COMPARISON OF THE EFFECT OF TRISODIUM PHOSPHONOFORMATE ON THE MEAN PLAQUE SIZE OF PSEUDORABIES VIRUS, INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS AND PIGEON HERPESVIRUS

By

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INTRODUCTION

Disodium phosphonoacetate and trisodium phosphonoformate are recently discovered antiherpetic compounds which are slightly cytotoxic and exhibit an important antiviral effect due to a specific inhibition of the virus-induced DNA-polymerase (Overby, Robishaw, Schleider, Rueter, Shipkowitz and Mao, 1974; Mao and Robishaw, 1975; Helgstrand, Eriksson, Johansson, Lannerö, Larsson, Misiorny, Noren, Sjöberg, Stenberg, Stening, Stridh, Öberg, Alenius and Philipson, 1978).

Susceptibility to these two compounds has been reported in all the herpesviruses so far studied (Helgstrand *et al.*, 1978; Reno, Lee and Boezi, 1978) and for several other viruses (Helgstrand *et al.*, 1978; Moreno, Carrascosa, Ortín and Vinũela, 1978). Some strains of herpesviruses were found to be resistant (Duff, Robishaw, Mao and Overby, 1978; Jofre, Schaffer and Parris, 1977; Reno *et al.*, 1978).

For therapeutic purposes, phosphonoformate has the advantage that it does not irritate the skin or the genital mucous membrane and has only a minor effect on normal cellular metabolism (Alenius and Norlinder, 1979; Stenberg and Larsson, 1978; Alenius, Dinter and Öberg, 1978). The use of phosphonoacetate is limited because of its topical irritative potential, its toxicity for laboratory animals and its accumulation in bones (Bopp, Estep and Anderson, 1977).

Phosphonoformate is active against several herpesviruses such as infectious bovine rhinotracheitis virus (*Bovid herpesvirus 1*, IBR) and pseudorabies virus (*Sus herpesvirus 1*, SHV) (Helgstrand *et al.*, 1978). These two viruses share biochemical and antigenic similarities (Pastoret, Aguilar-Setién and Schoenaers 1978; Aguilar-Setién, Pastoret, Burtonboy, Coignoul, Jetteur and Schoenaers, 1979; Aguilar-Setién, Vandeputte, Pastoret, Michaux, Pensaert and Schoenaers, 1979; Aguilar-Setién, Pastoret, Toma, Joubert, Michaux and Schoenaers, 1979), and like pigeon herpesvirus (*Pigeon herpesvirus 1*, PHV), produce plaques under agarose overlay (Pastoret *et al.*, 1978; Vindevogel, Pastoret, Burtonboy, Gouffaux and Duchatel, 1975; Vindevogel, Duchatel and Gouffaux, 1977; Vindevogel, Duchatel, Gouffaux and Pastoret, 1977).

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IBR virus can replicate in cell cultures of bovine origin, PHV in chicken embryo fibroblasts (CEF) and SHV in both types.

The present report deals with the comparison of the effect of various concentrations of phosphonoformate on the populations of plaques produced by SHV, IBR virus and PHV, under agarose overlay, by the use of previously described methods (Jetteur, Pastoret, Aguilar-Setién, Leroy, Godart and Schoenaers, 1979; Pastoret, Jetteur, Aguilar-Setién, Leroy, Schwers, Godart and Schoenaers, 1979). This investigation was performed with a view to a possible clinical application for the treatment of local lesions produced by PHV infection (Vindevogel and Duchatel, 1979).

MATERIALS AND METHODS

Virus and Cell Cultures

SHV, strain Cul, was isolated in our laboratory from infected piglets. IBR virus, international reference strain Los Angeles, was kindly provided by Professor McKercher, Davis, California. PHV strain Cul (Vindevogel *et al.*, 1975) was used.

Primary CEF monolayers were prepared and grown as previously described (Vindevogel *et al.*, 1975). GBK cells (Georgia bovine kidney cells) were cultured in Earle's minimum essential medium (MEM), supplemented with non-essential aminoacids (Flow laboratories), O·85 μg per ml sodium bicarbonate, 10 per cent foetal bovine serum (FBS), 100 units per ml penicillin and 10 μg per ml streptomycin. These cells were kindly provided by Professor Babiuk, University of Saskatchewan, Saskatoon, Canada.

Production and Titration of Plaques

Confluent cell monolayers, grown in Petri dishes (30 mm diameter), were rinsed with phosphate buffered saline (PBS) pH 7·4, inoculated with 0·2 ml of 10-fold viral dilutions, and incubated for 1 h at 37 °C. The infected cells were then covered with MEM medium containing 1 per cent agarose (indubiose) and 2 per cent FBS. This overlay medium was supplemented with trisodium phosphonoformate at the following final concentrations: 0, 10, 100, 500 and 1000 μM per ml. The same procedure was followed on uninfected cells as controls. The cells were fixed with formaldehyde after 4 days of incubation for CEF cells and 5 days for GBK cells, then stained with May-Grünwald-Giemsa (Vindevogel *et al.*, 1975; Jetteur *et al.*, 1979); plaques were counted with an inverted microscope.

The effect of phosphonoformate, at various concentrations, against IBR virus was tested on GBK cells, against PHV on CEF cells, and against SHV on both kind of cells.

Measurement of Mean Plaque Size

Cell cultures inoculated with the optimal viral dilution causing a sufficient amount of isolated plaques were examined according to a method previously described (Jetteur *et al.*, 1979; Pastoret *et al.*, 1979). Briefly, plaques were printed with an offset camera to obtain a final magnification of × 20. For each virus cultivated on each cell line, and at each concentration of phosphonoformate, the areas of 50 plaques taken at random were measured with an OH Kempton planimeter.

Statistical Tests

Normality test. The normality of the plaque size distribution was tested for skewness and kurtosis.

Analysis of variance. The effect of the addition of trisodium phosphonoformate, at increasing concentrations in the medium, was studied by a one-way analysis of variance. Student's t test was used to determine which concentration of phosphonoformate produced a significant reduction of the plaque size.

RESULTS

Comparison of the Effect of Phosphonoformate on IBR and SHV Plaques Produced in GBK Cells

The mean and the standard deviations of the plaque sizes are given in Table 1.

Normality tests. The plaque population induced by IBR virus was normally distributed in cell cultures devoid of phosphonoformate, or when 10 μM per ml of phosphonoformate was present in the medium, but not with higher concentrations (100, 500 and 1000 μM per ml). To secure a normal distribution in all cases, the square root of the data was taken before variance analysis.

TABLE 1

PLAQUE SIZE OF IBR VIRUS AND SHV ON GBK CELLS

Virus		*μM per ml phosphonoformate*				
		0	*10*	*100*	*500*	*1000*
IBR	Mean	13·313	8·995	3·390	1·482	0·791
	S.D.	7·183	4·693	2·196	1·087	0·637
SHV	Mean	34·044	25·047	6·753	—	—
	S.D.	14·219	6·978	3·909	—	—

Values are in mm², calculated from 50 plaques.

The plaque population produced by SHV was normally distributed in cell cultures devoid of phosphonoformate, or when 10 and 100 μM per ml of phosphonoformate was added to the medium (with the exception of a slight asymmetry at a concentration of 10 μM per ml). At higher concentrations (500 and 1000 μM per ml) a loss of titre was observed and the number of plaques produced was not sufficient to allow statistical analysis.

Analysis of variance. Addition of phosphonoformate to the overlay medium influenced the size of plaques for both viruses ($P < 0{\cdot}0001$).

The t test showed that, for both viruses, each of the various increasing concentrations of phosphonoformate allowing measurement produced a significant reduction of the mean plaque size compared with each previous one ($P < 0{\cdot}002$) (Fig. 1).

Effect on the titres. Addition of 10 and 100 μM per ml of phosphonoformate to the overlay medium did not change the titre of IBR virus and SHV.

Addition of 500 or 1000 μM per ml produced a decrease of titre, especially for SHV (Fig. 2).

Comparison of the Effect of Phosphonoformate on SHV and PHV Plaques Produced in CEF Cells

The mean and the standard deviation of plaque size are given in Table 2.

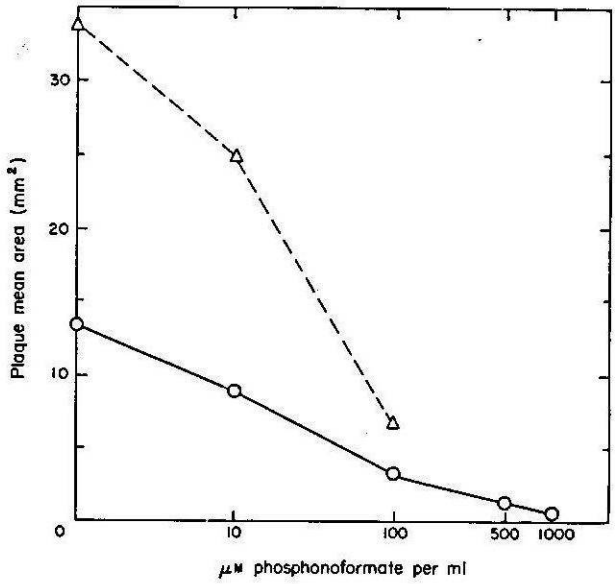

Fig. 1. Comparison between the reduction of the mean plaque size of IBR virus (○———○) and SHV (△- - - - -△) on GBK cells with increasing concentrations of trisodium phosphonoformate.

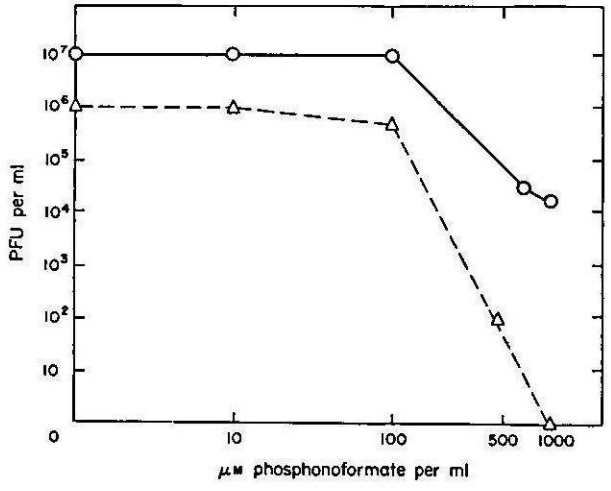

Fig. 2. Comparison between the decrease in the titres of IBR virus (○———○) and SHV (△- - - - -△) on GBK cells with increasing concentrations of trisodium phosphonoformate.

Normality test. The sizes of the plaque population produced by SHV followed a normal distribution in the cell cultures devoid of phosphonoformate, and when trisodium phosphonoformate was added at a concentration of 10 μM per ml. If 100 μM per ml of phosphonoformate was added to the medium, the distribution became slightly asymmetric. At higher concentrations (500 and 1000 μM per ml), the loss of titre was too great to permit statistical analysis. The square root of the data was taken to produce a normal distribution.

TABLE 2

PLAQUE SIZE OF SHV AND PHV ON CEF CELLS

Virus		*μM per ml phosphonoformate*				
		0	*10*	*100*	*500*	*1000*
SHV	Mean	28·842	23·005	3·888	—	—
	S.D.	7·908	7·700	2·870	—	—
PHV	Mean	14·432	11·179	4·502	4·861	3·445
	S.D.	7·051	6·128	3·747	4·420	3·001

Values are in mm², calculated from 50 plaques.

Although PHV plaque size distribution was far from normal if more than 10 μM per ml of trisodium phosphonoformate was added to the overlay medium, the square root of the plaque size always followed a normal distribution, except for a very slight asymmetry at a concentration of 500 μM per ml.

Analysis of variance. The presence of trisodium phosphonoformate induced a significant reduction in the mean plaque size ($P < 0{\cdot}0001$) for both viruses.

The *t* test showed a significant reduction in the mean plaque size of SHV at each concentration of phosphonoformate allowing the measurement to be made (0, 10 and 100 μM per ml) ($P < 0{\cdot}002$) (Fig. 3).

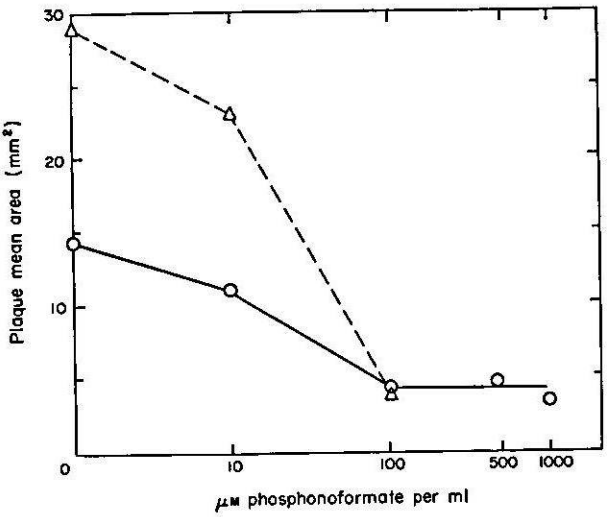

Fig. 3. Comparison between the reduction of the mean plaque size of PHV (○———○) and SHV (△- - - - -△) on CEF cells with increasing concentrations of trisodium phosphonoformate.

There was a significant reduction in the mean plaque size of PHV at a concentration of 10 and 100 μM per ml of phosphonoformate ($P < 0{\cdot}02$); higher concentrations did not induce a further reduction (Fig. 3).

Effect on the titres. Addition of 10 μM per ml of trisodium phosphonoformate had no effect on the titre of SHV but higher concentrations provoked a steady decrease.

The titre of PHV decreased only for phosphonoformate concentrations higher than 100 μM per ml, and to a smaller extent than SHV (Fig. 4).

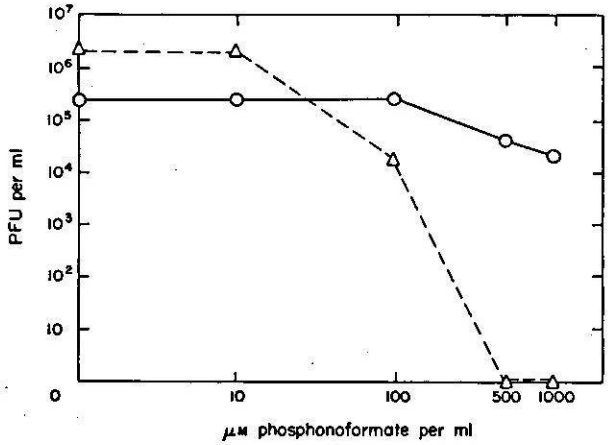

Fig. 4. Comparison between the decrease in the titres of PHV (○———○) and SHV (△- - - - -△) on CEF cells with increasing concentrations of trisodium phosphonoformate.

DISCUSSION

With the plaque assay, the numbers of plaques produced by IBR virus in GBK cells, and by SHV in GBK and CEF monolayers, were significantly reduced in the presence of high concentrations of phosphonoformate (500 and 1000 μM per ml). Our data agree with those previously published by Helgstrand *et al.* (1978), who found, with a different method of titration (end-point titration), a 90 per cent inhibition for SHV at a concentration of 100 μM per ml of phosphonoformate, an inhibition of 99 per cent at a concentration of 500 μM per ml, and for IBR virus an inhibition higher than 99·9 per cent at a concentration of 500 μM per ml.

For PHV, the decrease of the titre was less obvious and required higher concentrations of phosphonoformate than for SHV.

As far as IBR and SH virus are concerned, a significant reduction of the mean plaque size occurred in the presence of as low a concentration as 10 μM per ml of phosphonoformate and this reduction increased with the concentration of the drug.

Phosphonoformate was more inhibitory to SHV than to IBR virus, whereas Helgstrand *et al.* (1978) found no difference in their susceptibility to trisodium phosphonoformate.

PHV seems to be less susceptible to the effect of trisodium phosphonoformate than the other viruses tested, both in titre decrease and plaque size reduction. For instance, the increase of the concentration of phosphonoformate over 100 μM per ml did not further reduce the mean plaque size. This last finding might be important in view of a possible clinical application in the treatment of local lesions produced by PHV infection.

To explain the inhibition which has been observed, the question arises

whether the compound affects primarily viral replication or cellular metabolism.

No cytotoxic effect was observed in the uninfected control cells, as already described by Helgstrand *et al.* (1978), who showed that trisodium phosphonoformate at concentrations higher than 2·5 μM per ml has no effect on cellular RNA and protein synthesis. Trisodium phosphonoformate is a specific inhibitor of the DNA-polymerase (Reno *et al.*, 1978), but mammalian cells DNA-polymerase is far less susceptible to the action of the compound than is the herpesvirus-induced enzyme (Helgstrand *et al.*, 1978).

Moreover, resistance to phosphonoformate is described for some strains of turkey herpesvirus (Reno *et al.*, 1978).

The inhibitory effect present in our experiments seemed to be mainly due to a specific antiviral action since we observed that two different viruses grown in the same type of cell culture were differently inhibited, whereas one virus strain (SHV) was inhibited in the same way even when grown in cell cultures of various origins.

SUMMARY

The effect of various concentrations of trisodium phosphonoformate on the titre and the mean plaque size of pseudorabies virus (SHV), infectious bovine rhinotracheitis (IBR) virus and pigeon herpesvirus (PHV) was studied.

Phosphonoformate significantly reduced the mean plaque size of all three viruses whatever the concentration used. However, an increase of the concentration of phosphonoformate over 100 μM per ml in the overlay medium did not further reduce the mean plaque size of PHV.

Phosphonoformate also produced a decrease in the titre of the three viruses at the highest concentration.

SHV was the most susceptible to the effect of trisodium phosphonoformate and PHV was the least susceptible. This finding might be of significance for a possible clinical application in the treatment of local lesions produced by PHV infection.

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