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Restricted Replication of Herpes Simplex Virus in Neural Cells (39384)

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An intriguing property of the herpes simplex viruses (HSV) is their ability to establish a latent infection *in vivo*. Recent studies have demonstrated that the site of HSV persistence in animals and man is the neural elements of ganglia (1-4). During the course of latent infection the replication of the virus is restricted but an appropriate stimulus causes a shift in the host-virus relationship in the neural tissue, ultimately resulting in the replication of the virus and the recrudescence of a clinical infection (5, 6).

The efficiency of HSV replication *in vitro* varies with the host cell. For example, dog kidney cells and C6 cells fail to support (or actively restrict) the replication of HSV (7, 8). In addition, cells may respond to HSV infection with the production of interferon (7, 9, 10). Aurelian and Roizman (11) have documented the alternating suppression of the synthesis of interferon and HSV directed macromolecules during the cycle of infection in canine kidney cells. Thus, restrictive modulation of HSV replication may be the result of interferon production. This study sought to further characterize the interaction between HSV and C6 cells, which were derived from a glioma (12), specifically relating the cycle of infection to the potential cellular response of interferon synthesis.

Materials and methods. Cells and medium. The glioblastoma cell line, C6, purchased from American Type Culture Collection, was obtained from Dr. R. Grasso, University of South Florida. The rabbit skin (RS) and HeLa cell lines were obtained from Dr. L. McLaren, University of New Mexico. The composition of the medium was described in a previous report (13).

Viruses. Herpes simplex type 1 strain Seibert (HSV-1) was propagated in RS cells and concentrated by polyethylene glycol "precipitation" (14). The HSV was quantitated by plaque formation in RS cells employing an overlay which contained 0.5% methyl cellulose (15). Newcastle disease virus (NDV) was propagated in fertile hen's eggs and quantitated by plaque formation (13). Vesicular stomatitis virus (VSV) was propagated in and quantitated by plaque formation in C6 cells employing an overlay medium which contained 0.5% Noble agar.

Total HSV-1 content. Cell monolayers, infected at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (PFU) per cell, were frozen (−85°) at the times indicated for the different experiments. The samples were then thawed, duplicates were pooled, and the cells were removed by centrifugation (800 *g*; 8 min). Cells were resuspended in 1 ml of the supernatant fluid and sonicated for 30 sec (20,000 cps). The remainder of the supernatant fluid was then added and the virus content was determined.

Assay for poliovirus in the presence of HSV-1. Monolayers which were infected with HSV-1 and superinfected with polio RNA were frozen (−85°) at the times indicated for each experiment, and subsequently thawed. Trition X-100 was added to the mixture of medium and cells to a final concentration of 0.5% which disrupted the cells, released the poliovirus, and inactivated the HSV-1. Poliovirus was quantitated by plaque assay on HeLa cells (16).

Interferon determinations. Medium alone or medium containing sonicated infected cells was acidified to pH 2, incubated at 4° overnight, and then neutralized (17). Dilutions of these samples were prepared in fresh medium and incubated with cells overnight. These monolayers were then challenged with approximately 100 PFU of VSV. The next day the monolayers were stained (18) and the plaques were counted. One unit of interferon was defined as the reciprocal of the sample dilution which resulted in a 50% decrement in plaque number relative to controls.

Interferon preparation. Interferon was prepared by the NDV infection (m.o.i. = 10) of C6 cells. The medium was decanted at 24 hr postinfection (hpi), acidified, and subsequently assayed for interferon activity as described above.

Polio RNA preparation. Infected HeLa cells were frozen, thawed, and then sonically disrupted after infection by poliovirus. Cellular material was removed by centrifugation (800 *g*; 8 min) and the virus was pelleted by centrifugation (120,000 *g*; 90 min). The virus was resuspended in 0.02 *M* phosphate buffer, pH 7.1, and passed through a 9.5 × 0.8-cm column of DEAE cellulose previously equilibrated with the same buffer. Fractions containing poliovirus were pooled and sodium dodecyl sulfate was added to a final concentration of 0.25%. Predigested protease (Sigma Chemical) was added (100 μg/ml final concentration) and the sample was incubated at 37° for 30 min. The sample was then extracted twice with equal volumes of phenol, twice with equal volumes of chloroform, and once with twice the sample volume of ether. The sample was then dialyzed against 1 liter of phosphate buffered saline (PBS) for 24 hr at 4°.

Polio RNA infection. Cells to be infected were washed three times with Hank's balanced salt solution (BSS; 19) and incubated for 10 min with 1 ml of PBS containing 500 μg DEAE dextran. The PBS was decanted, the cells were exposed to the polio RNA for 15 min, and then the original tissue culture medium was replaced. Determination of the infectivity of the polio RNA preparation was achieved by plaque assay on HeLa cells in which the final medium added contained 0.4% ion agar and 30 m*M* $MgCl_2$. Incubation of polio RNA samples with ribonuclease (10 μg; 20 min) reduced the infectivity of the preparation to less than 10 PFU/ml.

Results. The temporal aspects of a single cycle of HSV-1 replication in C6 and RS cells were examined. A comparison of these replication cycles revealed the inefficient production of HSV-1 in C6 cells as shown in Fig. 1. The productive cycle of infection in RS cells indicated a 4-hr eclipse phase followed by the rapid synthesis of virus. By contrast, the eclipse phase noted in C6 cells was prolonged, through 12 hpi and was followed by an increase in HSV-1 levels. In addition, the adsorption of HSV-1 to both C6 and RS cells was equivalent as determined by the quantity of virus per 10^6 cells immediately following the adsorption period (Fig. 1, 0 hr). Thus, the low virus yield in C6 cells (1 PFU/cell) compared with 420 PFU/RS cell at 48 hpi reflected the restrictive nature of the HSV-C6 interaction.

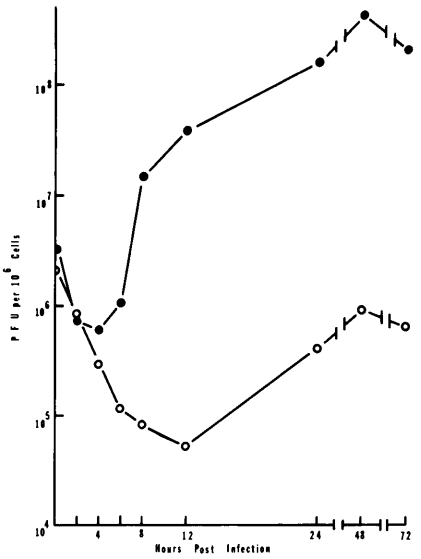

FIG. 1. Replication of HSV-1 in RS and C6 cells. Monolayers were infected with HSV-1 (m.o.i. = 10) as described above. At the times indicated, duplicate samples were frozen (−85°). All samples were subsequently processed as described in the Methods and the total virus content was determined (15). ●- - - -●, RS cells; ○- - - -○, C6 cells.

Since the production of interferon has been previously shown to suppress HSV directed biosynthetic metabolism (11), the relationship between interferon production and HSV-1 infection of C6 cells was examined. Medium from HSV-1 infected C6 cells as well as medium containing the total intracellular contents were assayed for interferon. In each instance, no interferon activity could be demonstrated in any of the samples of HSV-1 infected C6 cells tested (Table I). However, the infection of cells with NDV did result in the production of interferon. These data indicated C6 cells have the genetic capability to synthesize this antiviral protein, but they either failed to produce interferon in response to HSV-1 infection or synthesized quantities which

TABLE I. INTERFERON CONTENT IN MEDIUM AND INFECTED C6 CELLS.[a]

Hours postinfection	Interferon titer (units/ml) Cells infected with: HSV-1	NDV
0, 2, 4, 6, 8, 12, 24, 48, 72	<5	
24		300

[a] Monolayers of C6 cells were infected with HSV-1 or NDV (m.o.i. = 10). At the times indicated the interferon content in the mixture of cells and medium was determined as described in the Methods.

could not be detected by the assay employed.

If low levels of interferon were responsible for restricting the replication of HSV-1 in C6 cells, then these cells might be expected to be refractory to superinfection by a second virus. This possibility was tested by monitoring the replication of infectious polio RNA which was used to superinfect C6 cells at different times post HSV-1 infection. Preliminary experiments indicated treatment of uninfected C6 cells with 5 units/ml of C6 interferon resulted in a 95% decrease in poliovirus production (data not shown). The results indicated that poliovirus synthesis was not appreciably inhibited through 10 hpi (Table II). However, by 24 hpi the decrement in poliovirus production in HSV-1 infected C6 cells was equivalent to that observed by 3.5 hpi in RS cells. While the levels of restriction of polio RNA replication were comparable, the time post HSV-1 infection when the restriction was manifested differed with the host cells.

The physiologically restrictive state of the C6 cells was further examined by determining the effect of exogenously added interferon on the replication of HSV-1. Monolayers received interferon before or immediately after HSV-1 adsorption and the total HSV-1 content was determined at 24 hpi. The results indicated 5 units/ml of C6 interferon had no effect on the yield of HSV-1 even when added 19 hr before the infection of the cells (Table III). Pretreating the C6 cells with 50 units/ml of interferon did result in a substantial decrement in HSV-1 yield. However, 50 units/ml of interferon failed to inhibit HSV-1 replication when it was added immediately after virus adsorption.

TABLE II. EFFECT OF HSV-1 INFECTION ON THE REPLICATION OF POLIO RNA IN C6 AND RS CELLS.[a]

Cell line	Time post HSV-1 adsorption of polio RNA superinfection (hr)	Poliovirus yield (% 0-hr yield)
C6	0	100[b]
	4	134
	10	67
	24	4
RS	0	100[c]
	3.5	9
	6.5	2

[a] Monolayers were exposed to HSV-1 (m.o.i. = 10) and virus adsorption proceeded for 2 hr at room temperature. At the times indicated, the monolayers were washed three times with BSS and superinfected with polio RNA as described in the Methods. At 24 hr post polio RNA infection, the cells were lysed with Triton X-100 and total poliovirus content was determined (16).

[b] 100% = 2.5×10^5 PFU/ml.

[c] 100% = 2.5×10^4 PFU/ml.

TABLE III. EFFECT OF EXOGENOUSLY ADDED INTERFERON ON THE YIELD OF HSV-1 IN C6 CELLS.[a]

Time of interferon addition (hpa)[b]	Interferon concentration (units/ml)	HSV-1 yield (% of control)
Control	None	100[c]
0	5	93
−19	5	98
Control	None	100[d]
0	50	135
−19	50	16

[a] Monolayers were divided into three groups. One group received interferon 19 hr before (−19) as well as immediately after HSV-1 adsorption. The second group received interferon only after HSV-1 adsorption (0), and the third group received no interferon (control). The cells were infected with HSV-1 (m.o.i. = 10) and at 24 hpi, the monolayers were processed as described in the Methods, and the total HSV-1 content was determined (15).

[b] hpa: hr postadsorption.

[c] 100% = 2.5×10^6 PFU/10^6 cells.

[d] 100% = 5.6×10^6 PFU/10^6 cells.

Discussion. The restrictive nature of the HSV-1 C6 cell interaction was evident from the diminished replicative capability of the virus in these cells. The maximum HSV-1 yield in C6 cells at 48 hpi was equal to or slightly below the quantity of virus which was present immediately following virus adsorption (0 hpi). These observations differ from the results in a previous study which reported a substantially lower quantity of HSV-1 in C6 cells at 48 hpi (8). The reason

for this difference is not obvious and may be due to variation in the HSV-1 strains employed in the two studies. However, the equivalence of HSV-1 adsorption to both C6 and RS cells indicated that the restrictive mechanism(s) regulating HSV-1 replication in C6 cells occurred at the intracellular level.

The infection of cells by HSV generally does not result in the production of large quantities of interferon (20). In addition, the production of interferon has been shown to be multiplicity dependent, with maximum production occurring at a m.o.i. of 10 (11). However, no interferon activity was observed in the composite of cells and media from HSV-1 infected C6 cells even though the cells have the genetic capability to produce interferon, the m.o.i. employed was 10, and a sensitive challenge virus (VSV) was used in the interferon assay.

Polio RNA was employed as a probe to determine whether HSV-1 infected C6 cells established a state of viral refractoriness in the absence of producing readily detectable quantities of interferon. This state might be rapidly achieved since interferon synthesis occurs shortly following the transcription of interferon messenger RNA (21, 22). Thus, the slight inhibition of polio virus yield when HSV-1 infected C6 cells were superinfected at 10 hpi may be a consequence of the establishment of an interferon-mediated viral refractory state. However, the data indicated comparable decrements in poliovirus yield were observed in C6 and RS cells superinfected at 24 and 3.5 hr post HSV-1 infection, respectively. In addition, the RS cells support a productive cycle of HSV-1 replication, with no evidence of interferon production (data not shown). Thus, the inhibition of poliovirus replication was probably the result of HSV directed interference because the inhibition was manifest during the respective eclipse phases in C6 and RS cells, which was the time previously reported to be associated with HSV interference of poliovirus replication (23). These data further reflect upon the inefficient nature of the HSV-1 C6 interaction as the HSV-1 were unable by 10 hpi to establish control of the biosynthetic processes in these cells which would preclude the complete synthesis of poliovirus.

The pretreatment of C6 cells with 50 units/ml of interferon demonstrated the susceptibility of the HSV-1 to the antiviral protein. The unrestricted replication of the HSV-1 in the presence of 50 units/ml of interferon added after virus adsorption reflected the relative resistance of this virus to interferon as well as the importance of the temporal relationship between the addition of exogenous interferon and the development of the refractory state to HSV-1. However, if interferon were produced in HSV-1 infected C6 cells, the level must be less than 5 units/ml. This amount of interferon did not inhibit the yield of HSV-1 in C6 cells even when the cells were pretreated with 5 units/ml of interferon 19 hr before HSV-1 infection. Thus, the data presented in this report suggest that interferon is not responsible for the restrictive replication of HSV-1 in C6 cells. The nature of the biochemical events associated with the restricted cycle of HSV-1 infection of C6 cells is currently under investigation.

Summary. The relationship between the replication of HSV-1 in C6 cells and an interferon-mediated state of viral refractoriness was studied. The cycles of HSV-1 replication in RS and C6 cells reflected the restrictive nature of the HSV-1 C6 interaction and indicated that the restrictive event(s) occurred intracellularly. However, interferon activity could not be demonstrated in the composite of medium mixed with the total intracellular contents from HSV-1 infected C6 cells from 0 through 72 hpi. The lack of a viral refractory state was also reflected in only a slight inhibition at 10 hpi of the replication of poliovirus RNA which was used to superinfect HSV-1 infected C6 cells. Furthermore, the exogenous addition of 5 units/ml of interferon supplied either before or after HSV-1 adsorption as well as 50 units/ml added after virus adsorption failed to affect the production HSV-1 in C6 cells. These observations suggest that interferon is not mediating the restrictive replication of HSV-1 in C6 cells.

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