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Experimental HSV Latency Using Phosphonoacetic Acid (40655)[1]

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Herpes viruses characteristically produce diseases with recurrent manifestations. During the asymptomatic period, between initial infection with herpes simplex virus (HSV) and recurrent manifestation, it is hypothesized that the virus is maintained in a "latent" state (1, 2). The cell–virus interactions involved in latency and reactivation have not been studied extensively due to the lack of appropriate *in vitro* models. Some HSV persistence and latency models developed thus far include the addition of herpes virus-specific antibodies to the growth medium of HSV-infected cells (3–5), drastic alterations in incubation temperature of HSV-infected cells (6, 7), and use of cells that are semipermissive for HSV replication (8, 9). Recently, cytosine arabinoside (ara-C), an inhibitor of DNA synthesis, was utilized by O'Neill and co-workers to inhibit the replication of HSV type 2 (HSV-2) over a 7-day period (10, 11). Inhibition of HSV replication by ara-C during this period not only produced an interval wherein infectious virus was undetectable, but a period of 5–11 days after the removal of the inhibitor was required for the reappearance of infectious virus. Infectious virus remained undetectable when infected cultures were shifted from 37 to 40° at the time of ara-C removal and were maintained at this temperature. If, however, the infected cultures maintained at 40° were again placed at 37°, infectious virus reappeared 11–16 days after temperature shift down (10).

Our intentions were to study some of the requirements for establishment of latency by determining whether inhibition of virus DNA synthesis was sufficient to produce the latent state or whether inhibition of cellular DNA synthesis was required. With this goal in mind, we selected phosphonoacetic acid (PAA), a preferential inhibitor of HSV DNA synthesis, for our latency studies. HSV previously has been shown to be sensitive to PAA inhibition (12, 13) at concentrations as low as 20–40 μg of PAA/ml when low multiplicities of infection (MOI) are used. This paper describes the successful induction of a latent period in cultured human cells using PAA.

Materials and methods. Experiments were performed on monolayers of human embryo lung (HEL) cells grown to confluence in Falcon T-25 flasks in Dulbecco's medium supplemented with 10% fetal calf serum (FCS; Flow Laboratories, Rockville, Md.), 0.03% glutamine, 0.075% sodium bicarbonate, 25 U penicillin/ml, 25 μg streptomycin/ml, and 100 μg kanamycin/ml. Maintenance medium contained 2% FCS, 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 10% FCS, 10% tryptose phosphate broth, 0.075% sodium bicarbonate, 100 U penicillin/ml, and 100 μg streptomycin/ml. Stocks of HSV-2 (strain 186) were grown in Flow 5000 cells. Confluent monolayers were infected at an MOI of 0.01 plaque-forming unit (PFU)/cell and infected cells were harvested when 90% of the cells showed cytopathic effects (CPE). Virus was obtained by freezing and thawing infected cells three times and eliminating cell debris by centrifugation at 800 *g*. All virus stocks were maintained at −70° and titered on primary rabbit kidney (PRK) cells overlaid with 0.5% methylcellulose (14). PAA (ICN Pharmaceuticals, Plainview, N.Y.) and the disodium salt of phosphonoacetate hydrate (Abbott Laboratories, Ill.) were used as DNA synthesis inhibitors. Latency experiments were carried out as previously described (10) with the following modifications:

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PAA was substituted for ara-C during drug treatment and virus titers were determined by plaque formation on PRK cells overlaid with 0.5% methylcellulose.

Results. When HEL cells were pretreated with 100 μg/ml of PAA, virus replication was delayed, but not inhibited, over a 7-day period demonstrating that the concentration of PAA used was not sufficient to inhibit HSV-2. Low levels of resistant virus (0.07%) present in unselected virus stocks were responsible for the virus replication observed in the presence of PAA. When HEL cells were infected in the presence of 100 μg PAA/ml for 3 or 5 days (PAA replaced daily), and the lysates were subjected to plaque titration in the presence and absence of PAA (100 μg/ml), the level of PAA-resistant virus present was 18–28% representing a 250- to 400-fold increase in the amount of resistant virus present in the original HSV-2 stock.

Drug dose studies were carried out to determine the concentration of PAA required to inhibit replication of low levels of PAA-resistant virus within original stocks. Results showed that a minimum of 200 μg PAA/ml was required to reduce infectious HSV-2 below detectable levels for 7 days.

We have shown previously that 25 μg/ml of ara-C will produce a latent period in HEL cells infected with HSV-2 strain 186 (15) similar to that observed with HSV-2 strain 316-D (10, 11). Although results were similar, the time of virus reappearance after ara-C removal differed in our system. When cultures were maintained at 37°, virus reappeared 4–30 days after ara-C removal. O'Neill and co-workers (10, 11), however, reported the reappearance of infectious virus 5–11 days after reversal of ara-C treatment. In addition, when cells were maintained at 40° and then shifted down to 37°, a shorter period (5 days after shift down) than that previously reported (11–16 days after shift down (10)) was observed.

To induce latency, twice the minimum concentration of PAA required for inhibition of HSV-2 (186) was used. Two separate experiments produced results similar to those observed with ara-C treatment. HEL cells pretreated with PAA (400 μg/ml) and infected with HSV-2 (186) were examined for the presence of virus (Fig. 1). Infectious virus was undetectable in the presence of the drug and did not reappear until 12 days after PAA removal (Day 19 p.i.) whereupon virus reappeared rapidly and reached high titer. The mean time of reactivation (18.5 days p.i.) was longer than that observed using ara-C-treated cultures (14.5 days p.i.). The absence of detectable amounts of infectious virus was extended by increasing the incubation temperature from 39.5 to 40° at the time of PAA removal (Fig. 2). The results were similar to the latency periods produced by ara-C.

Our HSV latency model used 400 μg/ml of PAA. This PAA concentration inhibited both HSV and cell DNA synthesis, yet produced little, if any, detrimental effect on confluent monolayers of HEL cells.

Discussion. Using PAA to differentiate between the inhibition of virus and cell DNA synthesis required for the establishment of latency was not possible since concentrations of PAA sufficient to prevent HSV-2 replication over a 7-day period inhibited cell as well as virus DNA synthesis. The mechanism by which PAA inhibits DNA synthesis has been well established (16–18); PAA preferentially inhibits herpes virus DNA polymerase. In contrast, the mechanism of DNA synthesis inhibition by ara-C is not well understood (19). We were able to demonstrate that PAA,

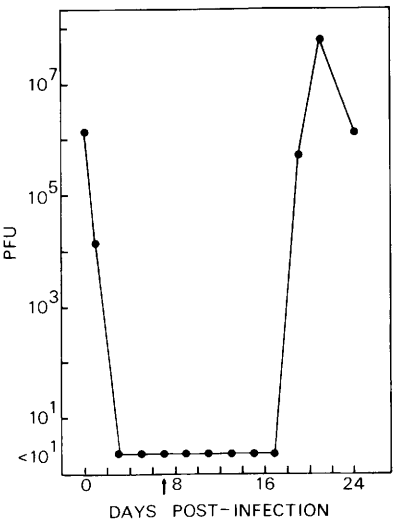

FIG. 1. Induction of HSV latency with PAA treatment (●). Cells were pretreated, infected, and treated with PAA (400 μg/ml) as described in Materials and Methods.

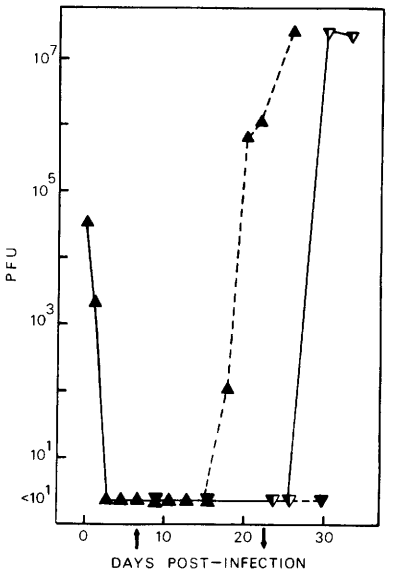

FIG. 2. Extension of the latency period by alteration of incubation temperature to 40° at the time of drug reversal. Cells were treated in a manner similar to that used in short-term latency experiments (Fig. 1) with the exception of alteration of incubation temperature to 40° 7 days p.i. Control cells were maintained at 37° during and after PAA treatment (▲) while parallel cultures were shifted to 40° and maintained at 40° throughout the experiment (▼). A group of infected cultures shifted to 40° at 7 days p.i. was subsequently shifted to 37° 23 days p.i. (▼).

an inhibitor of defined action, was capable of providing the appropriate conditions for the establishment of HSV-2 latency *in vitro*. In addition, PAA appeared to be as effective as ara-C in inducing a period in which infectious virus is undetectable. Studies in progress will attempt to characterize this period by examining the expression of virus products and the state of the virus during this latent period.

Summary. Treatment of herpes simplex virus type 2 (HSV-2)-infected human embryo lung cells with phosphonoacetic acid (PAA) at a concentration of 100 μg/ml did not inhibit the replication of HSV-2 over a 7-day period due to replication of low levels of PAA-resistant virus present in original virus stocks. Dose–response curves demonstrated that a minimum of 200 μg of PAA/ml was required to inhibit HSV-2 over this time interval. At twice the minimum concentration of PAA required, it was possible to induce a period during which infectious virus was undetectable after reversal of drug treatment. This latent period was longer than that observed previously using cytosine arabinoside, and was extended by increasing the incubation temperature to 40° at the time of drug removal.

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