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Decreased Antiviral Effect of Phosphonoacetic Acid on the Poikilothermic Herpesvirus of Channel Catfish Disease (40275)

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Recently, a characteristic sensitivity to the drug phosphonoacetic acid (PAA) has been demonstrated for representative herpesviruses of mammalian (1–8) and avian (9) species. In each reported system virus expression has been significantly inhibited in the presence of 100 μg/ml or less concentration of PAA. This mode of inhibition has been determined to be interference of virus-coded DNA polymerase activity (10, 11) and due to this specificity the therapeutic aspects of this drug in mammalian herpesvirus systems currently appear quite promising (12).

We have investigated PAA in a cold-blooded (poikilothermic) herpesvirus system for the eventual possibility of disease control. Channel catfish herpesvirus (CCV) is the etiologic agent (13, 14) of an economically devastating disease well known to the commercial aquaculture industry (15). We found CCV expression in cell culture to be inhibited by PAA. However, 10–20 times the drug concentration was required compared to that amount necessary to inhibit warm-blooded (homeothermic) herpesvirus systems.

Materials and methods. Viruses, cell, reagents. Channel catfish virus strain Auburn (CCV_A) originally received from Dr. John Plumb (Auburn University, Auburn, AL) was prepared at 25° in a continuous cell line of brown bullhead catfish (BB) cells. Channel catfish virus strain Homestead (CCV_H) was isolated from an epizootic of channel catfish virus disease which occurred in South Florida (R. Koment, unpublished). This strain differs from the Auburn strain in its plaque morphology and complete lack of syncytial cell-forming cytopathic effects in BB cell culture. BB cells were grown at 25° in 75cm^2 plastic tissue culture flasks under Eagle's medium supplemented with 10% fetal calf serum, 0.075% sodium bicarbonate, 100 units/ml of penicillin and 100 μg/ml streptomycin.

Stocks of herpes simplex viruses (HSV) type 1 (HSV-1) strain 2bb and herpes simplex virus type 2 (HSV-2) strain 196 were prepared in human embryo lung cell cultures (Flow 2000). Primary rabbit kidney (pRK) and baby hamster kidney (BHK) cells were cultured at 37° under the same growth medium as described above for BB cell cultures.

Disodium phosphonoacetate was obtained from Abbott Laboratories (Chicago, IL). Dilutions were prepared in either maintenance medium (Eagle's medium supplemented with 2% fetal calf serum, 0.075% sodium bicarbonate, 100 units/ml of penicillin and 100 μg/ml of streptomycin) or overlay medium (Eagle's medium supplemented with 0.5% methylcellulose, 5% fetal calf serum, 0.23% sodium bicarbonate, 100 units/ml of penicillin and 100 μg/ml of streptomycin).

Virus plaque assay, plaque reduction by PAA. A standard virus plaque assay was developed for channel catfish virus in BB cells under Eagle's medium containing 0.5% methylcellulose. This was with modifications based on procedures previously described for the *in vitro* assay of herpes simplex virus (16). Briefly, tenfold serial dilutions of CCV were prepared and inoculated onto confluent monolayers of BB cells in 35 mm plastic dishes. After 1 hr. incubation at 25° to allow virus adsorption, 2 ml of overlay medium was added per dish and cultures incubated at 25° in a 5% CO_2 atmosphere. After 72 hr the overlay medium was removed, monolayers washed once with phosphate buffered saline and stained with 1% crystal violet. Plaques formed by HSV at 37° were stained at 48 hr after inoculation. All plaques were counted with the aid of a stereomicroscope.

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To determine plaque reduction a known number of plaque forming units (PFU) was inoculated onto cell monolayers in 35 mm dishes and overlay medium containing increasing concentrations of PAA was added. The average number of plaques counted on replicate cultures without PAA was regarded as the 100% value of plaques formed.

Inhibition of virus by PAA-containing medium. For multiplicity of infection (MOI) studies BB cells were grown in 16 × 125 mm tissue culture tubes and monolayers were inoculated with different multiplicities of CCV_A. Maintenance medium containing increasing amounts of PAA was added, 1 ml per tube. Inoculated control tubes contained no PAA. Cultures were maintained at 25° for 1 week with daily observation for cytopathic effect (CPE). We define effective concentration of PAA as that amount of drug which completely inhibited the induction of detectable virus CPE.

Results. Virus plaque reduction by PAA. CCV in amounts of 200, 100 or 50 plaque forming units in separate experiments was inoculated onto confluent monolayers of BB cells in 35mm dishes. Concentrations of PAA ranging from 50 to 2000 μg/ml in overlay medium was applied for 72 hr. The resulting data listed in Table I indicates that greater than 95% of CCV_A plaques were inhibited at a final drug concentration of 1000 μg/ml. This relationship remains the same whether cultures were infected with 200, 100 or 50 virus plaque forming units. Likewise, the wild-type isolate, CCV_H, was similarly inhibited in the plaque reduction assay. However, plaques of this strain were reduced 100% by concentrations of 500 μg PAA/ml, half the amount required for the laboratory adapted CCV_A strain.

In similar experiments using HSV, 200 PFU were inoculated onto either BHK or pRK cell cultures and concentrations of PAA in overlay medium applied for 48 hr. Table II indicates that in all cases 97% or more of both HSV-1 and HSV-2 plaques were inhibited at a final PAA concentration of 50 μg/ml.

Effect of PAA on host cell viability. The effect of PAA in high concentrations on BB cells was determined as follows. At the beginning of each experiment viable cell counts, as calculated by trypan blue dye exclusion, were done on BB cells grown in 35 mm dishes. Representative cultures were randomly selected. Overlay medium containing PAA in final concentrations of 0, 500, and 2000 μg/ml was added to cell cultures containing no virus, and at 72 hr viable cell counts were done. The data in Table III demonstrate that the total number of viable cells was the same in PAA treated and untreated BB cell cultures. This indicates that no drug toxicity occurred during the 72 hr-CCV assay period. In addition, parallel BB cell cultures containing either 0, 500, or 2000 μg/ml of PAA were

TABLE I. CHANNEL CATFISH VIRUS PLAQUE REDUCTION BY PAA.

Virus[a]	PAA Conc[b]	No. plaques[c]	% Plaque reduction
CCV_A 200 PFU	0	183	0
	50	175	4
	100	191	0
	200	162	11
	500	84	54
	1000	8	96
	2000	4	98
100 PFU	0	75	0
	50	71	5
	100	79	0
	200	68	9
	500	23	69
	1000	3	96
	2000	4	95
50 PFU	0	29	0
	50	24	17
	100	27	7
	200	20	31
	500	9	69
	1000	0	100
	2000	0	100
CCV_H 200 PFU	0	195	0
	50	194	0
	100	134	33
	200	109	44
	500	1	100
	1000	0	100
	2000	0	100
50 PFU	0	69	0
	50	54	22
	100	28	59
	200	11	84
	500	0	100
	1000	0	100
	2000	0	100

[a] Channel catfish virus strains Auburn (CCV_A) and Homestead (CCV_H).
[b] In μg/ml final concentration.
[c] Average of four plates per PAA concentration.

TABLE II. HERPES SIMPLEX VIRUS PLAQUE REDUCTION BY PAA.

Virus[a]	Cell[b]	PAA Conc[c]	No. plaques[d]	% Plaque reduction
HSV-1	BHK	0	150	0
		50	0	100
		100	0	100
		200	0	100
	pRK	0	165	0
		50	0	100
		100	0	100
		200	0	100
HSV-2	BHK	0	199	0
		50	5	97
		100	0	100
		200	0	100
	pRK	0	165	0
		50	0	100
		100	0	100
		200	0	100

[a] Herpes simplex virus type 1 (HSV-1) strain 2bb and type 2 (HSV-2) strain 196.
[b] Baby hamster kidney (BHK) cells and primary rabbit kidney (pRK) cells.
[c] In μg/ml final concentration.
[d] Average of four plates per PAA concentration.

washed, trypsinized and successfully subcultured twice under PAA-free growth medium.

Relationship of PAA to multiplicity of infection. To determine if a PAA dose dependency existed for CCV similar to that reported (2) for HSV, CCV_A was prepared in various dilutions and inoculated onto BB cells grown in tissue culture tubes. These virus dilutions corresponded to multiplicities of infection of 0.01, 0.1, 1.0, and 6.0 plaque forming units per cell. Maintenance media containing the same PAA concentrations as listed in Table I were added to each MOI group of inoculated BB cell cultures. Viral CPE for all cultures did not progress beyond 4 days after inoculation, but cultures were observed for a period of 1 week. Results of these experiments indicated that a direct relationship does indeed exist between PAA concentration and CCV_A MOI. For every tenfold increase in virus input a twofold increase of drug was required for total inhibition of virus cytopathology. This ranged from 500 μg PAA/ml (MOI = 0.01 PFU/cell) to more than 2000 μg PAA/ml (MOI = 6.0 PFU/cell). The toxicity level of PAA in BB cells was evident at ≥2500 μg PAA/ml of culture medium.

Discussion. The herpesviruses are widely dispersed throughout animal phylogeny (17). Although they infect a range of species the resultant interaction may vary subtly from subclinical infection to severe disease to oncogenicity. For many reasons those herpesviruses that parasitize homeothermic animals, the mammals and birds, have received most research attention. It has been consistently found that PAA in amounts of 100 μg or less inhibits the expression of each herpesvirus tested. Likewise, our results agree with the results of others (2, 4) whereby HSV-1 and HSV-2 expression at 37° is inhibited by less than 100 μg PAA/ml.

The data presented in this report support the developing contention that susceptibility to inhibition by PAA is a new characteristic of the herpesviruses. Furthermore, this characteristic is apparent in poikilothermic as well as homeothermic animal-virus systems. Our findings indicate, however, that up to 20 times the amount of drug required for other herpesvirus systems is necessary to inhibit CCV.

Currently the precise mode of virus inhibition which occurs in our system is unclear. In homeothermic systems PAA has been shown to interfere with enzymes of viral DNA replication (10, 11). In view of the vast phylogenetic distance between the mammalian and teleostean cell however, there may be differences in metabolic reactions to antiviral drugs. If the mode of action is similar then the action of PAA may be dependent upon either temperature or, relatedly, the physiology and metabolic rate of the host cell. It is well known that enzyme-substrate reactions can be directly influenced by temperature, and the importance of temperature as a catalytic mechanism has been demonstrated in the regulation of many life functions of poikilothermic species (18). The importance of host cell physiology is also suggested by the increased tolerance of BB cells to PAA. We have observed drug toxicity to occur at

TABLE III. VIABLE BB CELL COUNTS AFTER EXPOSURE TO PAA.

PAA conc[a]	Time[b]	Viable cell count[c]
0	0	1.4×10^6
0	72	1.9×10^6
500	72	1.9×10^6
2000	72	2.3×10^6

[a] In μg/ml final concentration.
[b] In hours.
[c] Trypan blue dye exlusion, total number of cells per culture.

or about the 2500 μg/ml level as determined by loss of monolayer integrity with concurrent decrease in viable cell counts.

An alternative hypothesis is that the poikilothermic virus is itself responsible for the increased amount of drug required for inhibition of virus expression. One means to resolve this question would be a determination through a range of temperatures of PAA levels inhibiting homeothermic herpesviruses in BB cells or CCV in homeothermic cells. Unfortunately, these experiments are not now possible as BB cells will not support the replication of those broad host range homeothermic herpesviruses tested (HSV, pseudorabies virus) and CCV will only replicate in selected cells of catfish origin.

The investigation of anti-viral drugs serves a twofold purpose: The realization of potential for control of acute viral disease and the attainment of a further understanding of the mechanisms of virus host-cell interaction. A clearer insight into both these objectives may be obtained by study of the mechanism by which poikilothermic channel catfish herpesvirus is less sensitive than homeothermic herpesviruses to PAA.

Summary. Both the laboratory adapted Auburn strain and a recently isolated wild-type strain of channel catfish herpesvirus (CCV) were found to be inhibited by phosphonoacetic acid (PAA) when replicated in catfish cell cultures. The inhibition of virus cytopathic effect by PAA exhibited a direct relationship between the multiplicity of infection and amount of drug required. However, in this poikilothermic system up to 20 times the amount of PAA required for inhibition of homeothermic herpesvirus systems was found necessary to inhibit CCV cytopathology.

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