7) was significantly less than that observed with HSV-2 (Fig. 6). The sera with the highest titers to HSV-2 also had the highest titers to HSV-1.

The hamsters injected with the *in vitro* transformed 333-8-9 cells were newborn. The age of the hamsters at injection could have resulted in partial immune tolerance in these animals, and therefore antibodies against HSV-1 might not have developed. To test this possibility, weanling hamsters were injected with  $10^6$  333-8-9 cells from a primary tumor. Six weeks after injection, the serum from each animal was collected, and its HSV-neutralizing activity was determined. The three sera tested neutralized HSV-2 efficiently (Fig. 8). Again, the reduction in infectivity of HSV-1 was significantly less (Fig. 9). For example, the most efficient serum left less than 1% of the HSV-2 infectious, but 25% of the HSV-1 survived. Sera from control hamsters did not neutralize HSV-1 or HSV-2. The specificity of

TABLE 3. Specificity of sera by immunofluorescence from 333-8-9 tumor-bearing animals

Serum no. <sup>a</sup>	Virus infecting cells		
	HSV-1-Patton <sup>b</sup>	HSV-2-333 <sup>b</sup>	SV40 <sup>c</sup>
1	+	+	—
2	+	+	—
3	+	+	—
4	+	+	—
5	±	+	—
6	+	+	—
7	+	+	—
8	+	+	—

<sup>a</sup> Sera numbers 1 through 8 are from hamsters bearing tumors induced by 333-8-9 and indicate the order of appearance of the tumor.

<sup>b</sup> HSV-1-Patton and HSV-2-333 were used in rabbit kidney cells.

<sup>c</sup> Simian virus 40 was used in green monkey kidney cells at the time of assay.

this neutralizing activity further strengthens the hypothesis that the HSV-2 antigen originates from the infecting tumor and is not a result of accidental contamination.

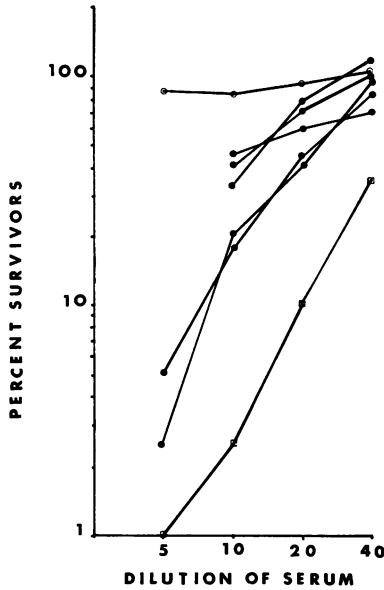


FIG. 6. Neutralization of HSV-2-333 by sera from Syrian hamsters with tumors induced by injection of 333-8-9 cells into newborn animals (●). Controls utilized pooled sera from hamsters immunized by injection of HSV-2 (□) and pooled sera collected from normal weanling hamsters (○).

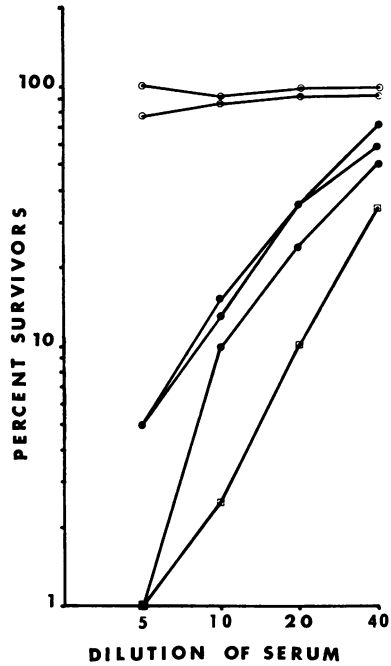


FIG. 8. Neutralization of HSV-2-333 by sera from three Syrian hamsters with tumors induced by injection of 333-8-9, T no. 1 cells into weanling hamsters (●). Controls utilized pooled sera from hamsters immunized by injection of HSV-2 (□) and sera collected from two Syrian hamsters received in the same lot as animals in which tumors were induced (○).

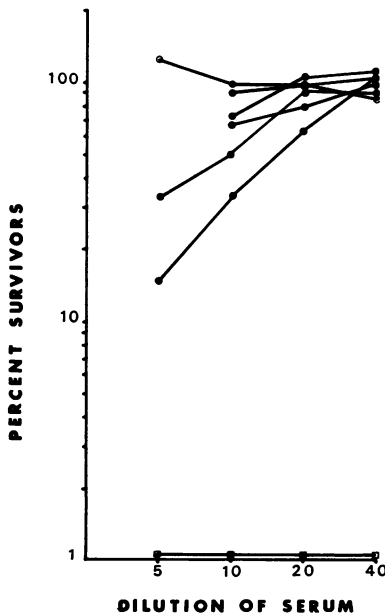


FIG. 7. Neutralization of HSV-1-Patton by sera from Syrian hamsters with tumors induced by injection of 333-8-9 cells into newborn animals (●). Controls utilized pooled sera from hamsters immunized by injection of HSV-2 (□) and pooled sera collected from normal weanling hamsters (○).

DISCUSSION

The results presented in this report and in a preliminary report [R. Duff and F. Rapp, *Nature* (London), *in press*] demonstrate the presence of HSV-2 markers in a transformed hamster cell line isolated after exposure to virus that had been inactivated by UV irradiation. The demonstration of these antigens in a small percentage of the cells in the culture and the cytoplasmic localization of these virus-specific antigens are reminiscent of observations made with Epstein-Barr virus antigens associated with cells from Burkitt lymphomas (8). It is not surprising that, in our system, attempts at virus recovery proved fruitless; the original irradiation of the virus to prevent cytopathic effects would make recovery of virus at a later date difficult, if not impossible.

The oncogenic potential of these cells in syngenic hamsters and the ease of transplantability enable determination of the host response to the cells and to possible virus markers in those cells. Antibodies capable of neutralizing HSV-2 that appear in the tumor-bearing animals would suggest (in the absence of detectable virus particles

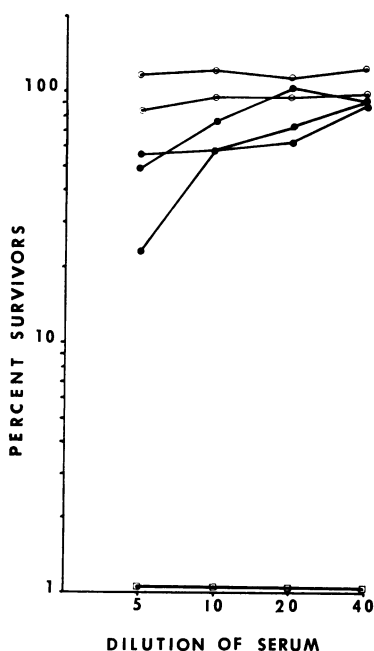


FIG. 9. Neutralization of HSV-1-Patton by sera from three Syrian hamsters with tumors induced by injection of 333-8-9, T no. 1 cells into weanling hamsters (●). Controls utilized pooled sera from hamsters immunized by injection of HSV-2 (□) and sera collected from two Syrian hamsters received in the same lot as animals in which tumors were induced (○).

in the cells) that cell surface changes may have occurred in which the antigens of HSV-2 are expressed and that the animal is reacting against these new antigens. Current knowledge concerning changes in the cell surface by HSV would agree with this hypothesis (23).

It is important to determine the role of the herpesvirus genome in the initiation or maintenance of the neoplastic state. Several hypotheses explain in part the results presented. Perhaps the most logical of these assigns to the herpes genome the role of at least partial control of the observed events concerned with transformation, in which case the virus would mimic other DNA-containing tumor viruses. The results presented are entirely compatible with those observed with Epstein-Barr virus and Marek's disease virus. Further credence to this hypothesis is lent by the recent observations of Munyon et al. (13) that the ability to synthesize thymidine kinase is transferred by UV-irradiated HSV-1 to the thymidine kinaseless L cells of mice. As in this study, the results seem to favor heritable transfer of herpesvirus genome to daughter cells in the culture after an original transformation event(s). Future ex-

periments to detect virus-specific nucleic acids are required to determine whether the cells transformed by HSV-2 in our laboratory continue to synthesize detectable amounts of virus-specific ribonucleic acid or DNA. At present, it is important to leave open the possibility that the virus genome detected in these cells plays no direct role in the maintenance of their oncogenic potential.

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