PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 431-434 (1977)

In Vitro Suppression of *Herpesvirus saimiri* Replication by Phosphonoacetic Acid[1] (39687)

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Phosphonoacetic acid (PAA) has been shown to inhibit the replication of several members of the herpesvirus group in model animal systems as well as in tissue culture (1-6). Several of these studies (2, 3, 6) have demonstrated that PAA exerts the effect by specifically inhibiting the virus induced DNA polymerase. The present report is concerned with the *in vitro* effect of PAA on the replication of the oncogenic simian herpesvirus, *Herpesvirus saimiri* (HVS).

Materials and methods. Cells, virus and reagents. Two continuous lines of owl monkey kidney (OMK) cells established in our laboratory, OMK 637-69 and OMK 210-68, were used. These cells were grown and maintained following standard procedures (7). HVS stock E940-F and *Herpesvirus hominis* HVH type 1, stock E-97-M prepared in OMK cells were used in these studies. Titers were calculated by the method of Reed and Muench (8). PAA was kindly provided by Abbott Laboratories, North Chicago, Ill.

PAA treatment. Unless otherwise stated, infected cells were incubated for 1 hr for virus adsorption, and treated with 100 μg/ml of PAA and then observed for 15 days.

Electron microscopy and staining procedures. Techniques to process infected OMK cells for electron microscopic (EM) examination have been described previously (9). The methods for staining infected cells with hematoxylin and eosin (HE) and the immunofluorescent antibody (IFA) test are standard procedures described elsewhere (10).

Results. Effect of PAA on HVS and Herpesvirus hominis type 1 (HVH). PAA at a concentration of 100 μg/ml was found to reduce the infectivity of HVH by 2.0 to 3.0 logs and that of HVS by over 5.0 logs which was a total inhibition of infectivity.

Determination of minimum effect doses of PAA to inhibit HVS replication and effect of drug on OMK cells. Groups of cultures infected with approximately 100 and 1000 $TCID_{50}$ of HVS were treated with PAA at varying concentrations as indicated in Table I and then observed for 15 days. Concentrations of 1-20 μg/ml had no effect on HVS replication. Forty μg/ml of PAA partially inhibited both concentrations of HVS and 60 μg/ml stopped virus replication.

The effect of variable concentration of PAA on fully grown OMK monolayers was also studied. For this, groups of OMK 637-69 cultures were treated with PAA doses of 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mg/ml. Cultures treated with up to 1 mg/ml remained apparently normal during the 15 days of observation. Higher concentrations caused rounding of cells with subsequent degeneration. OMK cells maintained on 100 μg/ml of PAA for 15 days were sequentially transferred in media containing the drug. They grew to confluency for two additional passages and thereafter slowed down and appeared affected by the drug. However, if growth media without PAA was fed to the cells they returned to their normal morphology and growth pattern.

Effect of PAA on HVS replication. As observed in preliminary experiments, PAA in effective and nontoxic concentrations completely inhibited HVS production (Table II). The compound had no effect on HVS replication when virus was treated

[1] This study was conducted under Contract Nos. NO1 CP 33390 and NCI-NO1 CP 43225 within the Virus Cancer Program of the National Cancer Institute, NIH, PHS.

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ISSN 0037-9727

with PAA for 24 hr and held at 4° before the adsorption period. Pretreatment of OMK cells for 24 hr before the addition of virus had no effect on HVS replication.

A representative growth curve of HVS in untreated and PAA treated cells, is shown in Fig. 1. In this experiment a group of 35 mm Falcon dishes containing confluent monolayers of OMK cells were infected at an input multiplicity of approximately 0.01/ per cell; after adsorption for 1 hr, the cultures were fed with fresh media and two cultures (cells plus media) were harvested every 24 hr to test for total virus. Seventy-two hours after infection, the normal maintenance media was replaced with PAA containing media in half the dishes, and parallel harvesting procedures were followed for an additional 15 days. Figure 1 shows that HVS replication in the untreated cells followed a predictable pattern. Maximum virus production with a CPE of three (approximately 75% of the cells involved) was

TABLE I. MINIMUM EFFECTIVE CONCENTRATION OF PAA TO CONTROL REPLICATION OF HVS IN OMK CELLS.

Concentration of PAA (μg/ml)	Degree of CPE HVS Concentration ($TCID_{50}$)	
	100	1000
200	0[a]	0
100	0	0
80	0	0
60	0	0
40	1	2
20	4	4
10	4	4
1	4	4
0	4	4

[a] = degree of CPE at day 15.

TABLE II. EFFECT OF PAA ON THE REPLICATION OF HVS IN OMK CELLS.

Treatment	Virus titer (log_{50} $TCID_{50}$/0.1 ml)		
	Control	PAA (100 μg/ml)	Reduction in titer
I. PAA treatment of infected cells for 15 days	5.5	0.0	5.5
II. Pretreatment of cells with PAA (24 hr)	5.0	5.0	0.0
III. Pretreatment of virus with PAA (24 hr)	4.0	4.5	0.0

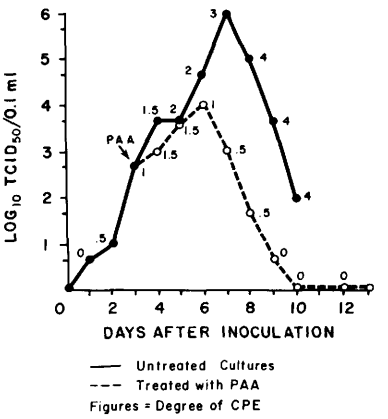

FIG. 1. Growth curve of HVS in PAA treated and untreated OMK cells. PAA was added 72 hr after the infection of the cells. Titer in untreated cultures ●——●, PAA treated cultures O- - -O, and degree of CPE (figures).

reached in 7 days and then the titer of HVS dropped steadily as expected.

The treated cells had a CPE of 1 and the titer was $10^{2.5}$/0.1 ml at the time when the PAA was added (72 hr). The increase in CPE and virus titers proceeded to a maximum of 1.5 and $10^{4.0}$/0.1 ml respectively in the next 2–3 days, then dropped steadily until reaching 0 between 9 to 12 days after infection. At this point the monolayers looked apparently normal and "cured" of HVS infection.

Effect of PAA on HVS replication when added at varying times. A group of OMK cells were infected with 1000 $TCID_{50}$ of HVS. PAA was added to these cultures at times 0, 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, 96, 120 and 144 hr postinfection. There were groups of five 35mm dishes for each time period. The cultures were kept until they were destroyed by the virus or observed for 15 days after the infected cultures recovered from CPE. Addition of PAA from 0 to 24 hr after virus adsorption resulted in no CPE. No virions, antigens or inclusions could be detected in these cells. Cocultivation of these cells with OMK cells did not result in recovery of infectious virus.

Recognizable CPE could be seen in cultures between 24 and 48 hr after infection. If PAA was added at this time the CPE disappeared. If these cultures were held in PAA containing media for 15 days and then

replaced with growth media without PAA, the CPE would reappear again. The infected cultures showing CPE involving a little more than 50% of the monolayer (CPE reading 2.5) recovered from CPE if PAA was added up to 120 hr after infection. Addition of PAA at 144 hr postinfection did not arrest further spread of infection and the monolayer was destroyed (Table III).

Long term persistence of HVS in OMK infected cells treated with PAA. A series of OMK monolayers were inoculated with 1000 TCID of HVS and maintained on media containing PAA, for a total of 63 days. During this time, at 7-day intervals the PAA media was replaced with normal growth media from two dishes and observed for the development of CPE. All these cultures developed CPE. The only difference observed was an increase in the lag period required for the appearance of CPE in cultures taken on days 7, 14, 21 and 28. However, from this point on, CPE appeared always between 19 and 23 days after withdrawal of PAA media.

Discussion. The oncogenicity of HVS in several species of monkeys and rabbits has been well established by several investigators. Our purpose in this study was to test the compound PAA on a known oncogenic virus, so as to conduct therapeutic trials in animal models if the compound was found to be effective in the *in vitro* system.

The results of our study presented in this report clearly shows that PAA inhibits the replication of HVS at a concentration of 60 μg/ml and above. Other investigators have shown that the replication of many herpesviruses are affected by PAA (1-6). To this growing list HVS may be added.

We have not looked into the mechanism of the action of PAA on HVS. In a very recent report PAA was found to affect the DNA polymerase of HVS (6). We found that pretreatment of cells or virus had no inhibitory effect on HVS replication. This would indicate that PAA did not affect virus adsorption or penetration of HVS in OMK cells. It also indicated that PAA did not affect the intact HVS.

Long term maintenance of PAA (63 days) on infected cells also had no effect on completely suppressing replication of HVS. Withdrawal of PAA after this period resulted in virus replication and destruction of infected monolayers. The presence of PAA

TABLE III. EFFECT OF ADDITION OF PAA AT DIFFERENT TIMES AFTER ADSORPTION OF *Herpesvirus saimiri.*[a]

Time for addition of PAA (hr)	Cytopathic effect				HVS detection in recovered cells[e]
	Degree at PAA addition time	Maximum CPE reached	Days to recover from CPE	Appearance in recovered cells withdrawn from PAA[d]	
Control		4.0[b]			+
0	0.0	0.0			−
1	0.0	0.0			−
2	0.0	0.0			−
3	0.0	0.0			−
4	0.0	0.0			−
6	0.0	0.0			−
9	0.0	0.0			−
12	0.0	0.0			−
24	0.0	0.5	3[c]	+	−
48	0.5	1.0	5	+	−
72	1.0	1.5	6	+	−
96	1.5	2.0	6	+	−
120	2.0	2.5	9	+	−
144	2.5	4.0			

[a] = infected with 10^3 $TCID_{50}$.
[b] = 100% destruction of monolayer.
[c] = days after PAA addition.
[d] = withdrawn 15 days after recovery.
[e] = tested by infecting OMK cells, electron microscopy and staining procedures for antigens by immunofluorescence and inclusion by HE staining.

permitted the virus to remain in a quiescent state which became reactivated on removal of the drug. Thus PAA does not have a virucidal effect on HVS. For its effectiveness to be felt, its continued presence appears to be necessary.

PAA has the remarkable property of being effective when added to HVS infected cultures even 120 hr postinfection. This was at a time when a little over 50% of the monolayer showed cytopathic effect. Addition of PAA at this point effected a "cure" and the cells appeared to have recovered. This shows that PAA was able to prevent further replication of the virus after the initial replication that took place during the 120 hr before the addition of PAA. This is somewhat similar to the effect of PAA when applied as an ointment to rabbit keratitis and mice dermatitis (1). However, in the tissue culture system removal of PAA from infected but apparently "cured" cell cultures results in reappearance of CPE.

Summary. Replication of *Herpesvirus saimiri* was inhibited by 60 μg/ml of phosphonoacetic acid (PAA) in owl monkey kidney cell cultures (OMK). OMK cells were not adversely affected by PAA. Concentrations over 1 mg/ml of PAA proved toxic to OMK cells. Pretreatment of virus or cells prior to infection had no inhibitory effect on HVS. The continued presence of PAA was required to suppress HVS replication. Removal of the drug from infected cell cultures even after 63 days resulted in the appearance of cytopathogenic effect. PAA had a greater degree of inhibition on HVS than *Herpes hominis* type 1.

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Received November 1, 1976. P.S.E.B.M. 1977, Vol. 154.