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Reactivation of herpes simplex virus type 2 from a quiescent state by human cytomegalovirus

(herpesviruses/*in vitro* latency/virus–virus interactions)

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ABSTRACT The ability of human cytomegalovirus to stimulate replication of herpes simplex virus type 2 (HSV-2) was examined. The system used involved HSV-2-infected human embryonic lung cells under conditions (39.5–40°C) in which HSV-2 remains undetectable. Reactivation of HSV-2 was maximal and persisted for the longest duration when cultures were superinfected with 0.02 plaque-forming unit of human cytomegalovirus per cell. Infectious HSV-2 appeared 2 days after superinfection with human cytomegalovirus and ranged from 10^2 to 10^6 plaque-forming units per culture. Virus reactivated from these cultures was neutralized by rabbit immune serum produced against HSV-2. The specificity of this interaction was demonstrated by various criteria: production of HSV-2 was not observed in cultures treated with mock infecting fluid, and inactivation of human cytomegalovirus by heat, ultraviolet irradiation, or immune serum prior to superinfection eliminated its ability to induce HSV-2 replication. These results suggest that interaction between these two human herpesviruses may be of importance in herpesvirus latency *in vivo*.

Herpes simplex virus (HSV) is maintained in a latent state (1) within the neural tissue of humans (2–6). The establishment of latency, the state of the virus, and the conditions leading to reactivation of virus are the current foci of research in this area. HSV virions and antigens in latently infected neural tissues were not detected by electron microscopy and immunofluorescence studies (1, 7). Later studies, however, demonstrated the expression of an HSV-specified enzyme, thymidine kinase (8), in the dorsal root ganglia of latently infected mice (9). This evidence suggested that the HSV genome was at least partially expressed during latency. Ganglia from latently infected mice (10) and humans (11) have also been examined by solution hybridization; HSV DNA was detected in neurons during both the acute and latent (or chronic) states (10). In contrast, virus mRNA was not detected during latent infection (10). The lack of detectable HSV mRNA contrasted strongly with the finding of HSV-specified thymidine kinase in the ganglia of latently infected mice. This paradoxical situation was resolved when *in situ* hybridization methods detected HSV mRNA in a small percentage (0.4–8%) of neurons in human sensory ganglia (11), substantiating that transcription of at least some regions of the HSV genome occurs during latency.

We were interested in studying the reactivation of HSV type 2 (HSV-2). Frequently, under natural conditions, the human host is simultaneously a reservoir for multiple animal viruses. The interaction among these harbored viruses may be crucial to the pathological state and may be of particular significance with regard to latent virus infection. Indeed, the replication of numerous viruses has been shown to be affected by the presence of coinfecting viruses such as human adenoviruses and simian virus 40 (12, 13) or simian adenovirus SV15 (14), Marek's disease virus and avian leukosis virus (15), and Moloney sarcoma and leukemia viruses (16). To rescue potentially defective HSV in neurons, Brown and coworkers (17) superinfected human ganglia in culture with HSV type 1 temperature-sensitive mutants. Wild-type HSV was recovered from explant cultures that had been negative for 45 days in culture.

The work reported in this paper was undertaken to examine the effects of human cytomegalovirus (HCMV) infection on cultures latently infected with HSV-2. The experiments presented demonstrate that superinfection with HCMV induces replication of HSV-2.

MATERIALS AND METHODS

Cells and Viruses. Experiments were performed with monolayers of human embryonic lung (HEL) cells grown to confluence in Falcon T-25 flasks in Dulbecco's medium supplemented with fetal calf serum (10%) (Flow Laboratories, Rockville, MD), glutamine (0.03%), sodium bicarbonate (0.075%), penicillin (25 units/ml), streptomycin (25 μg/ml), and kanamycin (100 μg/ml). Maintenance medium contained 2% fetal calf serum, 0.15% sodium bicarbonate, and additional supplements similar to those in the growth medium.

Flow 5000 cells (Flow Laboratories) were passaged in T-75 flasks in modified Eagle's minimum essential medium supplemented with fetal calf serum (10%), tryptose phosphate broth (10%), sodium bicarbonate (0.075%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Confluent monolayers were infected with HSV-2 (strain 186) at a multiplicity of infection (moi) of 0.01 plaque-forming unit (PFU) per cell, and infected cells were harvested when 90% of the cells showed cytopathic effects. Virus was obtained by freezing and thawing infected cells three times and eliminating cell debris by centrifugation at 1000 × *g*. HCMV (strain AD169) was also grown in Flow 5000 cells; HCMV (strain Eisenhart) was replicated in HEL cells. The viruses were harvested in a manner similar to that of HSV-2-infected cultures when 80% of the cells showed cytopathology.

To establish latent infection of HEL cells with HSV-2, we followed previously described protocols (18, 19). Briefly, confluent monolayers of HEL cells were pretreated for 5 hr with 25 μg of arabinonucleoside (cytosine arabinoside) (ara-C; Sigma) per ml, infected with HSV-2 at a low moi (0.025–0.1 PFU per cell), and treated daily with maintenance medium containing ara-C for 7 days. The cultures were washed, the medium was then replaced with fresh fluid without inhibitor, and the cultures were shifted to 39.5–40°C. For reactivation studies, cultures were superinfected with HCMV (strain AD169

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Abbreviations: HCMV, human cytomegalovirus; HSV, herpes simplex virus; HSV-2, HSV type 2; moi, multiplicity of infection; PFU, plaque-forming unit(s); ara-C, arabinonucleoside (cytosine arabinoside); PRK cells, primary rabbit kidney cells; HEL cells, human embryonic lung cells.

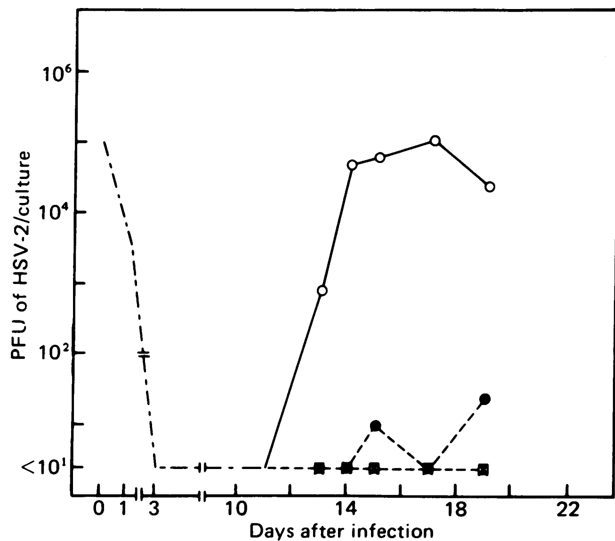

FIG. 1. Reactivation of HSV-2 from a quiescent state by superinfection with HCMV. Cultures were treated with ara-C and superinfected with HCMV 11 days after infection with HSV-2. HSV-2-infected cultures at 39.5–40°C were infected with HCMV (strain AD169) at a moi of 0.02 PFU per cell (O) or mock infected (□). Parallel cultures were infected with either UV-irradiated (●) or heat-inactivated (X) HCMV. The curve (- - -) symbolizes establishment of the quiescent state. Virus production decreased rapidly to below detectable levels in the presence of ara-C (19, *). The cultures were harvested at various times after superinfection and assayed for the presence of infectious HSV-2 by plaque formation in PRK cells.

or Eisenhart), maintained at 39.5–40°C, and harvested at various intervals thereafter; they were then titrated for HSV-2 and HCMV. Fluid from uninfected Flow 5000 cells harvested in parallel with stock HCMV was used to mock infect parallel cultures.

Virus Titrations. To detect HSV-2, we exposed primary rabbit kidney (PRK) cells to material from cultures frozen and thawed three times and overlaid them with 0.5% methylcellulose (20). Plaques were counted after incubation at 37°C for 3–4 days. To detect HCMV plaques, we used Flow 5000 cells with a 1% agarose overlay (21); plaques were counted after incubation at 37°C for 2 weeks.

In mixed infections, HCMV was titrated by neutralizing the HSV-2 with rabbit immune serum for 1 hr at 37°C. Neutralized virus was then adsorbed to confluent monolayers of Flow 5000 cells, overlaid with 1% agarose, and incubated for at least 2 weeks at 37°C. The rapidity of HSV-2 plaque formation and failure of HCMV to replicate in PRK cells enabled direct titration of HSV-2 in the presence of HCMV.

Virus Neutralization with Antiserum. Virus reactivated from a culture 6 days after superinfection was treated with medium, rabbit immune serum, or normal serum at 37°C for 1 hr in a shaking waterbath. Aliquots were plated immediately after neutralization on PRK cells. Immune serum was produced by repeated injections (five at 2- to 4-week intervals) of rabbits with HSV-2 (strain 186) purified in dextran-10 gradients and mixed with incomplete or complete Freund's adjuvant. Nonimmune serum was obtained from the rabbits prior to immunization with HSV-2.

Inactivation of HCMV with Antiserum. HCMV was pretreated with either nonimmune serum (diluted 1:4) or human anti-HCMV serum (diluted 1:4) at 37°C for 1 hr. This dilution of the immune serum reduced the titer of infectious HCMV four logarithms; the serum had an immunofluorescence titer of 1:36. The neutralized virus was then used to superinfect HSV-2-infected (quiescent) cultures at 39.5–40°C, and reactivation of HSV-2 was examined at various intervals.

UV and Heat Inactivation of HCMV. An aliquot of diluted HCMV (2×10^5 PFU/ml) was irradiated with shortwave UV light at 24 erg/sec per mm^2 (1 erg = 1.0×10^{-7} joules) for 15 min with periodic agitation. Another aliquot was incubated at 65°C for 2 hr.

RESULTS

To establish a state in which infectious HSV-2 is undetectable, we treated HSV-2-infected HEL cells daily with 25 μg of ara-C per ml for a 7-day period. On the seventh day after infection, ara-C was removed, the infected cells were washed, and medium without inhibitor was placed on the cells (18). At this time, infected cells were shifted to 39.5–40°C (19). Under these conditions, titers of infectious HSV-2 drop rapidly and the virus becomes undetectable by plaque formation in PRK cells for extended periods of time (>70 days); HSV-2 reappears only after shiftdown to 37°C.*

Superinfection with low levels of HCMV (strain AD169) of HSV-2-infected cultures after 4 days at 39.5–40°C (subsequent to ara-C treatment for 7 days) induced HSV-2 replication (Fig. 1). Cultures superinfected with HCMV showed cytopathic effects 2–3 days after superinfection. Cultures treated with mock-infecting fluid failed to yield infectious HSV-2. The ability of HCMV to reactivate HSV-2 was eliminated when HCMV was inactivated by heat prior to superinfection (Fig. 1). In cultures treated with UV-irradiated HCMV, small amounts of HSV-2 were reactivated presumably because the

* Colberg, A., Isom, H. C. & Rapp, F. (1978) *Annu. Meeting Amer. Soc. Microbiol.*, p. 220 (abstr.).

Table 1. Neutralization of virus reactivated from HCMV-superinfected cultures with rabbit anti-HSV-2 serum

Treatment	Serum dilution	No. of plaques*: HSV-2† (strain 186)	HCMV‡ (strain AD169)	Reactivated virus§
Medium		60, 66, 59, 65	38, 57, 51	31, 21, 32
Normal rabbit serum	1:10		33, 49, 42	
	1:100	77, 83, 76, 81		26, 26, 30
Rabbit anti-HSV-2 serum	1:10		34, 36, 34	
	1:100	3, 3, 0, 1		0, 0, 0

* Stock virus was diluted such that 50–100 plaques would be present in 0.2 ml of the final dilution. Each number represents the number of plaques per plate upon adsorption and incubation of 0.2 ml to the confluent indicator cells.

† After treatment with medium or with normal or immune rabbit serum, virus was assayed by plaque formation on PRK cells and overlaid with 0.5% methylcellulose.

‡ After treatment with medium or with normal or immune rabbit serum, virus was assayed by plaque formation on Flow 5000 cells and overlaid with 1% agarose.

§ Virus was harvested from cultures treated with ara-C and HSV-2 and superinfected with 0.2 PFU of HCMV (strain AD169) 19 days after infection with HSV-2. Cultures were harvested 6 days after HCMV superinfection.

Table 2. Reactivation of HSV-2 by superinfection with HCMV

Latency conditions: Days after infection with HSV-2*	Latency conditions: Days after superinfection with HCMV (AD169)	Titers of HSV-2 and HCMV after superinfection with HCMV, PFU/culture: HSV-2	HCMV†
23	4	5×10^5	1×10^4
24	5	3×10^6	2×10^4
25	6	6×10^5	2×10^4
25	6‡	$<1 \times 10^1$	—

* Cultures were treated with ara-C and infected with HSV-2.
† To obtain HCMV titers, we treated aliquots of serial 1:10 dilutions of the harvested virus with rabbit anti-HSV-2 serum (1:10) for 1 hr at 37°C and assayed them on Flow 5000 cells for the presence of HCMV.
‡ Control cultures were mock infected.

UV irradiation dose used did not completely inactivate HCMV (Fig. 1). When HCMV was irradiated at 28 erg/sec per mm^2 for 15 min, HCMV was completely inactivated. This UV-irradiated HCMV failed to induce infectious HSV-2.

To identify the reactivated virus as HSV-2, we carried out virus neutralization tests. Virus reactivated from HCMV-superinfected cultures was neutralized only by the rabbit immune serum (Table 1). HSV-2 used to immunize the rabbit was similarly neutralized. Conversely, HCMV used for superinfection was not neutralized by immune serum at 10-fold the concentration used to neutralize HSV-2.

Cultures superinfected with HCMV were titered for the presence of HSV-2 and HCMV. (Table 2). The HCMV titers obtained were not significantly different from those obtained for the growth of HCMV in HEL cells at 39.5–40°C in the absence of HSV-2 infection. Exposure of HEL cells at 39.5–40°C to HCMV at a similar moi produced 2–5 $\times 10^4$ PFU per culture 5–11 days after infection. As previously described (Fig. 1), elevated titers of HSV-2 were obtained after superinfection with HCMV.

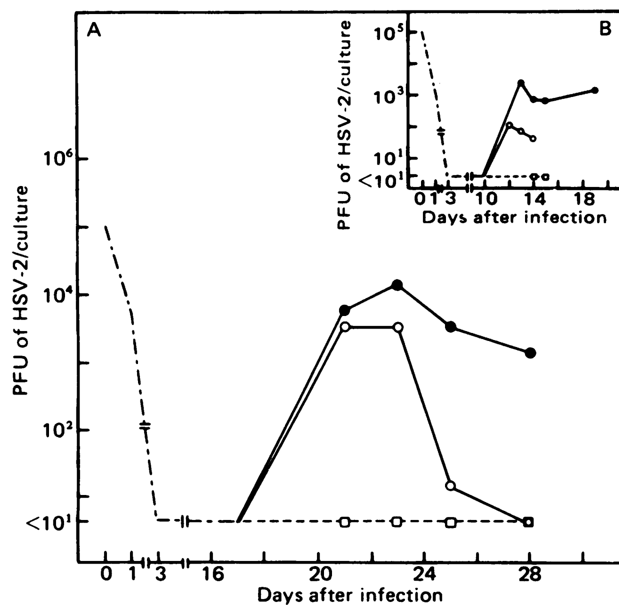

FIG. 2. Reactivation of HSV-2 from infected HEL cells at 39.5–40°C by superinfection with HCMV strain AD169 (*A*) or strain Eisenhart (*B*). Cultures were superinfected at 17 days (*A*) or 10 days (*B*) after infection at a moi of 0.2 PFU (○) or 0.02 PFU (●) of HCMV per cell. Mock-infected (□) and HCMV-superinfected cultures were harvested in parallel and assayed for the presence of HSV-2 by plaque formation in PRK cells. The curve (- - -) symbolizes establishment of the quiescent state. Virus production decreased rapidly to below detectable levels in the presence of ara-C (19, *).

Table 3. Effect of neutralization of HCMV on reactivation of HSV-2

Treatment	HSV-2 titer, PFU/culture: 4 days*	5 days*	6 days*
Normal human serum†	4×10^3	2×10^2	2×10^2
Medium†	1×10^2	1×10^2	ND
Human anti-HCMV serum†	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$
Mock infection	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$

ND, not done.
* Time after superinfection with HCMV.
† Diluted HCMV to be used for reactivation was pretreated with medium or with normal or immune human serum (1:4) at 37°C for 1 hr. HCMV treated in this fashion was then used to superinfect HSV-2-infected cultures at 39.5–40°C. Aliquots of the neutralized samples were assayed for the presence of HCMV by plaque formation on Flow 5000 cells and overlaid with 1% agarose. Titers of HCMV after the various treatments shown in this table were as follows: normal human serum, 5×10^5 PFU of HCMV per ml; medium, 2×10^5 PFU of HCMV per ml; human anti-HCMV serum, 4×10^1 PFU of HCMV per ml.

The effect of various multiplicities of HCMV on the amount of HSV-2 reactivated from the cultures was examined. Stimulation was maximal when cultures were superinfected with a moi of 0.02 PFU of HCMV per cell (Fig. 2). A rise in the level of infectious HSV-2 was apparent 3 days after superinfection and remained elevated through 11 days after superinfection (Fig. 2*A*). Stimulation was also observed when cultures were superinfected with 0.2 PFU of HCMV per cell; however, the titers of HSV-2 reactivated were not as high as those observed with 0.02 PFU of HCMV per cell and the amount of HSV-2 produced by superinfected cultures was greatly diminished 8 days after superinfection. In addition, a moi as low as 0.0002 PFU of HCMV per cell was capable of reactivating HSV-2 replication; 6 days after superinfection, 2×10^2 PFU of HSV-2 per culture were detected (data not shown). These data are consistent with results using UV-irradiated HCMV (Fig. 1). Another strain (Eisenhart) of HCMV produced results (Fig. 2*B*) strikingly similar to those obtained with strain AD169.

The specificity of HSV-2 reactivation by HCMV was corroborated by neutralization studies. HCMV pretreated with HCMV immune human serum prior to superinfection of HSV-2-infected cultures failed to induce detectable levels of infectious HSV-2 (Table 3). Pretreatment with either normal human serum or medium at 37°C for 1 hr did not inhibit the ability of HCMV to stimulate HSV-2 replication.

DISCUSSION

In this report, an *in vitro* culture system for the experimental manipulation of HSV-2 replication developed by O'Neill (19) and modified in our laboratory* was used. After ara-C removal and alteration of the incubation temperature to 39.5–40°C, infectious HSV-2 became undetectable by plaque formation in PRK cells for extended periods of time (19, *). Indeed, when HSV-2-infected cultures treated in this fashion were frozen and thawed into a small volume of medium and the total volume was placed on PRK cells, no plaques were observed (unpublished observations). Infectious HSV-2 reappeared only after shiftdown to 37°C (19, *).

Reactivation of quiescent HSV-2 at 39.5–40°C was initially attempted in our laboratory by using hormones (unpublished data). Repeated treatment of cultures with dexamethasone phosphate (100 mM) or dibutyryl cyclic AMP (0.5 mM) in the presence and absence of theophylline (0.5 mM) did not stimulate replication of HSV-2 to detectable levels at 39.5–40°C.

As this report demonstrates, HCMV, a heterologous herpesvirus, is capable of stimulating replication of HSV-2. HCMV DNA shares little or no base-sequence homology with HSV-2 DNA (22). Thus, HSV-2 production observed at 39.5–40°C would be difficult to explain simply as a result of complementation of the two herpesviruses. However, further support for the significance of interactions between herpesviruses comes from the recent findings of Y. Nishiyama and F. Rapp (unpublished observations) that demonstrate an enhancement in the survival of UV-irradiated HSV-2 in cells previously infected with HCMV.

Specificity of the HCMV reactivation of HSV-2 from its quiescent state was established in several ways. First, parallel cultures were exposed to mock-infecting fluid. By nature of its composition, such fluid would detect reactivation caused by factors present in serum, medium, or lysed cells; HSV-2 was not detected in any mock-infected culture. Second, inactivation of HCMV by various means prior to superinfection abrogated or reduced its ability to induce HSV-2 replication. The phenomenon of HSV-2 reactivation by HCMV was not strain specific; strain Eisenhart produced results virtually identical to those observed with strain AD169. Cells induced to replicate by subculture at 39.5°C in this system also failed to yield HSV-2 in the absence of HCMV.

Studies of HCMV superinfection may provide information on the state of HSV-2 at 39.5–40°C in this system. To establish the system, we used very low multiplicities of HSV-2 to infect HEL cells. This procedure avoids excessive cell death and permits retention of the HSV-2 genome (19). As indicated by O'Neill (19) and our own studies (unpublished observations) using the infectious center assay, very few cells in the population are capable of producing infectious HSV-2. Our values of infectious centers ranged from 1 in 6000 cells to below detection levels (<1 in 10^5 cells). The titers of HSV-2 produced early after superinfection with low levels of HCMV were relatively high for the incubation temperature used. These observations suggest several possibilities concerning the state of HSV-2 during this period and its reactivation. (*i*) Perhaps more cells than indicated by the infectious center assay contain the HSV-2 genome, but for some reason these are not capable of producing infectious HSV-2 without the stimulation provided by HCMV. (*ii*) Perhaps only a few cells contain the HSV-2 genome (as indicated by the infectious center assay). These are triggered by HCMV to amplify HSV-2 followed by either cell-to-cell spread of genetic information or extracellular spread of virus progeny.

Knowledge that both HSV and HCMV are ubiquitous in the human population and persist, probably for life, suggests the possibility that activation of the type reported here may occur *in vivo*. In fact, HSV induces synthesis of retrovirus gene products in rodent cells (23–26) and HCMV also appears to possess this property (23).

The ratio of particle to infectious virus counts for HCMV is large, suggesting an excess of noninfectious particles. Because very low levels of HCMV (as low as 0.0002 PFU per cell) were capable of inducing HSV-2 replication and HSV-2 was detected relatively early after HCMV superinfection, it is possible that defective HCMV virions may play a role in reactivation of HSV-2. It is already known that HCMV (27, 28) and its defectives (29) induce host cell DNA synthesis, and they may also be effective in stimulating HSV-2 production. Temperature-sensitive mutants, restricted at 39–40°C, are available in our laboratory (30, 31). One practical approach to further our understanding of the phenomenon discussed in this report would be the use of such mutants of HCMV to induce synthesis of HSV-2 in the system described.

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