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Use of [^{125}I]Deoxycytidine to Detect Herpes Simplex Virus-Specific Thymidine Kinase in Tissues of Latently Infected Guinea Pigs

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The footpad skin and the lumbosacral dorsal root ganglia were removed from inbred guinea pigs at different times after subcutaneous infection with herpes simplex virus type 2 (HSV-2) in both hind footpads. These tissues, shown by our previous study to harbor latent HSV, were dispersed into single cells. The presence of virus-specific thymidine kinase (TK) in these cells was assayed by the uptake and phosphorylation of [^{125}I]deoxycytidine in culture. [^{125}I]deoxycytidine was shown to be a specific substrate for the HSV-coded TK. The method could detect herpes TK activity in a culture of 10^6 cells with less than 0.1% of the cells being virally infected. The enzyme was readily detected in footpad cells of acutely (24 h) but not of latently (14 days to 1 year) infected guinea pigs. No herpes TK was found either in the sensory ganglionic cells of guinea pigs during the early and late phases of latent infection. It is concluded that HSV-2, while residing in the footpads and the lumbosacral ganglia of the guinea pig during latent infection, does not express any viral TK function.

Latent herpes simplex virus (HSV) infection is found to be associated with the nervous system in humans (1-3) and in experimentally infected animals (7, 8, 10, 11, 13, 14). Herpes DNA can be detected in the ganglionic neurons of mice (9) during the latent infection by DNA-DNA hybridization. It remains, however, unclear whether any virus-specific functions are being expressed during latent infection. Thus, the same authors were not able to detect any virus-specific RNA in the latently infected mouse ganglia by reassociation kinetics. In contrast, Galloway et al. demonstrated the presence of RNA in human sensory ganglia by in situ hybridization, using nick-translated ^{3}H-labeled viral DNA as probe (5). Of the virus-specific proteins, viral thymidine kinase (TK) has been reported to be detectable in latently infected mouse ganglia, but only for up to 8 weeks post-infection (17).

In the present study, we examined the synthesis of the herpes TK in guinea pigs latently infected with HSV type 2 (HSV-2). Unlike the murine and rabbit systems in which latent virus resides exclusively in the nervous tissue, the guinea pig has been shown to harbor the virus not only in the sensory ganglia, but also in the footpad at the site of the primary infection (12). Both the guinea pig footpad and ganglia were therefore investigated for the presence of herpes TK by the selective uptake and phosphorylation of [^{125}I]deoxycytidine ([^{125}I]dC) in culture.

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MATERIALS AND METHODS

Synthesis of [^{125}I]dC. [^{125}I]dC was synthesized by a modification of the procedure described by Summers and Summers (15). A 1-mCi amount of Na^{125}I (Amersham Corp.) was mixed with a freshly prepared solution containing 23 μg of deoxycytosine (Serva), 20 μg of thallium trichloride (Merck & Co., Inc.), and 0.2 mM potassium iodide. The reaction mixture was adjusted to pH 5 with glacial acetic acid and incubated at 55°C for 2 h. A 25-μl amount of a 2% Na_2SO_3 solution and 3 μl of concentrated NH_4OH were added to stop the reaction. [^{125}I]dC was separated from other products by paper chromatography in 50% methanol and 1% NH_4OH, eluted from the paper with water, further purified by chromatography on a small column of DEAE (Whatman DE 52), equilibrated, and eluted with 0.2 M NH_4HCO_3. The first peak of radioactivity eluted from the column was [^{125}I]dC. Purified [^{125}I]dC was stored at 4°C and used within 2 weeks.

Cells and viruses. Primary guinea pig fibroblasts (GPF) were prepared from guinea pig fetuses and grown on minimum essential medium (MEM) containing antibiotics and 10% fetal calf serum. HSV-2, strain 72, was employed in the present study. Virus stock was prepared in primary rabbit kidney (PRK) cells and quantitated by plaque assay, using a liquid overlay medium containing anti-HSV hyperimmune serum.

To isolate HSV-2 mutants lacking in TK, the wild-type virus was grown in the presence of 5 μg of bromodeoxyuridine per ml. Virus resistant to bromodeoxyuridine was selected and plaque purified. These virus mutants were ascertained to be negative in TK by two criteria. First, the mutants could grow in the

presence of 5 μg of bromodeoxycytidine per ml. Second, cell lysates were prepared from GPF infected with the TK-negative mutants and were assayed for viral TK activity directly. There was no detectable virus-specific TK.

Infection of cells and animals. Confluent cultures of GPF containing 10^6 cells per culture were infected with 0.2 ml of HSV-2 virus suspension in Hanks medium at various multiplicities of infection ranging from 1 to 10^{-5}. After 30 min at room temperature, the virus inoculum was removed and the cells were washed three times with phosphate-buffered saline. One milliliter of MEM containing 5% fetal calf serum was added, and the cells were incubated at 36°C.

Inbred guinea pigs, strain 2, were inoculated subcutaneously in both hind footpads with 10^4 PFU of HSV-2 in 0.2 ml of Hanks solution.

Infectious center assay. Monolayer cultures of GPF (10^6 cells per culture) infected with HSV at various multiplicities of infection were dispersed into single cells with 0.02% EDTA at 36°C. The cells were washed and suspended finally in MEM containing 10% fetal calf serum and 0.1% anti-HSV hyperimmune serum. Serial dilutions of this cell suspension were made in the same medium. One milliliter of the original or diluted cell suspensions was inoculated onto monolayers of Vero cells. After adsorption for 4 h at 36°C, 2 ml of MEM containing 0.1% anti-HSV hyperimmune serum was added. The cultures were further incubated for 2 days at 36°C before staining with carbolfuchsin (Merck) and counting of the HSV plaques.

Preparation of cells from guinea pig footpads and ganglia. Animals were sacrificed at different times after infection, and the skin of the hind footpads and the dorsal root ganglia of the fifth lumbar to the third sacral segment were removed. The tissues were finely minced and treated with 0.2% collagenase at 36°C to produce single-cell suspensions. The dispersed cells were washed well with phosphate-buffered saline to remove the collagenase. Cells (10^6) were dispensed into each Falcon culture tube and were maintained at 36°C in 1 ml of MEM containing antibiotics and 5% fetal calf serum.

Uptake and phosphorylation of [^{125}I]dC. [^{125}I]dC (10^6 cpm) and 100 μg of tetrahydrouridine (kindly supplied by Harry B. Wood, Jr., National Cancer Institute, Bethesda, Md.) were added to a culture containing 10^6 cells. After incubation at 36°C for indicated time periods, the cells were scraped into the medium and pelleted by centrifugation. The cell pellet was washed three times with phosphate-buffered saline by suspension and centrifugation to remove any residual medium. [^{125}I]dC radioactivity in the washed cell pellet was determined by counting in an automatic gamma counter and represented the total [^{125}I]dC uptake by the cells.

Phosphorylated [^{125}I]dC was determined in the trichloroacetic acid (CCl_3COOH)-precipitable and in CCl_3COOH-soluble material. Therefore, the cell pellet was washed twice with cold 5% CCl_3COOH, and the precipitate was counted in the gamma counter. To measure the free form of phosphorylated [^{125}I]dC, 40-μl portions of the pooled CCl_3COOH-soluble material were spotted in duplicate on separate DEAE-cellulose disks (Whatman DE 81; 2.4 cm in diameter). The disks were air dried and washed three times with 4 mM ammonium formate, once in distilled water, and once in ethanol and were dried and counted in a gamma counter. The sum of the radioactivity in the CCl_3COOH precipitate and that remaining on the cellulose disks after washing measured the total amount of [^{125}I]dC phosphorylated by the viral TK in the infected cells.

RESULTS

Specificity of [^{125}I]dC for herpes TK. In an attempt to ascertain that [^{125}I]dC is a specific substrate for the herpes TK and not for the cellular TK, the utilization of [^{125}I]dC by GPF cultures infected with either the wild-type or the TK-negative HSV-2 were examined. The cells were infected at a multiplicity of infection of one with the wild-type or mutant virus. At different times after infection, 10^6 cpm of [^{125}I]dC and 100 μg of tetrahydrouridine were added to the medium, and the cultures were incubated at 36°C for 1 h. The uptake and phosphorylation of [^{125}I]dC by the cells were measured as described above. Uninfected cells served as the control. In this case, less than 0.1% of the [^{125}I]dC added was taken up into the cells and no [^{125}I]dC radioactivity was detected in the CCl_3COOH-precipitable material or on the Whatman DE 81 cellulose disk after washing.

Cells infected with wild-type HSV-2, however, exhibited a detectable increase in the uptake of [^{125}I]dC by 2 to 3 h postinfection (Table 1). About one-third of the [^{125}I]dC taken up at this time was found to be phosphorylated, being free or incorporated into CCl_3COOH-precipitable material of the infected cell. [^{125}I]dC uptake and phosphorylation were observed to be highest at 14 to 15 h after infection by HSV-2. In contrast, cells infected with the TK-negative mutant showed extremely low uptake and no phosphorylation of [^{125}I]dC, at a time when maximum

TABLE 1. *[^{125}I]dC uptake and phosphorylation by GPF infected with wild-type or TK-negative HSV*

Virus	Time after infection (h)	[^{125}I]dC (cpm × 10^{-3})[a]	
		Uptake	Phosphorylation
Wild type	1-2	1.0	0
	2-3	5.9	1.7
	7-8	46.6	14.7
	14-15	167.0	71.7
	22-23	155.3	77.9
TK$^-$ mutant	14-15	2.4	0

[a] Counts per minute obtained above uninfected control.

TK activity was found to occur in cells infected with the wild-type virus. We therefore conclude that [^{125}I]dC can be used to measure specifically the activity of HSV-coded TK. The uptake of [^{125}I]dC itself seems to depend on the presence of a functioning TK. Similar observations that an increase of thymidine transport into the cells is correlated with the induction of a virus-coded TK have also been reported by others (4, 6).

Sensitivity of [^{125}I]dC assay. To determine the sensitivity of the present herpes TK assay, confluent GPF (10^6 cells per culture) were infected at various multiplicities of infection with HSV-2. The cells were washed with phosphate-buffered saline to remove unattached virus and incubated at 36°C for 14 h before [^{125}I]dC labeling. Parallel unlabeled cultures were set up for the infectious center assay so as to determine the number of virus-infected cells in the culture at the time of [^{125}I]dC labeling. The results are shown in Table 2. It was observed that with 300 virus-infected cells in a culture containing a total of 10^6 cells, the level of [^{125}I]dC uptake and phosphorylation was still significantly higher than that of uninfected control.

Presence of herpes TK in infected guinea pig footpad skin. [^{125}I]dC was used in subsequent experiments to detect herpes-coded TK in cells obtained from skin and ganglia of guinea pigs acutely and latently infected with HSV-2. Earlier studies on the HSV-guinea pig model (11) have shown that the titer of virus in the footpads is highest 24 h after infection and declines by day 11 to a level undetectable by plaque assay. The virus, however, persists in both the footpads and the dorsal root ganglia of these infected animals and can be recovered by cocultivation of the footpad and ganglia explants with PRK cells.

Guinea pigs inoculated in each hind footpad with 10^4 PFU of HSV-2 were therefore sacrificed at 24 h, 2 weeks, and 1 year after infection. The latter animals had all shown previous recurrent herpes infections but not at the time when the experiment was performed. Two animals were employed for each group, and the hind footpads were pooled for preparing single-cell suspensions as described above. The uptake and phosphorylation of [^{125}I]dC by these cells were determined as an indication for the presence of the viral TK.

Figure 1a and b shows the results of herpes TK activity found in infected guinea pig footpads. Cells from the footpads of animals infected for 24 h exhibited a significantly higher level of [^{125}I]dC uptake and phosphorylation as compared to the control cells from uninfected animals. Whereas the uptake of [^{125}I]dC by the former (24-h-infected) cells leveled off after 1 h,

TABLE 2. *Sensitivity of [^{125}I]dC assay*

Multiplicity of infection	No. of infected cells[a]	[^{125}I]dC (cpm)[b]	
		Uptake	Phosphorylation
1	10^5	9.5×10^4	1.4×10^4
10^{-1}	10^4	6.7×10^4	1.5×10^4
10^{-2}	4×10^4 (4%)[c]	1.4×10^4	3.5×10^3
10^{-3}	4×10^3 (0.4%)	2.6×10^3	6.5×10^2
10^{-4}	3×10^2 (0.03%)	2.5×10^2	1.0×10^2
10^{-5}	0	0	0

[a] Determined by infectious center assay.
[b] Counts per minute obtained above uninfected control.
[c] Number of infected cells as a percentage of the total cells in culture.

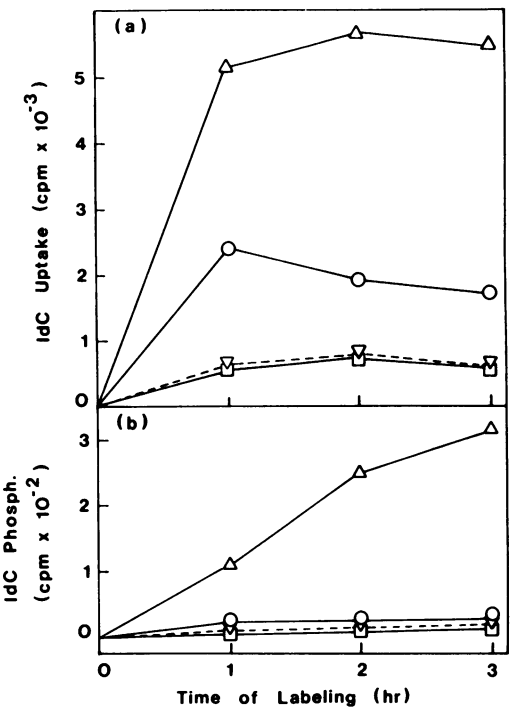

FIG. 1. *[^{125}I]dC uptake (a) and phosphorylation (b) by guinea pig footpad cells in culture. Cells (10^6) in 1 ml of MEM were incubated with 10^6 cpm of [^{125}I]dC at 36°C for 1 to 3 h. Each point represents the average of duplicate experiments. ▽, Uninfected; △, 24-h-infected; ○, 14-day-infected; and □, 1-year-infected guinea pig footpads.*

the amount of phosphorylation was found to increase with increasing time of incubation. In footpad cells from animals infected for 14 days, [^{125}I]dC uptake was also significantly higher than in uninfected controls, but the level of phosphorylation was not demonstrably different. It has been observed, however, that an increase in [^{125}I]dC uptake correlated with the presence of a viral TK (Table 1). Hence, one could infer that a small amount of the enzyme

probably still existed in the guinea pig footpads 2 weeks after infection. This might represent the residual activity of viral TK synthesized during the acute phase of the infection when the virus was multiplying in the footpad.

No viral TK could indeed be detected in the footpad cells from animals latently infected for 1 year (Fig. 1). To be sure that the latter observation was not due to an absence of the virus in the footpad cells, attempts were made to activate the latent virus and to follow the synthesis of the viral TK. The footpad cells were therefore cultivated for various lengths of time at 36°C before labeling with [^{125}I]dC. Figure 2a and b shows that the viral TK activity began to appear in these footpad cells between the first and second day in culture and continued to increase with further incubation. This increase in TK activity was accompanied by an obvious cytopathic effect in the culture. It was observed that [^{125}I]dC uptake, but not phosphorylation by uninfected footpad cells, also increased with time in culture. This could be due to an increase in the number of cells upon cultivation.

Presence of herpes TK in infected guinea pig ganglia. Similar [^{125}I]dC labeling experiments were carried out with the lumbosacral sensory ganglia obtained from guinea pigs at the early (14 days) and late (1 year) phases of latent infection. Thirty ganglia pooled from five animals were employed for each study. No difference in [^{125}I]dC uptake and phosphorylation was observed between the ganglionic cell cultures from latently infected animals and from uninfected controls (Fig. 3a and b). Incubating the cultures at 36°C for a period of 6 days before [^{125}I]dC labeling failed in this case to induce virus multiplication and viral TK synthesis (data not shown). Attempts were made to maintain the ganglionic cells in culture for a longer period of time to activate the virus. A large percentage of the cells, however, began to degenerate after 6 days at 36°C, and the experiment was discontinued.

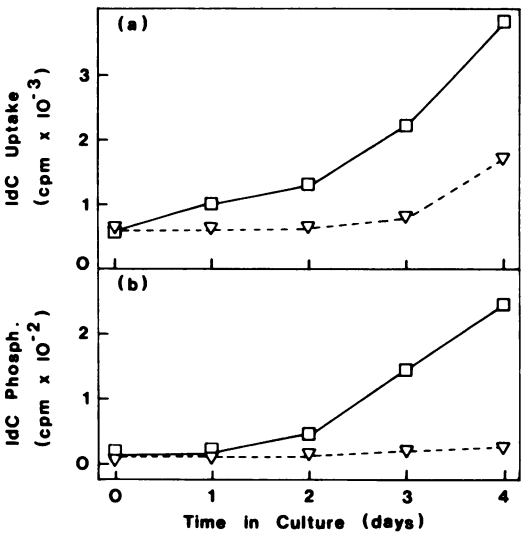

FIG. 2. *[^{125}I]dC uptake (a) and phosphorylation (b) by footpad cells of latently infected guinea pigs after virus activation. The footpad cells were incubated at 36°C for 1 to 4 days to activate virus growth before labeling with [^{125}I]dC for 1 h. Each point represents the average of duplicate experiments. ▽, Uninfected; and □, 1-year-infected guinea pig footpads.*

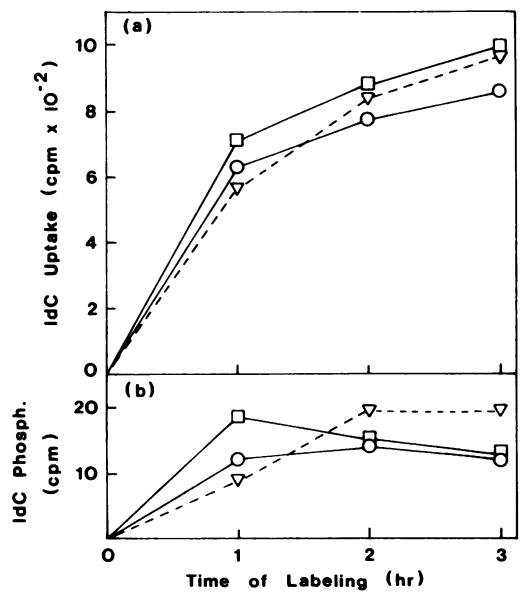

FIG. 3. *[^{125}I]dC uptake (a) and phosphorylation (b) by guinea pig ganglionic cells in culture. Each point represents the average of duplicate experiments. ▽, Uninfected; ○, 14-day-infected; and □, 1-year-infected guinea pig ganglia.*

DISCUSSION

Not much is known about the state and activity of the HSV within the ganglia during latent infection. This type of infection cannot be detected by any direct assay, such as immunofluorescence, electron microscopy, or tests for infectivity, but is only demonstrated after cultivation of the ganglia in vitro (13, 14) or by assays for viral DNA (9). It is therefore assumed that HSV persists in ganglia in a latent, nonreplicating state. If any viral functions were expressed during such latent infection, it would most likely be one or several of the early proteins.

One of the well-studied and easily detectable early viral proteins is the HSV-coded TK (4, 6). This enzyme was found to be synthesized in GPF as early as 2 to 3 h after infection by our

present assay measuring the uptake and phosphorylation of [^{125}I]dC in the infected cells (Table 1). In addition, we have shown that the sensitivity of this assay was such that it could detect HSV-coded TK activity in a culture of 10^6 cells with 0.03% of its cells being virally infected (Table 2). Any method employed in examining the expression of a virus function in latently infected tissue would indeed be required to be highly sensitive as the virus is found in only a small fraction of the cells. Walz et al. (16) have estimated by infectious center assay that in mice latently infected with HSV-1, approximately 0.1% of the cells in the ganglia harbor the virus. If a similar situation were to exist in HSV-2-infected guinea pigs and if the infected cells were synthesizing viral TK, the latter would be readily detected by our assay.

The [^{125}I]dC assay was therefore applied to measure HSV-coded TK in latently infected guinea pigs in the present study. Our data showed, however, that no viral TK was detectable by this test in the cells of the animals' ganglia or footpad skin, where the virus is known to persist (11, 12). Maintenance of footpad skin cells in vitro led, however, to detectable levels of viral TK activity within 1 to 2 days, followed by development of a cytopathic effect. No such effect could be observed in ganglionic cell cultures for up to 6 days. These findings are in accordance with our observation that infectious virus can be recovered from explants of footpad skin of latently infected guinea pigs within 2 to 7 days, whereas reactivation in ganglia explants takes an average of 2 weeks (unpublished data). In contrast to ganglia explants, suspended ganglionic cells did not remain in a healthy state for such a period of time under our cultural conditions. Therefore, we could not ascertain that these cultures harbored reactivable virus. We can estimate, however, that two to three of the five animals whose ganglia were pooled in the present experiments should have carried the latent infection, since usually about 50% of infected guinea pigs harbor latent HSV in their ganglia (12).

The negative findings in ganglionic cells, assayed immediately or after cultivation, could be taken to mean that the enzyme is not present in latently infected ganglia and, thus, to support the hypothesis that the virus resides there in a nonreplicating state. Alternatively, it could suggest that HSV-2 latency in guinea pig ganglia involves a much smaller fraction of infected cells than that reported for HSV-1 in mice. The level of viral TK expressed in these cells might consequently be too low to be detected by the present assay.

The early induction of TK activity and production of infectious virus in the skin cells, on the other hand, might be due to a larger fraction of latently infected cells as compared to ganglia. Alternatively, the virus might be maintained in the peripheral tissue in a different state, possibly in a replicating form. The level of this productive infection would then be too low to be detectable by any direct assay, including the TK assay. If the latter hypothesis is true, this would mean that fewer than 1 in 3,000 cells within the footpad skin harbors HSV during the clinically quiescent infection. Whereas virus replication is effectively restricted in vivo, probably by the host's immune response, it rapidly increases after explantation of the tissue or suspended cells in vitro.

The possibility that HSV persists in different states within nervous and extraneural tissues will be further investigated.

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