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Herpes Simplex Virus Infection of Mouse Neuroblastoma Cells (39880)

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Latent herpes simplex virus (HSV) infection is considered to be harbored in neurons of peripheral ganglia and CNS (1-3). The concept of a nonreplicating latent HSV infection is based on, inter alia, the recovery of infective virus by cocultivation of nervous tissue with cells permissive to cytocidal HSV infection, when isolation of virus from specimens of homogenized nerve tissue has failed to demonstrate virus (4, 5). In reactivated neuronal infection, virions are observed in nerve cells and, in addition, in adjacent supporting cells, probably as a result of the spread of infection (6, 7). How nonreplicating HSV is maintained in latently infected nerve cells is unknown. Possibly the controlling conditions may be analogous to those observed in latent EB virus infection. EB virus DNA can be demonstrated by electron microscopy in latently infected cells as circular, nonintegrated episome-like structures (8).

Mouse neuroblastoma cells (C 1300) have been extensively studied because *in vitro* they maintain several characteristics of differentiated neurons (9, 10). They were considered to offer interesting possibilities in studies of neuron-virus interactions. The present study reports the specific reaction pattern observed after infection of C 1300 cells with HSV.

Material and methods. Cells. The following cells lines were used: clone 41 A_3 of mouse neuroblastoma cells C 1300 (11),[2] mouse L 929 cells, MCG 101 mouse sarcoma cells from a methylcholantrene-induced tumor in C 57 black 6 J mice (12), primary embryonic mouse kidney and brain cells, human glioma cells 138 MG (13), human embryonic lung cells, and green monkey kidney cells (GMK, AH-1). All cells were cultured as monolayers in 16-oz precipitation bottles and in tubes with Eagles MEM medium supplemented with 10% fetal calf serum, 100 IU of penicillin, and 100 μg of streptomycin/ml. As maintenance medium, the same medium but supplemented with only 2% serum was used.

Passage of C 1300 cells. Confluent monolayer cultures of C 1300 cells were treated with ethylenediamine tetraacetic acid (200 μg/ml, Triplex III, Merck, in phosphate-buffered saline, pH 7.2) for 15 min at 36.5°. The cells were then suspended in growth medium and seeded into new bottles.

Cell counting. Two or three monolayer cultures of each batch of cells were randomly chosen and trypsinized. The suspended cells were diluted and four samples from each cell suspension were counted by phase-contrast microscopy in a cell-counting chamber.

Viruses. The following virus strains were studied: adenovirus type 2 (Huebner F 2850), Coxsackie B5 (ET 652), HSV types 1 and 2 (strains F and G),[3] mumps (Gbg 1371/68), and vaccinia (SBL, Stockholm).

Infectivity titrations. Plaquing of virus was carried out in GMK cells grown in 5-cm plastic dishes as previously described (14).

Fifty % endpoint titrations were carried out in monolayer cultures grown in test tubes. Samples (0.1 ml) of 10-fold dilutions of virus were added to the cultures. Five cultures were inoculated with each dilution. $TCID_{50}$ was calculated according to Reed and Muench (15).

HSV antisera. Rabbit anti-HSV sera were prepared utilizing strain F virus. The cytoplasmic fraction of HSV-infected rabbit kidney cells was centrifuged on a dex-

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tran T 70 gradient (11–38%) at 41,000*g* for 2 hr. The banded virions were inoculated onto both eyes of a rabbit. Three weeks later, when the acute conjunctivitis had subsided, 0.1 ml of the same banded virions was injected intramuscularly. These injections were repeated every 3 weeks and the rabbits were bled after 6 months. The antisera used showed titers in complement fixation and neutralization tests of 256 and 128 to 256, respectively.

Immunofluorescence (IF) studies. Indirect IF was carried out on cells grown on microscopic slides. Slides with infected or uninfected cells were washed three times with phosphate-buffered saline, pH 7.2 (PBS), and fixed in acetone for 5 min at 4°. Membrane fluorescence was studied with cells not submitted to acetone treatment. Acute and convalescent sera of a patient with herpes simplex encephalitis or pre- and postimmunization rabbit sera, prepared as described above, were used. Slides were incubated with serum for 30 min at 37° and then washed three times in PBS. Sheep anti-human IgG or sheep anti-rabbit IgG FITC-conjugate (SBL, Stockholm) was then applied for 30 min at 37°. Slides were again washed in PBS, mounted with buffered glycerol, and examined using a Leitz Ortholux microscope.

Results. Yields of HSV in C 1300 cells. HSV type 1 was inoculated onto bottle cultures of C 1300 cells [multiplicity of infection (m.o.i.) = 1 pfu/cell]. The cultures were incubated at 36.5° for 1 hr. The medium, 4 ml, was discarded, the cells were washed four times in prewarmed Hanks' BSS, 10 ml of new medium was added, and the cultures were incubated further for 23 hr. The cultures were then frozen and thawed and the cells were scraped off the harvested together with the culture fluid. Cells and fluid were sonicated at 1.35 A for 60 sec in a Raytheon sonicator. Altogether eight cultures were studied. The number of C 1300 cells per culture was $4.48 \pm 0.33 \times 10^6$. The amount of infective virus per culture was determined by plaque titrations in GMK cells. For comparison, a number of other mouse, as well as human and monkey, cell lines were infected in the same manner with the same pool of HSV and the virus yields were assayed. The procedures followed were identical with those used with C 1300 cells. The results are presented in Table I.

The yield of HSV in C 1300 cells was hundred-fold to several thousand-fold less than that in the other cell lines. All cell lines but the C 1300 cells developed cytopathic changes of varying degrees of severity.

C 1300 Cells with persistent HSV infection. C 1300 Cells, although relatively resistant to HSV infection, were able to replicate the virus. HSV type 1, at m.o.i. of 1, 0.01, or 0.001 pfu/cell, was added to each of six bottle cultures. The cells were subcultured by dividing one culture into two the day after infection, and then every third or fourth day. Culture fluids were regularly tested for HSV at the time of subcultivation by inoculating 0.5-ml aliquots on GMK cell monolayers. Two milliliters of the same fluid was frozen at −70° for plaque titrations later on.

Cultures inoculated with 1 and 0.01 pfu/cell survived only a limited number of subcultivations. Those cultures receiving a concentration of virus corresponding to

TABLE I. YIELDS OF HSV TYPE 1 IN C 1300 MOUSE NEUROBLASTOMA AND OTHER CELL LINES OF MURINE, SIMIAN, AND HUMAN ORIGIN.[a]

Cells and origin of cells	pfu/Culture	pfu/Cell[b]	Relative yield
C 1300, Mouse neuroblastoma	5.4×10^3	0.0012 ± 0.0002	1
MCG 101, Mouse sarcoma	1.7×10^8	33 ± 8	27,000
L 929, Mouse connective tissue	6.1×10^6	1.2 ± 0.2	1,000
Primary emryonic mouse brain	3.8×10^7	5.6 ± 0.3	4,700
Primary embryonic mouse kidney	1.2×10^8	23 ± 3	19,000
138 MG, Human glioma	1.1×10^6	0.48 ± 0.2	400
AH-1, Green monkey kidney	2.7×10^8	130 ± 10	110,000

[a] Multiplicity of infection = 1 pfu/cell.
[b] Means and standard errors of means (SEM).

m.o.i. = 1 could be maintained for a maximum of three passages; with m.o.i. of 0.01 up to six passages could be made. HSV was recovered from all cultures at every passage and gradually the virus content increased from 10^3 to 10^5 pfu/ml when the cells demonstrated cpe. However, when culture fluids titered below 10^5 pfu/ml no gross difference could be detected in the outgrowth of cells in infected cultures as compared to uninfected cells.

A persistent state of infection was achieved when cultures were inoculated with 0.001 pfu/cell. Cells of these cultures have been successfully subcultivated more than 46 times during a period of approximately 6 months. HSV has been regularly recovered from the culture fluids in titers up to 250 pfu/ml. Occasionally, single subcultures started to produce more virus and degenerate after one to three passages.

HSV type 1 antiserum was added to the medium of eight cultures of persistently infected C 1300 cells. The final serum concentration was 5%. After two successive passages with antiserum, the cells were cultivated without the antiserum and tested for production of infective HSV. No virus was detected in fluid from these cultures during 20 subsequent passages.

Occasional release of virus from HSV-infected C 1300 cultures. Cultures of C 1300 cells were infected with HSV type 1 at a m.o.i. of 0.0001 and then were subcultivated as described above. Randomly selected subcultures were individually passaged with 1:2 splits. Culture fluids were again assayed for infective virus using GMK cells.

Virus was recovered from all cultures in the first two subcultivations; in the third series of subcultures infective virus was found in only 5 out of 24 cultures (Table II). These five cultures showed extensive cellular degeneration and were therefore discarded. In the 7th passage virus was isolated from 3 out of 36 cultures, and in the 11th passage from 1 out of 22 cultures. Subsequently, no virus was detected until passage 25, when again infective virus was isolated from 5 out of 44 cultures.

At the 30th passage, attempts to isolate virus were performed daily for 14 days from 30 cultures. Infective virus could not be detected in any of these cultures. Cells from one of the cultures was passaged; the other cultures were discarded. After four subsequent passages, 4 out of 16 subcultures again yielded virus. Passages 41 and 48 resulted in recovery of infective virus from 3 out of 60 and 19 out of 32 cultures, respectively. Thus, production of virus was observed in cells that had been cultured during a period of 6 months by repeated passages without previously detectable virus.

TABLE II. Occasional Release of Virus from HSV-Infected C1300 Cultures[a]

Passage No.[b]	No. of cultures tested	No. of positive virus isolations
1	6	6
2	12	12
3	24	5
4-6	114	0
7	36	3
8-10	98	0
11	22	1
12-24	534	0
25	44	5
26-33	210	0
34	16	4
35-40	245	0
41	60	3
42-47	272	0
48	32	19

[a] Multiplicity of infection = 0.0001 pfu/cell.
[b] The cells were infected at passage No. 0.

In all cases the virus isolated was identified as HSV type 1. The C 1300 cultures from which HSV was isolated all showed total cellular degeneration at the time of virus isolation. It should be emphasized that extreme precautions were taken to avoid viral contamination.

Occurrence of HSV antigens in infected C 1300 cells. HSV antigens were looked for in C 1300 cell cultures with cytocidal or persistent HSV type 1 infection. Acetone fixed and nonfixed HSV-inoculated C 1300 cultures were studied by IF using human or rabbit antisera. HSV antigens were observed in nuclei and membranes of C 1300 cells demonstrating cpe. In cultures characterized as persistently infected, showing no cytopathic changes but consistently releasing virus to the culture medium, approximately 1% of the cells contained demonstrable HSV antigens. No HSV anti-

gens were observed in cultures which did not release infective virus.

Failure to reactivate possibly latent HSV infection in C 1300 cells. Cell cultures which, as described above, occasionally released virus into the culture medium after inoculation with a low concentration of HSV type 1 (m.o.i. = 0.0001) were exposed to 5-iodo-2′-deoxyuridine, actinomycin D, cortisone, or irradiation. IUdR (100 μg), actinomycin D (0.25 μg), or cortisone (50 to 2.5 mg) were added per milliliter of the cell culture medium. The IUdR- and actinomycin D-containing medium was replaced with fresh medium after 12 hr to 3 days. Ultraviolet irradiation was performed for 1 to 20 min according to standard procedures using an Osram HNS 30-W emmitter at a distance of 10 cm, or cells were submitted to X-ray irradiation of 4000 rad. Attempts to isolate infective virus from treated and untreated cultures were made repeatedly by inoculation of GMK cultures with culture fluid and by cocultivation of the C 1300 cells with GMK cells. No infective virus was detected.

Sensitivity of C 1300 cells to viruses other than HSV. Adenovirus type 2, Coxsackie B5, mumps, and vaccinia viruses, as well as HSV types 1 and 2, were titrated on cultures of C 1300, GMK, and human embryonic lung (HEL) cells. Cpe, supplemented in the case of mumps virus infection with hemadsorption using sheep erythrocytes, was determined after 6 days of incubation at 36.5° and the results, log $TCID_{50}$ values, are listed in Table III.

In general, somewhat lower virus titers were obtained in C 1300 cells than in the other two types of cells. However, in comparison with the other viruses tested, the titers of the HSV viruses were 3–4 log units lower in C 1300 cells.

Production of Coxsackie B5, vaccinia, and HSV type 1 in C 1300 and GMK cells, respectively, was studied by inoculation of groups of eight bottle cultures with each virus. After incubation at 36.5° for 1 hr, the medium was removed and the cell cultures were washed four times. Fresh medium was added and the cultures were incubated at 36.5°. At 24 hr after infection, samples of the culture assayed were assayed for infective virus in GMK cells (Table IV). The 24-hr yields per cell of Coxsackie and vaccinia viruses were of the same order of magnitude in the C 1300 cells as in the GMK cell cultures. With HSV, a difference of more than 4 log units was observed between the two types of cultures.

TABLE III. VIRUS TITERS (log $TCID_{50}$ PER 0.1) IN C 1300, GMK, AND HEL CELL CULTURES.

	C 1300	GMK	HEL[a]
HSV 1	1.5	7.2	7.5
HSV 2	0.0	5.7	6.4
Vaccinia	3.5	5.7	6.4
Adeno 2	3.3	ND[b]	4.5
Coxsackie B5	4.5	8.5	6.5
Mumps	5.5	6.5	3.5

[a] HEL = human embryonic lung cells.
[b] ND = Not done.

Discussion. Observations of intra-axonal transport mechanisms for HSV (16, 17), findings indicating latent, nonreplicative HSV infections in ganglia and neuronal cells of CNS (1–3), and induced changes in turnover of neurotransmitters associated with HSV infection of CNS (14, 18) all suggest extraordinary characteristics of HSV–neural cell interactions. HSV multiplies in all the different specific cells of the CNS with characteristic cytopathic alterations (7). However, HSV infections in organized cultures of mammalian nerve tissue (19–21) as well as of dissociated neuron tissue (22) have pointed out the relatively slow progression of HSV infection in neural cells. Restricted replication of HSV in neural cells, C 6 rat glioma cells, has been reported (23).

Mouse neuroblastoma cells (C 1300) display many properties characteristics of differentiated neurons (9, 10). On prolonged cultivation or treatment with dibutyryl cyclic AMP, they extend long processes (24). Clones of C 1300 cells demonstrate marker enzymes of both cholinergic and adrenergic nerves (25).

We have observed the reaction pattern of C 1300 cells to HSV infection. In comparison with a number of other cell lines of mouse origin, human glioblastoma, and green monkey kidney cells, C 1300 cells yielded small amounts of HSV. Murine

TABLE IV. 24-HOUR YIELDS OF COXSACKIE B5, VACCINIA, AND HSV TYPE 1 IN C 1300 AND GMK CELLS.[a]

	C 1300 Cells		GMK cells	
	pfu/Culture	pfu/Cell	pfu/Culture	pfu/Cell
Coxsackie B5 (m.o.i. = 1)	1.6×10^7	4.0 ± 0.2	1×10^7	6.7 ± 0.6
Vaccinia (m.o.i. = 0.001)	4.5×10^2	$11 \pm 1 \times 10^{-5}$	7.0×10^2	$36 \pm 3 \times 10^{-5}$
HSV (m.o.i. = 1)	4.3×10^3	$11 \pm 0.4 \times 10^{-4}$	1.4×10^8	70 ± 4

[a] Means of yield from eight cultures and SEM.

cells, other than C 1300, whether tumor derived, established, or in primary tissue cultures, whether of neural origin or not, all produced hundredfold to several thousand-fold more virus. Thus, there seemed to be restricted replication of HSV in the C 1300 cells. Furthermore, this restriction seemed specific for HSV and not for the single representatives of adeno-, entero-, myxo-, and poxvirus groups tested.

By adjusting the concentration of HSV inoculated, C 1300 cell cultures persistently or occasionally releasing infective virus into the culture medium could be obtained. Persistent infection with HSV in the absence of anti-HSV serum has been achieved in other cell systems. Coleman and Jawetz (26) obtained persistently HSV-producing adenocarcinoma lung cells by lowering the temperature to 31°. O'Neill *et al.* (27) achieved latently infected human embryonic lung cells by treating the cells with cytosine arabinoside. When the antiviral inhibitor was removed there was a delay of 5 to 6 days before infective virus and cpe reappeared.

However, in some systems persistent HSV infection has been established without culture manipulations. Robey *et al.* (28) observed persistent HSV infection in two Burkitt lymphoma-derived cell lines. Only a few cells were actively producing virus despite the presence of immunofluorescent HSV antigens in many cells.

Hampar and Copeland (29) were able to establish a persistent HSV infection in a Chinese hamster cell line, characterized by cycles of cell destruction and regrowth. The maintenance of persistent infection appeared to be dependent upon a genetically determined cell-virus equilibrium (30).

In certain respects the persistently infected C 1300 cells behaved as the Earle's L cells studied by Nii (31). He also achieved a balanced state of virus and cell growth. Occasionally the production of infective virus increased and was accompanied by cell destruction.

The mechanisms behind the persistently and occasionally HSV-producing C 1300 cell cultures are at present under investigation and will be reported later. From the observations made in the present study it would seem that only a small number of cells per culture were productively infected. The possible occurrence of nonpermissively infected cells could not be verified. Addition of antiserum cleared the cultures of infection. These findings do not support the possibility of spread of virus by cell-to-cell contact. Possibly there are both ineffective attachment and production of HSV.

Summary. Herpes simplex virus infection was studied in *in vitro* cultured mouse neuroblastoma (C 1300) cells displaying many properties characteristic of differentiated neurons. High multiplicity of infection caused a cytocidal type of infection. At a low multiplicity of infection, cultures persistently or only occasionally releasing virus into the culture fluid were established. These cultures have been subcultured more than 40 times. C 1300 Cells produced hundred- to thousand-fold less HSV per cell than a number of other cell cultures of murine, simian, or human origin. About 1% of the C 1300 cells in a culture persistently releasing virus was found to carry HSV-induced antigens. Since HSV antiserum added to the persistently infected cultures efficiently cleared cultures of infective virus, cell-to-cell spread of virus seemed of minor importance.

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