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In vitro and *in vivo* Inhibition of Myxoma Virus by Treatment with Phosphonoacetic Acid

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Summary

Both myxoma and fibroma viruses were found to be sensitive *in vitro* to the effects of phosphonoacetic acid. Detectable myxoma virus replication was inhibited at a drug concentration of 100 μg/ml. Fibroma virus replication was inhibited at a concentration of 500 μg/ml. Because of this difference in sensitivity, myxoma virus was used to infect rabbits to test the efficacy of phosphonoacetic acid in the treatment of a systemic viral disease. Rabbits were given 400 mg kg^{-1} day^{-1} of phosphonoacetic acid subcutaneously in two injections. Phosphonoacetic acid-treated animals showed a reduction in the severity of disease. Neither serum viral antigen nor infectious virus could be detected. In phosphate buffered saline-treated animals both serum viral antigen and infectious virus were found. All animals treated with phosphate buffered saline died of myxomatosis.

Introduction

Phosphonoacetic acid (PAA) has been found to be a potent inhibitor of several herpesviruses [2, 10, 12, 16, 22, 23]. Although initial studies indicated that the inhibitory effect of PAA was limited to the herpesvirus-induced DNA polymerase [17], later work has shown this drug to be inhibitory to the α polymerase of mammalian cells [21], and to certain other viruses, *e.g.*, vaccinia virus (VV). Bolden *et al.* [3] found PAA to be an effective inhibitor of VV nuclear DNA synthesis and VV-induced DNA polymerase.

PAA was used to treat skin lesions in rabbits inoculated with VV or Shope Rabbit Fibroma virus (SRF). When given in an ointment, PAA was very effective in reducing the severity of the lesions of VV and the tumors of SRF. Intralesional injection of PAA completely suppressed tumor formation of SRF, but systemic administration of PAA by the i.p. route had no significant effect on the pustular lesions of VV nor the benign tumors caused by SRF [8].

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In this report we examine the effect of PAA upon replication of myxoma and fibroma viruses. Furthermore, we look at the efficacy of systemic administration of PAA in the treatment of rabbits infected with myxoma virus.

Materials and Methods

Cell Cultures

A continuous cell line (CRK) was derived from a primary cottontail rabbit kidney culture *(Sylvilagus floridanus)*. CRK cells were used in all assays and for the preparation of stock virus. Growth medium consisted of medium 199 and 10 percent fetal calf serum. Established or virally infected monolayers were maintained in medium 199 in which the concentration of fetal calf serum was reduced to 3 percent. All media contained 100 units of penicillin and 100 μg of streptomycin per ml. The pH of the medium was adjusted to 7.6 with 7.5 percent sodium bicarbonate.

Virus

Stock preparations of myxoma virus (Moses strain) and fibroma virus (Patuxent strain) were grown in CRK cells and frozen at −70° C. The titer of the myxoma stock virus was 1.5×10^5 PFU/ml. The titer of the fibroma stock virus was 3.9×10^6 PFU/ml.

Reagents

PAA was purchased from Richmond Organics (Richmond Organics, Richmond, VA) and converted to the disodium salt by the method of Shipkowitz [22]. A stock solution of PAA for the *in vitro* experiments was made in medium 199 and 3 percent fetal calf serum to a final concentration of 10 mg/ml. PAA used in animal experiments was dissolved in phosphate buffered saline (PBS) to a concentration of 200 mg/ml.

Virus Replication in the Presence of PAA

Monolayers of CRK cells were grown in six-well disposable trays (Linbro Scientific Co., New Haven, CT) and infected with 100 PFU of virus. PAA was added at the time of inoculation of virus to a final concentration of 500, 100, 50, 25, 10 or 1 μg/ml. All cultures were incubated at 36° C in a CO_2 incubator. Cultures were refed with PAA-containing medium at 5 days and harvested when virus controls showed 75 percent CPE, approximately 6 to 7 days. The cultures were frozen and thawed twice to disrupt cells and were stored at −70° C until assayed.

Quantitation of Virus in Blood

Blood was obtained from rabbits by bleeding from the marginal ear vein using heparin as an anti-coagulant and serially diluted in medium 199 and 3 percent fetal calf serum. Medium was supplemented with 300 units of penicillin per ml, 300 μg of streptomycin per ml and 3 μg of fungizone per ml. Ten-fold dilutions were plated in quadruplicate on CRK cells grown in 24 well disposable trays (Linbro Scientific Co., New Haven, CT). Inoculated cultures were incubated for 7 days at 36° C in a CO_2 incubator.

Virus Neutralization Assay

Virus neutralization assays were performed by mixing two-fold dilutions of serum with equal volumes of medium containing 400 PFU of virus and incubated for 1 hour at room temperature. The serum-virus mixtures were then inoculated onto CRK cells, incubated at 36° C for 2 hours and overlayed in the same manner as the virus plaque assay.

Virus Plaque Assay

Virus plaque assays were plated in triplicate on monolayers of CRK cells grown in six-well disposable trays. All inoculated cultures were held at 36° C for 2 hours to facilitate virus adsorbtion and covered with an overlay containing 0.5 percent lact-albumin hydrolysate, 0.1 percent bovine albumin, 0.1 percent yeast extract, 6 percent

horse serum and 1 percent purified Seaplaque agarose (Marine Colloids, Inc., Rockland, ME) in Hanks' Balanced Salt Solution. One hundred units of penicillin and 100 μg of streptomycin per ml were added and the pH was adjusted to 7.4 with 7.5 percent sodium bicarbonate before overlaying cultures. After incubation at 36° C in a CO_2 incubator for 6 to 8 days, monolayers were fixed through the agarose with a solution of 95 percent ethanol, glacial acetic acid and formalin (6:2:1). The overlay was removed and the cells were stained with a 0.02 percent solution of crystal violet.

Detection of Viral Antigen in Serum

Viral antigen levels were determined by the semiquantitative gel precipitation technique described by ARNASON [1]. Levels of viral antigen were reported as the reciprocal of the highest serum dilution producing a distinct precipitin band with rabbit anti-myxoma serum. The neutralizing titer of this serum was 2048.

Animals and Experimental Protocol

New Zealand white rabbits, weighing approximately 2 kilograms, were randomized to three treatment groups; i) virus infected and PBS treated, ii) virus infected and PAA treated, iii) mock infected and PAA treated. Five hundred PFU (0.5 ml) of virus or diluent were given intradermally in the left hind quarter of each animal. PBS or PAA, at a dosage of 400 mg kg^{-1} day^{-1}, was given subcutaneously in two injections 12 hours apart. Administration of PAA or PBS started immediately after inoculating virus and continued for 10 days. Blood and serum samples were obtained on days 0, 4, 6 and 10. Blood was assayed for virus as previously described. Serum samples were assayed for neutralizing antibody and virus antigen.

Results

Virus Inhibition by PAA in vitro

Replication of both myxoma and fibroma was susceptible to the effect of PAA *in vitro*. In the case of myxoma virus, no replication could be detected at drug concentration of 100μg/ml (Table 1). The presence of drug at this concentration in cultures resulted in a 3.8 log decrease in titer between drug treated and control cultures.

Table 1. *Myxoma and fibroma virus replication in the presence of phosphonoacetic acid*

Concentration of PAA (μg/ml)	Titer $\log_{10} \pm$ SE	
	Myxoma	Fibroma
1	4.35±0.00	6.13±0.27
10	3.29±0.27	5.85±0.11
25	3.55±0.27	5.02±0.13
50	3.64±0.01	4.97±0.04
100	*	3.15±0.03
500	*	*
Virus control	3.82±0.34	5.42±0.57

CRK cells were grown to confluency in six-well disposable trays. The growth medium was removed and maintenance medium containing virus together with the indicated concentration of PAA was added. Incubation was continued until control cultures showed 75 percent cytopathic effect. Cultures were freeze-thawed three times and assayed. Each drug concentration was run in quadruplicate. Data presented represent the mean titers of the four replicates

* No virus detected

In contrast to myxoma, fibroma virus replication was only partially inhibited at a concentration of 100 μg/ml of PAA (Table 1). There was a 2.5 log decrease in virus titer between the cultures treated with a drug concentration of 100 μg/ml and control cultures. Inhibition of detectable virus replication was achieved at a concentration of 500 μg/ml. Due to this difference in susceptibility to drug, myxoma virus was chosen for further experimentation *in vivo*.

PAA Treatment of Experimental Myxomatosis

The pathogenesis of myxomatosis involves a stepwise systemic invasion. After an initial period of multiplication at the site of injection, the virus probably multiplies in the regional lymph node and is then disseminated to various parts of the body [6].

In this experiment, rabbits were infected intradermally at multiple sites in the left hind quarter with 500 PFU or mock infected with diluent. Animals were observed twice daily for signs of disease.

One of the first changes observed was erythema and induration at the site of injection of virus in the untreated group. This was seen as early as 2 days post-challenge. The sites of inoculation fused to form one lesion 3—5 cm in diameter which became grossly necrotic by 7 days after challenge. In addition, this group showed a serous exudate from the eyes by day 5 along with several macules appearing randomly on the skin. By day 7, additional skin lesions could be found and the eyes exuded a thick, creamy exudate. All animals by day 8 exhibited respiratory distress and died between day 8—10 post-challenge.

In contrast, the physical signs in the PAA treated group were less severe than in the untreated group. In treated animals there was moderate erythema and induration at the site of injection of virus. In three of five animals the site of inoculation could not be demonstrated by day 8. In four of five animals, there was no evidence of secondary skin lesions but a slight serous discharge was observed from the eyes by day 5. No other signs of disease were observed. Four of five treated animals did not develop generalized myxomatosis while all untreated animals did.

Humoral Response

Both treated and untreated groups showed low levels of neutralizing antibody by 4 days after challenge (Tabel 2). A rise in neutralizing antibody could be seen in the untreated group through day 6 and this continued in the one remaining animal through day 10. This rise in antibody titer did not correlate with survival.

Neutralizing antibody titers in the treated group were characterized by an initial rise recognized by day 6. These titers did not change appreciably by day 10 with the exception of one animal which had a neutralizing titer of 160. None of the animals in the treated, uninfected group showed a neutralizing titer.

Appearance of Viral Antigen in Serum

Several investigators working with myxoma virus found that infectious virus could not be recovered from the serum but was associated with the leukocyte fraction [6, 11, 19]. In addition, soluble viral antigen was demonstrated in the serum of infected animals.

In our experiment, virus antigen could not be detected by double diffusion gel precipitation on day 4 in treated and untreated animals. On day 6 virus antigen was detected in the serum of all five animals in the untreated group. Two animals showed a precipitin reaction with undiluted serum while three animals showed a reaction at a serum dilution of 1:2. No viral antigen was detected in sera collected from the treated group on day 6 or day 10.

Virus Titers

In a separate but similar experiment virus was assayed in the blood of treated and nontreated groups. Virus could not be detected in the blood of nontreated animals on day 4 or 6 but was detected on day 10 with titers ranging from 10^3 to 10^4 per ml of blood. No virus was found at any of these times in the treated group.

Mortality of Treated and Non-Treated Groups

All animals which were infected and not treated with PAA died of myxomatosis with a mean survival time of 8.6 days and a range from 6 to 10 days. Four of five PAA treated animals also died. Of the four animals that died only one developed myxomatosis with death at 22 days. The other three animals showed loss of weight, anurea and failure to defecate but showed no distinct signs of myxomatosis. At necropsy, the most noticeable gross pathology was that of renal damage. The kidneys from treated animals were enlarged and hemorrhagic with a mottled appearance. Of the four animals that died in the treated group, the mean survival time was 13.7 with a range from 8 to 22 days. One animal survived the experiment and was refractile to re-challenge with myxoma. None of the animals in the treated, non-infected group died.

Table 2. *Serum neutralizing antibody titers of rabbits challenged with myxoma virus and treated with PAA or PBS*

	Day		
Animal	4	6	10
PAA-treated			
1	20	20	20
2	20	40	20
3	5	20	20
4	<5	5	160
5	<5	40	*
PBS-treated			
6	20	10	80
7	<5	40	*
8	10	80	*
9	<5	80	*
10	10	20	*

Serum samples were collected on days 0, 4, 6, 10. Titers were determined by the 50 percent plaque-reduction end point and expressed as the reciprocal of the dilution

* Dead

Discussion

The use of PAA as an inhibitor of herpesvirus replication has been well documented. The inhibitory effect of PAA on the poxvirus family has not been well studied. Bolden *et al.* [3] was the first to show that PAA inhibited vaccinia virus nuclear DNA synthesis in isolated HeLa cell nuclei. In addition, it was shown that PAA inhibited purified vaccinia virus-induced DNA polymerase.

The first objective of our investigation was to study the effect of PAA upon different poxviruses. Our findings show the inhibitory action of PAA upon poxviruses is not confined to VV. Phosphonoacetic acid was found to inhibit detectable replication of myxomavirus at a concentration of 100 μg/ml while fibroma virus was inhibited at 500 μg/ml. Although virus inhibition may be explained by cytotoxic effects of the drug, the difference in drug inhibition of myxoma and fibroma virus argues against it. No cytotoxicity has been seen with a drug level of 500μg/ml [10]. This is the first *in vitro* demonstration of inhibition of poxviruses other than VV. However, PAA was found effective *in vivo* against SRFV [8].

The second objective of our investigation was to study the efficacy of PAA administered parenterally for treatment of a systemic viral disease. Previous work has shown PAA to be effective when applied topically for the treatment of herpes simplex virus infections of skin or mucous membranes [9, 13, 15, 22]. Phosphonoacetic acid has been much less effective when administered systemically for the treatment of virus infections [7, 8, 13, 14, 20]. Much of this work has been done with mice which show a high tolerance to the effects of PAA [20]. In other animals species, intramuscularly administered PAA effectively prevented disease in patas monkeys infected with simian varicella virus [5].

In our studies, we have shown a reduction in the severity of the disease produced by myxoma virus using PAA administered parenterally. This is supported by three lines of evidence: i) Absence or reduction of clinical signs associated with myxomatosis, ii) Failure to isolate virus from the blood of animals, iii) Absence of detectable viral antigen in the serum of treated animals.

Because the pathogenesis of myxomatosis has been well delineated [6], one may speculate on the mechanism by which treatment with PAA altered the pathogenesis of disease. It appears that PAA partially inhibited the replication of the virus at the site of injection and significantly reduced the spread to target organs and subsequent multiplication. In the first place, lesions in the infected, treated group showed pathological changes indicative of virus multiplication but these were less than in the infected, PBS treated group. There was also a rise in antibody titer and a minimal exudate was observed from the eyes of infected, treated animals on day 5. The treated, mock infected group did not show any of these signs which leads us to believe that these changes are due to virus replication. Because the assay is insensitive, our failure to detect virus in the blood does not exclude the possibility of a low-grade viremia or minimal virus replication in target organs, but certainly there was less viremia than in controls. There is some support for the idea that phosphonoacetic acid inhibits the multiplication of virus in target organs in that Overall *et al.* [18] found that treatment of mice with PAA after infection with murine cytomegalovirus reduced virus titers in the liver.

In this work, several problems became obvious concerning the administration of PAA. Investigators have noted problems with the systemic administration of

PAA in animals other than mice. FRIEDMAN-KIEN *et al.* [8] observed that rabbits became highly agitated when administered PAA i.p. In initial experiments in this laboratory, PAA was given to rabbits i.p., but was found to be too irritating for continued administration by this route. Repeated s.c. injections resulted in obvious discomfort for the animals. FELSENFELD *et al.* [5] found it necessary to anesthetize patas monkeys with ketamine hydrochloride before im administration of PAA presumably due to an adverse reaction.

Furthermore, in our study disease was not completely prevented. Three of five animals in the infected, treated group died without showing physical signs of myxomatosis and without detectable virus or virus antigen in the blood. However, the death of these animals cannot be attributed to the toxicity of PAA since no animals died in the mock infected, treated group. Based on these observations there may be a synergistic effect between infection with myxoma virus and administration of PAA that leads to a heightened susceptibility to either virus or drug. One possibility may be that virus multiplication in the kidney may interfere with excretion of the drug allowing it to reach toxic levels. In a previous experiment, PAA was given twice a day to uninfected rabbits at a dosage of 250 mg kg^{-1} day^{-1}. One animal died after three injections of PAA. At necropsy, the animal was found to have only one functional kidney. This observation suggests the importance of a fully functional renal system in animals given PAA.

PAA was initially an attractive antiviral agent because of its specificity on the molecular level. Its use as other than an experimental tool must be questioned due to its accumulation in the bones of experimental animals [4] and its toxicity. It would appear that more promising results might be found with the analogs of PAA such as phosphonoformate. In this system, phosphonoformate was found to be impractical due to its low solubility.

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References

1. ARNASON, B. G., DE VAUX ST.-CYR, C., RELYVELD, E. H.: Role of the thymus in immune reactions in rats. IV. Immunoglobulins and antibody formation. Int. Arch. Allergy Appl. Immunol. **25**, 206—224 (1964).
2. BARAHONA, H., DANIEL, M. D., BEKESI, J. G., FRASER, C. E. O., KING, N. W., HUNT, R. D., INGALLS, J. K., JONES, T. C.: *In vitro* suppression of *Herpesvirus saimiri* replication by phosphonoacetic acid. Proc. Soc. exp. Biol. Med. **154**, 431—434 (1977).
3. BOLDEN, A., AUCKER, J., WEISSBACH, A.: Synthesis of herpes simplex virus, vaccinia virus, and adenovirus DNA in isolated HeLa cell nuclei. I. Effect of viral-specific antisera and phosphonoacetic acid. J. Virol. **16**, 1584—1592 (1975).
4. BOPP, B. A., ESTEP, C. B., ANDERSON, D. J.: Disposition of disodium phosphonoacetate-^{14}C in rat, rabbit, dog and monkey. Fed. Proc. **36**, 939 (1977).
5. FELSENFELD, A. D., ABEE, C. R., GERONE, P. J., SOIKE, K. F., WILLIAMS, S. R.: Phosphonoacetic acid in the treatment of simian varicella. Antimicrob. Agents Chemother. **14**, 331—335 (1978).
6. FENNER, F., WOODROOFE, G. M.: The pathogenesis of infectious myxomatosis: The mechanism of infection and immunological response in the European rabbit *(Oryctolagus cuniculus)*. Brit. J. Pathol. **34**, 400—411 (1953).

7. Fitzwilliam, J. F., Griffith, J. F.: Experimental encephalitis cause by herpes simplex virus: Comparison of treatment with tilorone hydrochloride and phosphonoacetic acid. J. inf. Dis. **133** (Suppl.), 221—225 (1976).
8. Friedman-Kien, A. E., Fondak, A. A., Klein, R. J.: Phosphonoacetic acid treatment of Shope fibroma and vaccinia virus skin infections in rabbits. J. Invest. Dermatol. **66**, 99—102 (1976).
9. Gerstein, D. D., Dawson, C. R., Oh, J. O.: Phosphonoacetic acid in the treatment of experimental herpes simplex keratitis. Antimicrob. Agents Chemother. **7**, 285—288 (1975).
10. Goodrich, J. M., Lee, K. W., Hinze, H. C.: *In vitro* inhibition of *Herpesvirus sylvilagus* by phosphonoacetic acid and phosphonoformate. Arch. Virol. **66**, 216—264 (1980).
11. Hobbs, J. R.: Studies on the nature of the infectious myxoma virus of rabbits. Amer. J. Hyg. **8**, 800—839 (1928).
12. Huang, E.-S.: Human cytomegalovirus IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. **16**, 1560—1565 (1975).
13. Kern, E. R., Richard, J. T., Overall, J. C., Glasgow, L. A.: Genital *Herpesvirus hominis* infection in mice. II. Treatment with phosphonoacetic acid, adenine arabinoside, and adenine arabinoside 5'-monophosphate. J. inf. Dis. **135**, 557—567 (1977).
14. Kern, E. R., Richard, J. T., Overall, J. C., Glasgow, L. A.: Alteration of mortality and pathogenesis of three experimental *Herpesvirus hominis* infections of mice with adenine arabinoside 5'-monophosphate, adenine arabinoside, and phosphonoacetic acid. Antimicrob. Agents Chemother. **13**, 53—60 (1978).
15. Klein, R. J., Friedman-Kien, A. E., Fondak, A. A., Buimovici-Kein, E.: Immune response and latent infection after topical treatment of herpes simplex virus infection in hairless mice. Infect. Immun. **16**, 842—848 (1977).
16. May, D. C., Miller, R. J., Rapp, F.: The effect of phosphonoacetic acid on the *in vitro* replication of varicella-zoster virus. Intervirology **8**, 83—91 (1977).
17. Mao, J. C.-H., Robishaw, E. E., Overby, L. R.: Inhibition of DNA polymerase from herpes simplex virus-infected WI-38 cells by phosphonoacetic acid. J. Virol. **15**, 1281—1283 (1975).
18. Overall, J. C., Kern, E. R., Glasgow, L. A.: Effective antiviral chemotherapy in cytomegalovirus infection of mice. J. inf. Dis. **133** (Suppl.), 237—243 (1976).
19. Rivers, T. M., Ward, S. M.: Infectious myxomatosis of rabbits. J. exp. Med. **66**, 1—14 (1937).
20. Roboz, J., Suzuki, R., Hunt, R., Bekesi, G.: Preclinical toxicological study of phosphonoacetic acid: Determination in blood by selected ion monitoring. Biomed. Mass Spectrometry **4**, 291—296 (1977).
21. Sabourin, C. L. K., Reno, J. M., Boezi, J. A.: Inhibition of eukaryotic DNA polymerases by phosphonoacetate and phosphonoformate. Arch. Biochem. Biophys. **187**, 96—101 (1978).
22. Shipkowitz, N. L., Bower, R. R., Appell, R. N., Nordeen, C. W., Overby, L. R., Roderick, W. B., Schleicher, J. B., von Esch, A. M.: Suppression of herpes simplex virus infection by phosphonoacetic acid. Appl. Microbiol. **26**, 264—267 (1973).
23. Summers, W. C., Klein, G.: Inhibition of Epstein-Barr virus DNA synthesis and late gene expression by phosphonoacetic acid. J. Virol. **18**, 151—155 (1976).

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