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Characterization of Herpes Simplex Virus Persistence in a Human T Lymphoblastoid Cell Line

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Persistent, dynamic-state infection with herpes simplex virus (HSV) type 1 has been maintained in human T lymphoblastoid (CEM) cells for many months after initial infection with the wild-type virus (HSV_0) (input virus/cell multiplicity of 1.0). Persistently infected cells grew as well as uninfected cells, except during occasional periods of crisis (increased viral replication and cytopathic effect). Cells could survive the crisis when they were maintained for twice the usual time interval (8 to 10 rather than 4 to 5 days) before subculture. Interferon was not detectable in the cultures. HSV_0 was compared with HSV_{p1}, a small plaque-forming isolate from persistently infected CEM cells. Primary infection of CEM cells with HSV_0 at a low input multiplicity (0.01) led to abortive replication, whereas infection with HSV_{p1} at the same multiplicity resulted in either rapidly lytic or persistent infection depending upon the time interval of subculture. Approximately 55% of plaque-purified clones of HSV_{p1}, as compared with only 5% of HSV_0 clones, displayed temperature-sensitive growth in Vero cells. Defective interfering virus was not detectable in uncloned HSV_{p1} by interference assay. Persistently infected cultures "cured" by treatment with HSV antiserum or incubation at 39°C were resistant to reinfection with HSV but permissive for vesicular stomatitis virus replication, suggesting that these treatments modulated a shift from the dynamic-state to the static-state, latent infection. These studies provide a model for characterization of HSV persistence and latency in a highly differentiated human cell line.

One of the most striking aspects of herpes simplex virus (HSV) is its ability to persist in a latent or subdetectable form in the host after primary infection (33). It has long been hypothesized that HSV resides in either a dynamic or static state during persistent infection (29). During the dynamic state, continuous low-level production of infectious virus occurs with little cytopathic effect (CPE) in the host. In the static state, viral DNA persists within cells in a nonreplicating or "latent" form. Subsequent reactivation or enhancement of virus replication by mechanisms as yet unknown may then lead to recrudescence of disease.

In vitro models are required to systematically analyze virus persistence in human cells. We have previously shown that HSV type 1 can persist in a dynamic state in a highly differentiated, human T lymphoblastoid cell line, CEM (1), for prolonged periods after initial infection (26). Further studies demonstrated that in separate experiments HSV persisted in CEM cells for 202 and 401 (27), and 169 days (unpublished results). There were no major differences in cell viability between infected and uninfected cells.

In the present studies we have further characterized the mechanisms of HSV persistence in the CEM cell line. CEM cells persistently infected with HSV were shown to undergo periodic crises with enhanced viral replication and CPE that could be overcome by increasing the time between subculture. More importantly, we present evidence that treatment of persistently infected cells with anti-HSV serum or elevated temperature appeared to "cure" the cells of virus, but actually may have modulated a shift from the dynamic-state to the static-state, latent infection. The persistent virus infection was further characterized by comparison of wild-type, parental virus with HSV isolated from persistently infected CEM cells. Although this isolate displayed restricted, temperature-sensitive replication in human and monkey fibroblasts, it was much more virulent than our wild-type virus for the CEM cells.

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MATERIALS AND METHODS

Cells and media. Human foreskin (FS) cells (originated from primary tissue obtained from Magee Womens Hospital, Pittsburgh, Pa.) were cultured for 9 to 25 passages in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2 mM glutamine, 250 U of penicillin per ml, 250 μg of streptomycin sulfate per ml, 0.025 U of nystatin per ml, 0.002 g of $NaHCO_3$ per ml, and 10% heat-inactivated (56°C for 30 min) fetal calf serum (Microbiological Associates, Bethesda, Md.). Vero cells (African green monkey kidney cells; American Type Culture Collection, Rockville, Md.) were cultured in Eagle minimum essential medium (GIBCO) with the same supplements as medium 199. FS and Vero cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

The CEM lymphoblastoid cell line (1) has T-cell characteristics, which include human T lymphocyte-specific surface antigen and the ability to rosette sheep erythrocytes, and lacks B-cell properties (surface immunoglobulin, complement receptors, Epstein-Barr virus nuclear antigen) (17). The CEM cell line was obtained from H. Lazarus, Sidney Farber Cancer Institute, Boston, Mass., and maintained as a stationary suspension culture at 37°C in upright, sealed 30-ml tissue culture flasks with RPMI 1640 medium (GIBCO) (with the same supplements as medium 199). The cells were shown to be free of mycoplasmal contamination by absence of growth in mycoplasmal broth and agar.

Virus. HSV type 1, strain 75-0876, was initially isolated in primary human embryonic kidney cells (9) and subsequently grown for three passages in human embryonic lung (HEL) cells; the virus pool had a titer of 1.3×10^8 plaque-forming units (PFU) per ml in Vero cells. This wild-type virus pool is referred to in the text as HSV_0. Uninfected HEL cell harvest was used as a control preparation. During the course of this study, HSV was assayed by a plaque titration method on Vero or FS cells in 4.5-cm^2 plastic cell culture wells by using an overlay medium containing 1% methyl cellulose and minimum essential medium supplemented with 2% fetal calf serum. After 4 to 5 days of incubation, the cells were stained for 10 min with gentian violet dye-fixative solution (2) and then washed with tap water. Virus titer was determined as the number of PFU per milliliter. The titer of HSV was the same in both the Vero and FS cell systems.

HSV_{p1} is a small plaque-forming isolate of HSV that had evolved during 401 days of persistent infection of CEM cells (27). The virus isolate was initially grown for two passages in FS cells and subsequently grown for one passage in CEM cells; the virus pool had a titer of 9.5×10^4 PFU/ml in FS cells. The plaque size of HSV_{p1} ranged from 0.5 to 1.0 mm in diameter as compared with the wild-type virus (HSV_0) plaque size of 2 mm in Vero cells under the 1% methyl cellulose overlay.

Vesicular stomatitis virus (VSV), Indiana strain, was grown in chicken embryo cell cultures as previously described (2) and had a titer of 6.2×10^8 PFU/ml on primary passage chicken embryo cell monolayers.

Viral replication. At 3 to 4 days after subculture, the CEM cells were washed twice with 10 ml of RPMI 1640 medium without serum and centrifuged at 300 $\times g$ for 10 min. The cell pellet was infected with HSV_0 (input virus/cell multiplicity of infection, 1.0) or a mock viral preparation in the presence of 25 mM of HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Cal Biochem-Behring Corp., La Jolla, Calif.). The cells were incubated for 1 h at 37°C in tightly capped tubes (50-cm^3 plastic centrifuge tube) on a shaker apparatus. After viral adsorption, the cells were washed twice with RPMI 1640 medium (without serum) to remove excess, unadsorbed virus.

Infected and uninfected cells were resuspended to a final concentration of 5×10^5 cells per ml in RPMI 1640 medium (with the standard supplements) in upright 30-ml plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.). HSV_0 was also incubated in medium alone as a control for thermal inactivation of virus. At 4- to 5-day intervals, cell viability counts were performed (trypan blue dye exclusion), and a portion of each cell line was diluted to 5×10^5 viable cells per ml with fresh medium. Additional cell counts were periodically done (without subculture of the cells) to monitor cell viability. Doubling time of the CEM cells was 36 to 38 h as calculated from growth curves of daily viability counts plotted on a semilog scale. Another portion of the infected cell suspension was lysed by sonication (3 min at 140 W; Raytheon Sonic Oscillator; Raytheon Co., Waltham, Mass.) and assayed for infectious virus.

In certain experiments, CEM cells were treated with 10 μg of phytohemagglutinin (PHA) (PHA-P; Difco Laboratories, Detroit, Mich.) or 5-iodo-2'-deoxyuridine (IUdR) (Sigma Chemical Co., St. Louis, Mo.) per ml. Uptake of [^{3}H]thymidine was measured in PHA-treated cells by the addition of 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) to 3×10^5 cells for 4 h in flat-bottom, 0.32-cm^2 microwells (Costar, Cambridge, Mass.) (26).

For the immunofluorescence test, infected and uninfected cell samples were washed three times in Hanks balanced salt solution (GIBCO). A 0.05-ml sample of each cell suspension (approximately 10^5 cells) was air dried on glass microscope slides, fixed for 10 min with acetone, and stained with fluorescein isothiocyanate-conjugated rabbit anti-HSV serum (Flow Laboratories, Rockville, Md.) as previously described (27). The percentage of HSV antigen-positive cells (nuclear and cytoplasmic fluorescence) per 500 total cells was counted in each sample.

Interferon assay. Supernatants of persistently infected and control cell cultures were assayed for interferon by the semimicro, dye-binding method (2) by using FS cells and VSV as the challenge virus. To remove HSV from the samples, supernatants from infected and uninfected CEM cells, as well as a human leukocyte interferon standard (lot M-76 P-IF; K. Cantell, Helsinki, Finland), were treated with human HSV

antiserum (titer 1/350 by microneutralization) at a final dilution of 1/20 at 37°C for 30 min, followed by ultracentrifugation at 100,000 × g for 2 h at 4°C. Supernatants were removed and stored at −20°C until assay. This procedure resulted in complete elimination of infectious virus and did not alter the antiviral activity of the interferon standard. The interferon titer was estimated by plotting absorbance of eluted dye as a function of $\log_{10}$ interferon dilution and estimating the endpoint as that dilution of interferon which inhibited cytopathic effect by 50% (2). The titer of the human leukocyte interferon standard was 1.5×10^7 IU/ml (geometric mean titer of four determinations) as corrected to an International Standard Preparation (human leukocyte interferon 69/19; Medical Research Council Research Standard B; defined as 5,000 IU/ml) during our interferon assays.

Screening of HSV clones for temperature sensitivity. Plaque-purified clones of HSV_0 and HSV_{p1} were prepared and screened for temperature-sensitive mutants by an adaptation of the methods of Youngner et al. (39, 40). FS cell monolayers grown in 2.0-cm^2 plastic cell culture wells were infected with 0.1 ml of HSV_0 or HSV_{p1} containing approximately 1 to 3 PFU. The infected cells were incubated at 37°C for 1 h, and 1.5 ml of methylcellulose overlay medium was added to each well. After 4 days of incubation at 37°C in a 5% CO_2 humidified atmosphere, isolated plaques were removed with a Pasteur pipette and transferred directly to FS cell monolayers in 2.0-cm^2 wells. The infected cells were overlaid with minimum essential medium supplemented with 2% fetal calf serum and incubated at 37°C in a 5% CO_2 humidified atmosphere. Fluids were removed from the wells when the cell monolayers displayed 3+ to 4+ (75 to 100%) CPE and were frozen at −70°C.

For temperature sensitivity screening, plaque-purified clones were thawed and sonicated, and 0.1 ml of serial 10-fold dilutions was added to Vero cell monolayers in 4.5-cm^2 wells. After adsorption of virus for 1 h at 37°C, the cells were overlaid with minimum essential medium containing 2% fetal calf serum and 25 mM HEPES buffer. Replicate wells were incubated in a humidified, non-CO_2-supplemented atmosphere at 34 and 39°C (±0.2°C). Cell supernatants were harvested from wells at both temperatures after 2 (HSV_0) or 4 to 5 (HSV_{p1}) days of incubation, at which time the lower dilutions usually displayed 3+ to 4+ CPE at 34°C. Virus yields at each temperature were assayed by standard plaque titration in Vero cell monolayers at 37°C. Efficiency of plating (EOP) was calculated for each clone as the ratio of virus yield at 39°C compared with that at 34°C. Virus clones with an EOP of ≤0.01 were considered temperature sensitive.

RESULTS

Persistent infection of CEM cells with HSV. HSV has previously been shown to persist in CEM cells for 202 and 401 days (27) and 169 days (unpublished results) in separate experiments. There were no major differences in cell viability between virus-infected and uninfected CEM cells. In the present study, it was observed that HSV_0 infection of CEM cells at an input virus/cell multiplicity of 1.0 resulted in different patterns of acute and persistent infection. The acute phase (first 2 weeks of infection) was characterized by differences in the production of infectious virus. For example, in one experiment, the titer of HSV_0 increased 0.9 $\log_{10}$ PFU/ml by day 1, followed by a gradual decrease in viral titer over the next 9 days of infection (from 4.9 to 3.5 $\log_{10}$ PFU/ml) (Fig. 1A). Subsequently, the viral titer increased and remained at a mean level of 5.5 $\log_{10}$ PFU/ml from day 14 to day 52 of the experiment. In a separate experiment, the acute phase of infection was characterized by a rapid decrease in titer to an undetectable level (<1.0 $\log_{10}$ PFU/ml) by day 3 postinfection (Fig. 2A). Infectious virus was detectable at 5 days and persisted at a mean level of 5.9 $\log_{10}$ PFU/ml from day 14 to day 67 of the experiment.

During the persistent phase of HSV_0 infection, the infected cell cultures underwent a period of crisis as evidenced by a sharp decrease in the number of viable cells. Titers of virus increased from a mean of 5.2 (days 0 through 33) to 6.2 (day 38) $\log_{10}$ PFU/ml (Fig. 1A) and from a mean of 3.8 (days 0 through 18) to 6.0 (day 22) $\log_{10}$ PFU/ml (Fig. 2A) in two separate experiments. The percentage of antigen-positive cells rose from a mean of 3.6% (days 0 through 33) to 28.5% (day 38) (Fig. 1B) and from a mean of 0.4% (days 0 through 18) to 36% (day 22) (Fig. 2B). It was noted that complete CPE occurred in cultures that were passed at normal intervals (4 to 5 days) into fresh medium during the period of crisis (Fig. 1C). The infected cell cultures could survive the crisis when they were maintained for longer time periods (9 instead of 4 to 5 days) during the crisis before passage into fresh medium (Fig. 2C). In contrast to our previous results with HSV-infected B and myeloid cell lines (27), the viability of the infected CEM cells could not be maintained during the crisis by concentration of the cells in small volumes of fresh medium. After the crisis was resolved, HSV persisted in the CEM cells for over 200 days postinfection (data not shown). During this time the cells were maintained through another crisis period (days 186 through 196) by increasing the length of time before subculture to 10 days during the crisis.

To determine whether endogenous interferon was involved in the maintenance of persistent HSV infection in the CEM cells, supernatant samples of both uninfected and virus-infected CEM cells taken at the same times as the virus samples were assayed for antiviral activity. No interferon activity could be demonstrated in either uninfected or infected CEM cells throughout the course of viral persistence in these experiments.

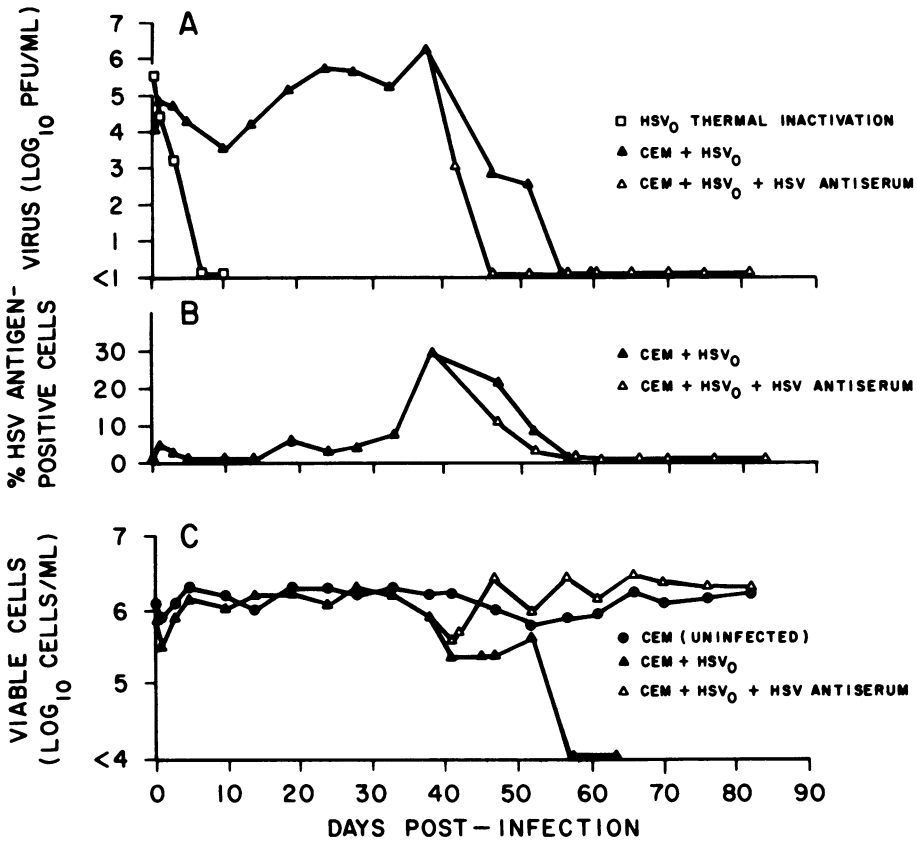

FIG. 1. *HSV_0 infection of CEM cells and effect of HSV-antiserum on virus persistence. Cells were infected at an input multiplicity of 1.0 and subcultured at 4- to 5-day intervals. HSV antiserum was added to the cultures on day 38 and removed on day 70. A, Infectious virus, log_{10} PFU/ml; B, percentage of HSV antigen-positive cells; C, viable cells, log_{10} cells per ml.*

Thus, CEM cells persistently infected with HSV_0 and maintained by routine passage at 4- to 5-day intervals, rather than the 5- to 7-day intervals employed in earlier studies (27), displayed periods of crisis, i.e., rapid cell death. Persistently infected cells could survive this crisis period when they were incubated for twice the usual time interval before subculture.

Treatment of HSV-infected CEM cells with antiviral serum. Anti-HSV serum was added to a persistently infected cell culture to detect whether extracellular virus was significant in maintenance of persistent infection. Uninfected and persistently infected CEM cells were subcultured from day 38 to day 70 postinfection in RPMI 1640 medium (with standard supplements) containing 5% heat-inactivated, anti-HSV human serum (titer 1/350 by microneutralization) (Fig. 1). The persistently infected CEM cells not treated with antiserum entered into a crisis period in which total CPE occurred by 56 days postinfection (Fig. 1A). In contrast, antiserum-treated cells were negative for infectious virus after 9 days of treatment (day 47 postinfection) and negative for viral antigen after 23 days of treatment (day 61 postinfection) (Fig. 1B); antiserum-treated cells grew as well as untreated, uninfected CEM cells (Fig. 1C). The antiserum was removed after 32 days of treatment (day 70 postinfection), and the cells remained free of infectious virus and viral antigen for the duration of the experiment.

Studies were undertaken to examine the antiserum-"cured" cells (CEM_{AC}) for the presence of latent HSV by using agents known to enhance herpesvirus replication. The CEM_{AC} cells were suspended to approximately 5×10^5 cells per ml and treated with PHA (26) or IUdR (34) (final concentration, 10 μg/ml) for 4 days (PHA, days 87 through 91; IUdR, days 108 through 112 postinfection) and monitored daily for cell viability, virus titer, and percentage of antigen-positive cells. Treatment with PHA resulted in clumping of the CEM cells and a 25% increase

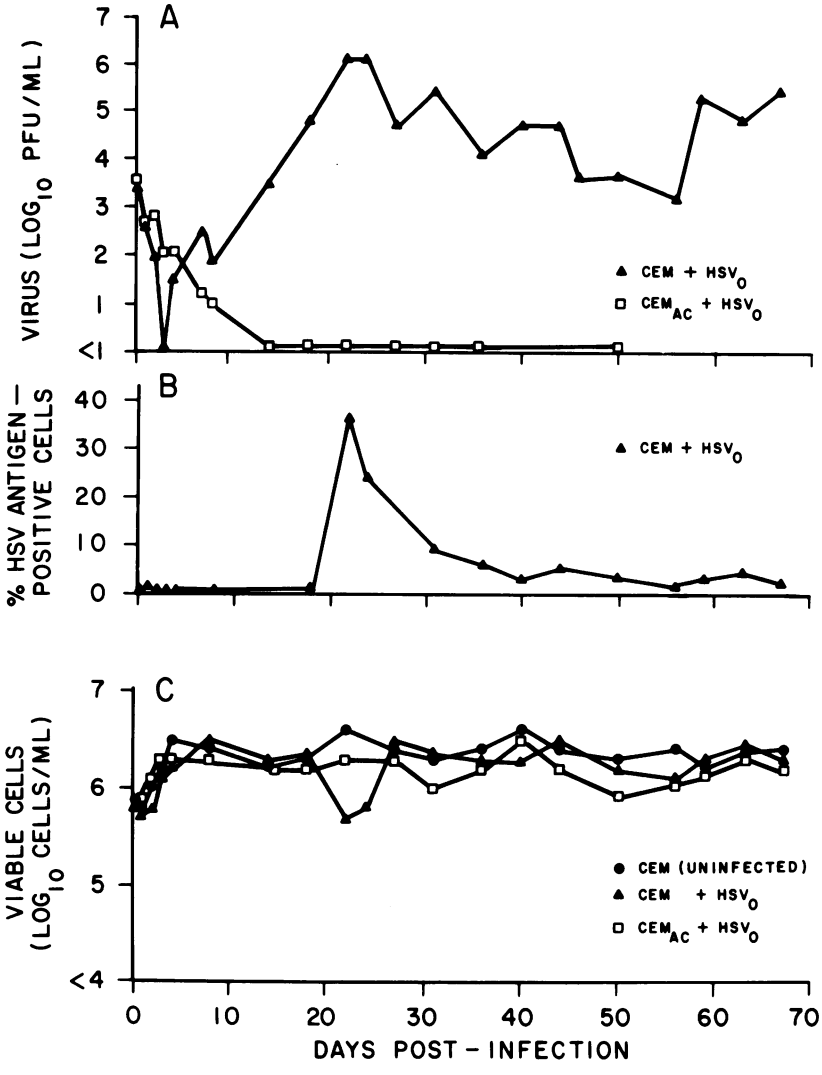

FIG. 2. *HSV0 infection of CEM and CEMAC cells. Cells were infected at an input multiplicity of 1.0 and subcultured at 4- to 5-day intervals, except for a 9-day interval from day 18 to day 27 for the CEM cells only. A, Infectious virus, log10 PFU/ml; B, percentage of HSV antigen-positive cells; C, viable cells, log10 cells per ml.*

in uptake of [^{3}H]thymidine by 3 days after treatment as compared with untreated cells (data not shown). IUdR had a toxic effect on the cells with a gradual decrease in cell viability from 4.8×10^5 to 1.4×10^5 cells per ml by day 4 of treatment. Infectious HSV or HSV antigen was not detected in PHA- or IUdR-treated CEM_{AC} cells. Cocultivation of untreated, PHA-treated, and IUdR-treated CEM_{AC} cells (2×10^5 cells) with FS cells (4×10^5 cells) for 10 days after treatment also failed to reveal presence of virus. The CEM_{AC} cells cultured under standard conditions have not shown evidence of infectious virus or viral antigens during over 200 additional days of incubation. Thus, treatment of the persistently infected cells with human anti-HSV serum prevented cell death, i.e., crisis, and appeared to "cure" the cells of HSV infection.

Reinfection of CEM_{AC} cells with HSV. Studies were done to examine the susceptibility of CEM_{AC} cells to reinfection with HSV. CEM and CEM_{AC} cells were infected with HSV_0 at an input multiplicity of 1.0. The virus replicated in the CEM cells, with an increase of 2.5 $\log_{10}$ PFU/ml from day 3 to day 7 postinfection; HSV_0 persisted in the CEM cells at a mean level of 5.8

$\log_{10}$ PFU/ml from day 7 to day 67 postinfection (Fig. 2A). In contrast, CEM_{AC} cells infected at 31 days after removal of antiserum failed to replicate HSV_0, with infectious virus decreasing to undetectable levels by day 14 of infection (Fig. 2A). The percentage of antigen positive cells during the first 4 days of infection in CEM_{AC} cells was at a mean of 0.5% and decreased to undetectable levels by day 8 (data not shown).

In further studies, CEM_{AC} cells were shown to retain their resistance to HSV_0 replication at 153 and 206 days after removal of antiserum; HSV_0 replicated in CEM cells infected at the same time (input multiplicity, 1.0; data not shown). In contrast, concurrently infected CEM and CEM_{AC} cells were permissive for heterologous virus (VSV) replication (input multiplicity, 1.0). Titers of VSV increased 0.9 to 3.5 $\log_{10}$ PFU/ml from day 0 to day 5 postinfection in both CEM and CEM_{AC} cells.

Investigations were done to detect latent, static-state virus in the CEM_{AC} cells that were reinfected with HSV_0. In initial experiments, PHA or IUdR treatment with or without cocultivation with FS cells failed to result in detectable virus. Based on our results showing enhanced replication of dynamic state virus at 34°C (described below), the cells were transferred from 37 to 34°C at 100 days after reinfection (306 days after removal of antiserum). The cell cultures underwent irreversible crisis after 43 days of incubation at 34°C, with a sharp, 98% decrease in the number of viable cells. Approximately 90% of the cells displayed HSV antigen by direct immunofluorescence, although infectious virus was not recovered from cell lysates by plaque assay at 34 or 37°C. Replicate cultures as well as CEM_{AC} cells concurrently incubated at 37°C did not show evidence of altered cell growth or viral activation.

Effect of temperature shifting on HSV persistence in CEM cells. To study the effect of temperature on persistent HSV infection in the CEM cell line, persistently infected cells from the experiment shown in Fig. 2 were shifted to either 34 or 39°C at 44 days of infection. Persistently infected cells shifted to 34°C displayed nearly a 10-fold rise in viral titer by 6 days, with a markedly enhanced CPE and complete cell lysis by 15 days (Table 1); control, uninfected CEM cells maintained normal growth at 34°C (data not shown). However, shifting of the cultures from 37 to 39°C resulted in complete shutoff of infectious virus replication by 12 days (Table 1) and viral antigen production by 15 days (data not shown); normal cell growth was maintained in these cultures (Table 1) and in uninfected, control cultures (data not shown) at the elevated temperature.

Studies were done to detect latent, static-state virus in cells "cured" of persistent, dynamic-state HSV infection by incubation at 39°C (CEM_{TC} cells). Portions of the CEM_{TC} cell cultures were shifted to 34 or 37°C after 35 days of incubation at 39°C. No evidence of infectious virus or viral antigen was detected in the CEM_{TC} cells at either of these temperatures. Further studies showed that CEM_{TC} cells did not support replication of HSV_0 upon reinfection at 37°C, whereas they were fully permissive for VSV replication (Table 2). Therefore, CEM_{TC} cells are similar to CEM_{AC} cells in their resistance to reinfection with HSV and their permissiveness for VSV replication.

Detection of temperature-sensitive mutants and defective interfering particles during persistent HSV infection. The role of

TABLE 1. *Effect of temperature shift on persistent HSV infection of CEM cells*[a]

Day after temperature shift	Day postinfection	34°C		37°C		39°C	
		Virus[b]	Cell[c]	Virus	Cell	Virus	Cell
0	44	4.7	5.7	4.7	5.7	4.7	5.7
2	46	4.5	NT[d]	3.6	NT	1.0	NT
6	50	5.6	6.1	3.6	6.1	1.0	6.3
12	56	<1.0	5.0	3.2	6.1	<1.0	6.0
15	59	<1.0	<4.0[e]	5.2	6.3	<1.0	6.4

[a] CEM cell cultures were shifted from 37 to 34 and 39°C 44 days after infection with HSV_0. Cells at each temperature were subcultured as described in the text on day 6 after the temperature shift; cells at 37 and 39°C were also subcultured on days 12 and 15. Samples were taken for virus and cell count before subculturing.

[b] $\log_{10}$ PFU/ml.

[c] $\log_{10}$ viable cells per ml.

[d] NT, Not tested.

[e] No viable cells were detected in the culture.

TABLE 2. *Replication of HSV and VSV in CEM_{TC} cells*[a]

Day postinfection	HSV		VSV	
	CEM cells	CEM_{TC} cells	CEM cells	CEM_{TC} cells
0	4.0[b]	4.5	5.4	3.9
1	4.2	3.3	6.1	5.0
3	6.4	2.9	6.3	4.8
5	5.7	2.7	6.6	6.6
7	5.8	<1.0	6.9	6.1
10	5.3	<1.0	6.2	6.9
14	4.2	<1.0	6.9	5.9

[a] CEM_{TC} cells were infected with HSV_0 or VSV (input multiplicity, 1.0) at 133 days after the cells were temperature "cured" of persistent HSV infection.

[b] $\log_{10}$ PFU/ml.

temperature-sensitive mutants in persistent HSV infection was examined by comparing virus isolated from CEM cells after 401 days of infection (HSV_{p1}) with parental, wild-type HSV_0. Initial studies showed that plaque titers of uncloned HSV_{p1} or HSV_0 did not differ at 34 or 37°C in Vero cells. However, further investigations demonstrated that the mean EOP at 34 and 39°C of 31 plaque-purified clones of HSV_{p1} in Vero cells was 0.064 (Fig. 3). Of these 31 HSV_{p1} clones, 17 had an EOP of ≤0.01, with six of these being <0.001. Thus approximately 55% of virus clones isolated during persistent infection displayed temperature-sensitive replication. In contrast, the mean EOP of 21 plaque-purified clones of parental, wild-type HSV_0 was 8.0, significantly greater than that of HSV_{p1} ($P < 0.0025$, Student's t test). Only 1 (5%) of the 21 HSV_0 clones had an EOP of <0.01.

Virus from persistently infected CEM cells was examined for the presence of defective interfering particles by an interference assay. Uninfected Vero cells and Vero cells infected with uncloned HSV_{p1} (1×10^4 PFU/0.1 ml per well) were coinfected with uncloned HSV_0 or VSV (1×10^6, 1×10^5, or 5×10^4 PFU/0.1 ml per well). Cell fluids were harvested after 20 h of incubation at 37°C. Yields of HSV_0 and VSV were easily distinguished from HSV_{p1} by their earlier formation of larger plaques on Vero cell monolayers. No evidence of interfering particles was detected, as both HSV_0 and VSV replicated to similar titers in the presence or absence of HSV_{p1} (Table 3).

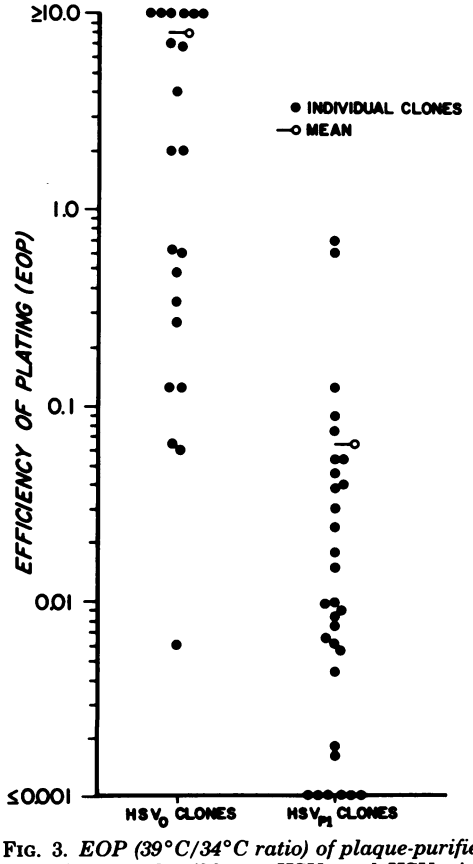

FIG. 3. *EOP (39°C/34°C ratio) of plaque-purified clones of parental, wild-type HSV_0 and HSV_{p1} isolated from CEM cells persistently infected for 401 days.*

Enhanced virulence of HSV_{p1} for CEM cells. The virulence of HSV_{p1}, the small plaque-forming virus isolated from persistently infected CEM cells (27), was compared with that of the parental, wild-type virus (HSV_0). CEM cells infected with a low input multiplicity (0.01) of the wild-type, large plaque-forming HSV_0 failed to replicate virus and have remained negative for infectious virus (Fig. 4A) and viral antigen (data not shown) since day 1 of infection.

In contrast, HSV_{p1} was highly lytic for the CEM cells at the same low input multiplicity, resulting in complete cellular destruction by days 10 through 16 in three separate experiments when the cells were maintained for the usual time interval (5 to 6 days) before the first subculture (passage interval I shown in representative experiment in Fig. 4). CEM cells in-

TABLE 3. *Examination of virus isolated from persistently infected CEM cells (HSV_{p1}) for evidence of defective interfering particles by the interference assay*[a]

Virus added	PFU	HSV_{p1} added[b]	Virus yield (PFU/ml)
HSV_0	1×10^6	+	3.3×10^8[c]
		−	6.6×10^8[c]
	1×10^5	+	4.4×10^8[c]
		−	1.5×10^8[c]
	5×10^4	+	3.7×10^8[c]
		−	2.1×10^8[c]
VSV	1×10^6	+	1.3×10^7[d]
		−	4.7×10^6[d]
	1×10^5	+	5.3×10^6[d]
		−	3.7×10^6[d]
	5×10^4	+	2.1×10^6[d]
		−	1.7×10^6[d]

[a] Vero cell monolayers grown in 2-cm^2 wells were infected with 0.1 ml of uncloned HSV_0 or VSV alone or simultaneously coinfected with uncloned HSV_{p1} (10^4 PFU in 0.1 ml). Virus yield after 20 h was determined by plaque assay of cell fluids in Vero cells at 37°C.

[b] +, HSV_{p1} added to cell cultures; −, HSV_{p1} not added to cell cultures.

[c] Yield of HSV_0.

[d] Yield of VSV.

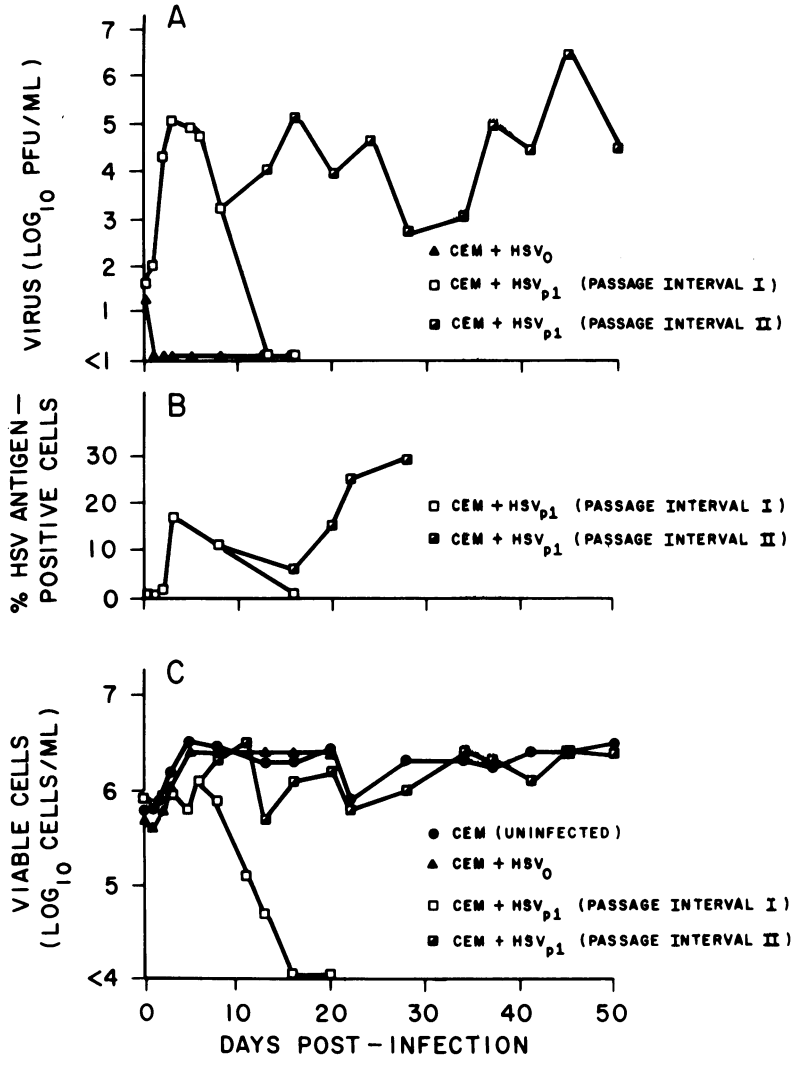

FIG. 4. *Infection of CEM cells with wild-type HSV (HSV_0) and a small plaque isolate from persistently infected CEM cells (HSV_{p1}) at an input multiplicitty of 0.01 and the effect of the interval of cell passage on persistence of virus. Passage interval I refers to CEM cells subcultured 6 days after primary infection with HSV_{p1}. Passage interval II refers to CEM cells subcultured at 8, 16, 20, 28, and 34 days after infection with HSV_{p1} and at 4- to 5-day intervals from day 34 to day 50. A, Infectious virus, log_{10} PFU/ml; B, percentage of HSV antigen-positive cells; C, viable cells, log_{10} cells per ml.*

fected with HSV_{p1} were able to survive the acute crisis when they were held for longer time intervals (passage interval II, Fig. 4) before subculture during the crisis period. After recovery from crisis, the CEM cells were subcultured at 4- to 5-day intervals and supported persistent infection for the remainder of the experiment. The virus isolate retained its small plaque size during this persistent infection.

DISCUSSION

In the present studies, a persistent infection with HSV was established in CEM cells, a human T-lymphoblastoid cell line. During the course of persistence the CEM cells entered a crisis in which there was a sharp rise in HSV titer with a subsequent decrease in the number of viable cells. We have previously shown that cell crises occur in human B and myeloid cell lines persistently infected with HSV (27). Similar cell crises have been reported for persistent infection of mouse L cells with VSV (25, 39) and parvovirus infection of another human T lymphoblastoid (Molt-4) cell line (3). In contrast to these results, HSV persistence in certain human B lymphoblastoid cell lines is apparently not

characterized by such crucial periods of enhanced CPE and viral replication (19, 28, 30, 31).

Several factors may be involved in the initiation of the crisis period. There may be a sudden enhanced virulence of HSV that results in increased CPE and cell death. In support of this hypothesis, we have shown that HSV isolated from persistently infected CEM cells (HSV_{p1}) (27) resulted in a highly lytic, primary infection of CEM cells; infection of CEM cells with the wild-type virus (HSV_0) at the same low input multiplicity (0.01) led to an abortive infection with insignificant CPE. Of interest is the observation that HSV_{p1} exhibits restricted growth (small plaque size) in human and monkey fibroblasts. Similarly, rabies (18) and Newcastle disease (36) viruses isolated from persistently infected cells have enhanced virulence for the parental cell types.

An abrupt decrease in interferon activity could also lead to a period of crisis (32). Interferon has not been detected in the HSV-CEM cell system. However, subdetectable levels of interferon could be involved in maintenance of the persistent virus. Consistent with this hypothesis is the finding that antiinterferon globulin treatment of mouse L cells persistently infected with VSV (24, 25, 40) and Sindbis virus (15) resulted in a marked increase in viral titer and CPE, i.e., crisis, even though interferon levels were very low or undetectable in the cultures. Alternatively, there could be an enhanced permissiveness of the T cells for HSV replication, possibly due to a temporary predominance of genetically susceptible cells (12, 38) or a sudden decrease in defective interfering virus particles (14). Although we have not detected defective interfering virus in HSV_{p1}, it may be of significance in establishment of persistent HSV infection and may be present at other times during infection of the CEM cells. We have previously noted that B lymphoblastoid cells persistently infected with HSV undergo a cyclic pattern of virus production and cell viability that could be related to the generation of defective interfering virus (27).

CEM cells infected with HSV_0 or HSV_{p1} survived the crisis when they were held for twice the usual time interval (8 to 10 rather than 4 to 5 days) before subculture during the crisis period. Passage at 4- to 5-day intervals during the crisis period may further the selective advantage of HSV, possibly because of enhanced permissiveness of CEM cells for viral replication during their exponential growth phase. In contrast, incubation for longer intervals during crisis may lead to inhibition of viral CPE due to decreased HSV replication in CEM cells at saturation density. Additional factors that may be operative include outgrowth of a subpopulation of CEM cells temporally resistant to HSV or enhanced activity of viral inhibitors (interferon, defective interfering particles, low pH). Other investigators have similarly shown that subculturing of cells (mouse and hamster fibroblasts) persistently infected with HSV often led to a crisis state; HSV persistence could be maintained by routine medium changes without subculturing (5, 8, 13).

The CEM cells were "cured" of persistent dynamic-state HSV infection by treatment with HSV-antiserum. Elimination of detectable virus in this and other virus-cell systems by antiserum (10, 11, 21, 37) suggests that extracellular virus is important in the maintenance of viral persistence. In contrast to these results, treatment with viral antiserum has failed to eliminate persistent HSV infection in human B lymphoblastoid (30), HeLa (35), rat (6), and hamster (13) cells. This may relate to differences in strains of virus, cell types, or antiserum treatment (concentration, time of exposure). Removal of antiserum from the CEM cultures has not resulted in the reappearance of infectious virus or viral antigen.

Shifting of CEM cell cultures from 37 to 39°C during dynamic-state, persistent infection also resulted in rapid shutoff of HSV production (both infectious virus and viral antigen) with normal growth of cells; CEM cell cultures shifted to 34°C displayed a significant rise in viral titer and complete CPE. In contrast to our results, production of infectious HSV, but not viral antigen, has been shown to be inhibited at 39°C in rat neuronal cells (20). Incubation at elevated temperature also has induced latent HSV infection in human fibroblasts, but induction of latent HSV infection required cotreatment of the cells with cytosine arabinoside (22). Our results on the effects of temperature shifting may be analogous to VSV-mouse L (23, 39) and measles virus-human B lymphoblastoid (16) cell systems, in which temperature-sensitive virus mutants are of significance in viral persistence. Further evidence for this is that approximately 55% of plaque-purified clones of virus isolated from CEM cells after 401 days of infection (HSV_{p1}) have temperature-sensitive growth characteristics. This represents a significant increase from the 5% level of temperature-sensitive clones detected in the parental, wild-type HSV. Further studies will examine virus isolates taken at various times after establishment of persistent infection for temperature sensitive mutants.

Antiserum and temperature-"cured" cells were resistant to reinfection with HSV, but were permissive for heterologous (VSV) virus replication. This suggests that HSV is still persisting in the CEM cells, and that antiserum and ele-

vated temperature have modulated a shift from dynamic to static-state, latent infection that renders the cells resistant to homologous virus. In this regard, cells transformed by HSV (7) and other deoxyribonucleic acid viruses (4) that are known to contain at least a portion of the viral genome in a latent state are often resistant to superinfection with homologous virus. A human lymphoblastoid cell line persistently infected with cytomegalovirus and "cured" by antiserum has also been shown to be resistant to homologous virus (11). In the present study we were unable to activate virus in the antiserum-"cured" cells with PHA, IUdR, or cocultivation. It is possible that the "cured" cells harbor only a portion of the HSV genome, or that other conditions are required to activate virus. Indeed, shifting of antiserum-"cured" cells to 34°C several months after abortive reinfection with HSV led to complete crisis. Approximately 90% of the cells contained HSV antigen as detected by immunofluorescence, but infectious virus was not recovered from cell lysates.

The ability of HSV to persist in a latent or subdetectable form after primary infection frequently leads to periodic reactivation and recurrence of disease. Knowledge of the methods by which HSV persists in host cells is critical to the development of controls for HSV infection. Persistent infection of the highly differentiated, CEM lymphoblastoid cell line with HSV provides an in vitro model for the study of mechanisms of HSV persistence and latency in human cells and of the effect of anti-viral agents on persistent and latent HSV infection.

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