Absence of Leukosis Virus Markers in Hamster Cells Transformed by Herpes Simplex Virus Type 2

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Hamster cells which transformed after exposure to herpes simplex virus type 2 previously irradiated with ultraviolet light were tested for the presence of hamster leukosis viruses or their virus-specific antigens. Three techniques that were used did not detect the presence of either hamster C-type particles or antigens commonly associated with such structures. The implication of these findings to current concepts of virus oncology is discussed.

The morphological and oncogenic conversion of normal hamster embryo fibroblasts by herpes simplex virus type 2 (HSV-2) previously inactivated by ultraviolet irradiation has recently been reported (3, 4). These transformed cells contained HSV-2 antigens and induced HSV-2-specific antibodies in tumor-bearing hamsters. The HSV-2 (strain 333) used for these transformation experiments was free from any detectable ribonucleic acid (RNA)-containing tumor viruses. However, the "oncogene" theory of cellular transformation postulates that the presence of a C-type particle or its genome within a cell is necessary for transformation and maintenance of the transformed state (8). Cells transformed after exposure to HSV-2 would therefore be expected to contain a hamster-specific RNA tumor virus if this virus played a major role in the oncogenic process. To determine this, the following experiments were designed to demonstrate the presence or absence of RNA tumor virus information within the HSV-2 transformed cells.

One method to detect C-type particles is the examination of cells for the presence of RNA-containing virus particles with a density of 1.16 g/cm^3 in sucrose gradients (13). Various cell lines established after transformation of hamster embryo fibroblasts (from LSH hamsters) by HSV-2 were grown in 10 ml of Eagle medium (BME) supplemented with 10% dialyzed fetal calf serum and 0.75% NaHCO_3 . This medium also contained 100 μCi of ^3H -uridine per ml. After 18 hr, this medium was removed and examined for ^3H -labeled C-type particles. The supernatant fraction was centrifuged at $27,000 \times g$ for 10 min. The pellet was discarded, and

the supernatant fraction was again centrifuged through 20 ml of a 10% sucrose gradient (Schwartz-Mann; ribonuclease-free) at $30,000 \times g$ for 2 hr. The pellet was suspended in 1 ml of ethylenediaminetetraacetic acid (EDTA) phosphate buffer (0.0015 M EDTA, 0.15 M NaCl, 0.01 M Na_2HPO_4 , 0.0015 M NaH_2PO_4 , 0.02 M KCl), and 0.1 to 0.2 ml was centrifuged through 15 to 60% sucrose at $190,000 \times g$ for 115 min. The radioactivity was determined on Whatman no. 3 filter paper after precipitation with tricarboxylic acid, using a scintillation cocktail of toluene and omnifluor; counts were performed in a Beckman LS-250 scintillation counter. Two transformed cell lines (333-8-9 and 333-2-26) were examined before passage in hamsters. Four cell lines [333-8-9 tumor (T) no. 1, 333-2-29 T no. 3, 333-2-21 T no. 1, and 333-8-9 T no. 5] were tested after one animal passage. Figure 1 represents an experiment carried out with the 333-8-9 transformed cell line and a cell line (J20-NRK) known to carry C-type particles of both a murine sarcoma virus and a murine leukemia virus (10). From the results graphed, it is evident that no detectable C-type particles were demonstrated in the 333-8-9 cell line; however, in the J20-NRK cell line, a large radioactive peak was identified at a density of 1.16 g/cm^3 . All HSV-2 transformed cell lines that were tested by the above technique were uniformly negative for the presence of C-type particles; however, the sensitivity of this procedure is such that less than 10^4 particles per ml would not be detected.

A more sensitive technique with which to detect the genome of C-type particles is to examine cells for the presence of RNA tumor virus-

FIG. 1. Sucrose density gradient sedimentation of virions with labeled RNA from 333-8-9 cells and from J20-NRK cells. Cells were labeled with 100 μ Ci of 3 H-uridine per ml for 18 hr. The medium was collected and centrifuged as described in the text. J20-NRK cells ($\square$) and 333-8-9 cells ($\bullet$) were assayed in a scintillation counter for 3 H counts per minute (CPM).

specific gs antigens (6, 7). Both the species-specific gs-1 and the interspecies gs-3 antigens can be detected by complement fixation or immunofluorescence techniques (9, 12). Cells were grown on round cover slips (18-mm diameter) for 24 hr. The cultures were then washed three times in tris(hydroxymethyl)aminomethane-buffered saline (pH 7.4) and air-dried or immediately fixed in methanol after the wash step. The methanol-fixed cultures were stored in methanol at -76°C . The cover slips were examined for gs antigens by the direct immunofluorescence test. Methanol-fixed cultures were used for the detection of gs-1 antigens with specific sera supplied by Berge Hampar (National Institutes of Health). Acetone-fixed cultures were tested for gs-3 antigen with sera from R. E. Wilsnack (Huntingdon Research Center). The results of this experiment are shown in Table 1. No gs antigens were detected in any of the five HSV-2 transformed cell lines. However, the J20-NRK cell line continuously producing C-type particles after transformation by the Kirsten sarcoma virus contained detectable antigens (10). A hamster cell line developed after treatment with HSV-2 inactivated with 7,12-dimethylbenz(a)anthracene was also used as a positive control (2). This cell line also contained

TABLE 1. Immunofluorescence test for the presence of hamster leukosis virus antigens in herpes simplex virus type 2 (HSV-2) transformed hamster cell lines^a

Cell line ^b	Anti-HaLV-gs serum ^c	Anti-MLV-gs serum ^d	Weanling hamster serum ^e
333-8-9	—	—	—
333-2-29	—	—	—
333-8-9 T no. 1	—	—	—
333-2-20 T no. 1	—	—	—
333-2-21 T no. 1	—	—	—
333-2-26 T no. 1	—	—	—
J20-NRK	+	+	—
HDC-22	+	+	—
HEF	—	—	—

^a Transformed hamster embryo cells were tested for the presence of gs antigens by the direct immunofluorescence technique as described in the text. +, Positive reaction; —, negative reaction.

^b Two cell lines transformed by HSV-2 (333-8-9 and 333-2-29) were tested before passage in newborn Syrian hamsters. Four cell lines transformed by HSV-2, [333-8-9 tumor (T) no. 1, 333-2-20 T no. 1, 333-2-21 T no. 1, and 333-2-26 T no. 1], were tested after one passage in newborn Syrian hamsters. The J20-NRK cell line, a rat cell line transformed by a murine sarcoma virus, was used as a positive control. Another hamster cell line (HDC-22) also yielded positive results. Normal hamster embryo fibroblast (HEF) cells were used as the negative control.

^c Anti-HaLV-gs serum was supplied by Berge Hampar and was directed against the hamster specific gs-1.

^d Anti-MLV-gs serum was supplied by R. E. Wilsnack and reacts against the gs-3 antigen common to mammalian C-type particles and infected cells.

^e Control weanling hamster serum was a pool of sera collected from 3-week-old Syrian hamsters.

detectable gs antigen. Results with this line revealed that our sera could detect gs antigen in hamster cells and that the cells derived from LSH hamsters are capable of producing this antigen.

It has recently been reported that synthesis of C-type particles and their antigens can be activated in previously negative cell lines by exposure to bromodeoxyuridine (BUdr) or iododeoxyuridine (1, 11). We therefore tested five HSV-2 transformed cell lines by this method for the induction of gs antigens. The cells were grown on cover slips in BME with 10% fetal calf serum and 0.75% NaHCO_3 , containing 30 μ g of BUdr per ml. This medium was removed after 24 hr and replaced by medium without BUdr. Twenty-four and forty-eight hours later, cultures were harvested, fixed, and examined by direct immunofluorescence for gs-1 and gs-3 antigens (Table 2). No gs antigens were detected

FIG. 2. Electron micrographs of thin sections of 333-8-9 cells which were transformed by irradiated HSV-2. (a) Intranuclear herpes-like virus particles, $\times 25,000$; (b) herpes-like particles in the cytoplasm of the cell at the periphery of a nucleus, $\times 44,000$.

TABLE 2. *Immunofluorescence tests for the induction of hamster leukosis viruses by bromodeoxyuridine (BUdr) in herpes simplex virus type 2 transformed hamster cells^a*

Cell line	Hr after BUdr induction ^b	Anti-HaLV-gs serum	Anti-MLV-gs serum	Weanling hamster serum
333-8-9	24	—	—	—
	48	—	—	—
333-2-29	24	—	—	—
	48	—	—	—
333-8-9 T no. 1	24	—	—	—
	48	—	—	—
333-2-20 T no. 1	24	—	—	—
	48	—	—	—
333-2-21 T no. 1	24	—	—	—
	48	—	—	—
333-2-26 T no. 1	24	—	—	—
	48	—	—	—
HEF	24	—	—	—
	48	—	—	—
J20-NRK	None	+	+	—
HDC-22	None	+	+	—

^a Transformed hamster embryo fibroblast (HEF) cells were tested for the presence of gs antigens as described in the text. The cells and sera used are described in Table 1. +, Positive reaction; —, negative reaction.

^b The cells were treated with medium containing 30 μ g of BUdr/ml for 24 hr. The BUdr medium was then removed, and the cells were harvested for immunofluorescence 24 and 48 hr later.

in the HSV-2 transformed cell lines, but the antigen was again detected in the J20-NRK and HDC-22 control cell lines.

The final method used to detect C-type particles in cell lines transformed by HSV-2 was the direct examination of cells by electron microscopy. Cell monolayers maintained in 8-oz (ca. 248.8 g) glass bottles were washed with phosphate-buffered saline (pH 7.3), scraped off the surface of the glass with a rubber policeman, and again washed once in phosphate-buffered saline. The cells were pelleted at 800 rev/min for 8 min and fixed overnight in cold 1% phosphate-buffered glutaraldehyde, pH 7.3. The cell cultures were postfixed in Dalton's chrom-osmium for 2 hr and embedded in Mollenhauer's araldite plastic. Sections were placed on naked copper grids, stained with 0.5% uranyl acetate in 50% methanol and Reynold's lead citrate, and examined with a Hitachi HU-12 electron microscope. Over 100 grids have been examined thus far, and no particles resembling C-type particles have been detected. These tests include five transformed cell lines which have been observed before and after passage in newborn hamsters. However, evidence of incomplete herpes-like particles were observed

in the nuclei of a small number of tumor cells (Fig. 2a). Nonenveloped herpes-like particles were also found at the outer edge of the nuclear membrane of a few cells (Fig. 2b). No enveloped herpes particles were detected.

The results presented in this communication suggest that C-type particles or their genome do not play an obvious role in the maintenance of HSV-2 transformed hamster cells. These data can best be explained in two ways. The first is that C-type viruses or RNA tumor viruses are not present in the HSV-transformed cells. This implies that the initial transformation event as well as the maintenance of transformation are independent of such information. The second possibility presumes that the HSV-2 activates the transforming potential of the C-type particles and then represses expression of detectable C-type markers in the oncogenic cells. Some cultures derived from cells transformed by simian virus 40 apparently also contain no detectable C-type markers (5; R. J. Huebner, *personal communication*). Perhaps several deoxyribonucleic acid-containing tumor viruses have the ability to initiate transformation of normal cells without intervention of RNA tumor viruses, a possibility that should be borne in mind by "oncogeneticists."

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