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SELECTIVE INHIBITION OF HERPESVIRUS DNA SYNTHESIS BY FOSCARNET

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The inhibition of cellular and herpesvirus DNA synthesis by phosphonoformate (INN; foscarnet sodium) has been determined after isopycnic separation of cellular and viral DNA in CsCl gradients. The DNA synthesis was determined as the incorporation of ortho[^{32}P]phosphate and [^{3}H]thymidine into DNA. A 50% inhibition of herpes simplex virus DNA synthesis was observed at 50 μM phosphonoformate. At this concentration cellular DNA synthesis was not inhibited. At 500 μM phosphonoformate more than 95% of the viral DNA synthesis was inhibited, while the cellular DNA synthesis in infected and uninfected cells were inhibited to about 10%. The same results were obtained in both Vero and GMK cells and using either ortho[^{32}P]phosphate or [^{3}H]thymidine to label the newly synthesized DNA. The 50% inhibitory concentration of phosphonoformate was similar for inhibition of herpes DNA synthesis and plaque reduction.

foscarnet phosphonoformate herpes simplex virus DNA synthesis isopycnic centrifugation

INTRODUCTION

Foscarnet (trisodium phosphonoformate, PFA) has been shown to inhibit the replication of several herpesviruses [7, 10, 13] and to inhibit the herpesvirus-induced DNA polymerase [3, 7, 10]. These inhibitions were seen at concentrations not inhibitory to cell proliferation [12] or inhibitory to cellular DNA polymerases [3, 7].

The selectivity of antiviral drugs on cellular and viral DNA synthesis has earlier been determined as the amount of [^{3}H]thymidine incorporation into viral and cellular DNA in infected cells after various drug treatments [2].

We have determined the effect of PFA on herpes simplex virus type 1 (HSV-1) DNA and on cellular DNA synthesis in two cell lines and with two different methods of isotope labelling.

MATERIALS AND METHODS

Chemicals

Trisodium phosphonoformate (PFA) was synthesized at the Research and Development Laboratories at Astra Lakemedel AB by the methods of Nylén [8]. Phosphorous-32

orthophosphate (carrier-free, 1 mCi/ml) was from Radiochemical Centre, Amersham, methyl[^{3}H]thymidine (49.5 Ci/mmol) and Econofluor scintillation solution were from New England Nuclear, Boston, MA. Pronase (nuclease-free) was from Calbiochem, San Diego, CA, and optical grade of CsCl and DNase I were from Sigma, St. Louis, Miss. All other chemicals were of analytical grade.

Cells and virus

African green monkey kidney (GMK) and Vero cells were grown in minimal Eagle's medium (MEM) and medium 199 with Earle's modified salts with 1% non-essential amino acids respectively. The media were supplemented with 7% foetal calf serum, 20 mM Hepes buffer (pH 7.2), penicillin (120 μg/ml) and streptomycin (100 μg/ml). The cells were grown at 37°C with 5% CO_2 and routinely screened for mycoplasma contamination by the uridine/uracil incorporation method [11] and were found negative. Herpes simplex virus type 1 (HSV-1) strain C42 and plaque reduction determination on GMK and Vero cells have been described earlier [4].

Incorporation studies

Confluent monolayers of cells growing in 6-well tissue culture cluster dishes (35 mm in diameter) were washed once with prewarmed phosphate-buffered saline (PBS) and exposed to 1–2 p.f.u./cell of HSV-1 for 1 h at 37°C. The cells were washed once with 2 ml PBS to remove unadsorbed virus and, when labelled with ortho[^{32}P]phosphate, with 3 ml of phosphate-free Eagle's medium containing 20 mM Hepes, 5% foetal calf serum, dialyzed against phosphate-free medium, and then the indicated concentrations of PFA were added. After 2 h, 5–10 μCi/ml of ortho[^{32}P]phosphate or [^{3}H]thymidine was added and the incubation continued for another 16 h. The same method was used for mock-infected cells. An identical incorporation of [^{3}H]thymidine into viral and cellular DNA was observed when cells grown in medium with phosphate and without phosphate were compared. The incorporation was terminated by removal of the medium and each well was washed twice with 2 ml of ice-cold PBS. The cells were loosened with 0.1 ml trypsin–EDTA (0.5 g/l trypsin, 0.2 g/l EDTA, 0.95 g/l NaCl), and transferred to test tubes by washing each well with 2 × 0.5 ml PBS. Two wells were used for each drug concentration.

Isopycnic centrifugation

The cells were pelleted at 2000 × *g* for 10 min and the pellets were resuspended in 0.9 ml buffer containing 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Sarcosyl and 0.2% sodium dodecyl sulfate (pH 7.4). After 10 min at room temperature, 100 μl pronase (1 mg/ml) was added and the suspensions were incubated at 37°C for 3 h. 0.5 ml of each suspension was mixed with 4.5 ml 10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA (pH 7.4) and 6.08 g CsCl giving a density of 1.715 g/cm^3. The gradients were centrifuged at 55,000 r.p.m. for 24 h in a Sorvall ultracentrifuge, OTD-65 using the vertical rotor, TV-865. The gradients were collected in 100 μl fractions from the bottom

of the tubes. To each fraction was added 100 μl 2 M KOH and the mixtures were incubated at 37°C for 2 h, to degrade ^{32}P-labelled RNA. 50 μl of each fraction was spotted on a Whatman cellulose paper, washed twice in 5% trichloroacetic acid (TCA) and twice in ethanol. The filter papers were dried and counted in 5 ml Econofluor. The DNA density was determined by measuring the refractive index of each fifth fraction. The amount of TCA-precipitable radioactivity in each peak was used as a measure of the DNA synthesis.

RESULTS

Comparison of the incorporation of [^{3}H]thymidine and ortho[^{32}P]phosphate into HSV and cellular DNA

Uninfected and HSV-1 infected Vero cells were labelled in parallel cell cultures with ortho[^{32}P]phosphate and [^{3}H]thymidine as described in Methods. Pronase-treated cell lysates from the differently labelled cultures were mixed and banded in CsCl as described in Methods. As shown in Table 1, no significant difference in the proportion of ^{3}H and ^{32}P incorporation into cellular and viral DNA could be observed. The identity of the ^{32}P-labelled peaks was determined by freezing and thawing ortho[^{32}P]phosphate-labelled infected Vero cells three times and then digesting this lysate with 100 μg/ml of DNase I at 37°C for 1 h in the presence of 4 mM $MgCl_2$ and 0.2% deoxycholate. After this DNase treatment, sarcosyl and pronase were added as described in Methods. When this material was centrifuged in CsCl gradients, no ^{32}P peaks at the density of cellular or viral DNA were seen (not shown).

Inhibition of cellular DNA synthesis by PFA

The effect of increasing concentrations of PFA on GMK cellular DNA synthesis is shown in Fig. 1. At 100 μM PFA, no effect on cellular DNA synthesis could be observed, but at 500 μM a slight inhibition was seen. The same result was obtained with Vero cells as shown in Table 2. In these experiments DNA synthesis was determined as the incor-

TABLE 1

Incorporation of [^{3}H]thymidine and ortho[^{32}P]phosphate into HSV-1 and cellular DNA

Label	HSV-1 infected cells		Uninfected cells
	HSV-1 DNA	Cell DNA	Cell DNA
[^{3}H]Thymidine, c.p.m.	10,976	21,083	31,337
Ortho[^{32}P]phosphate, c.p.m.	6,080	11,599	17,206
Ratio ^{3}H/^{32}P	1.81	1.82	1.82

DNA from Vero cells was banded in CsCl gradients and the amount of radioactivity in each peak was determined.

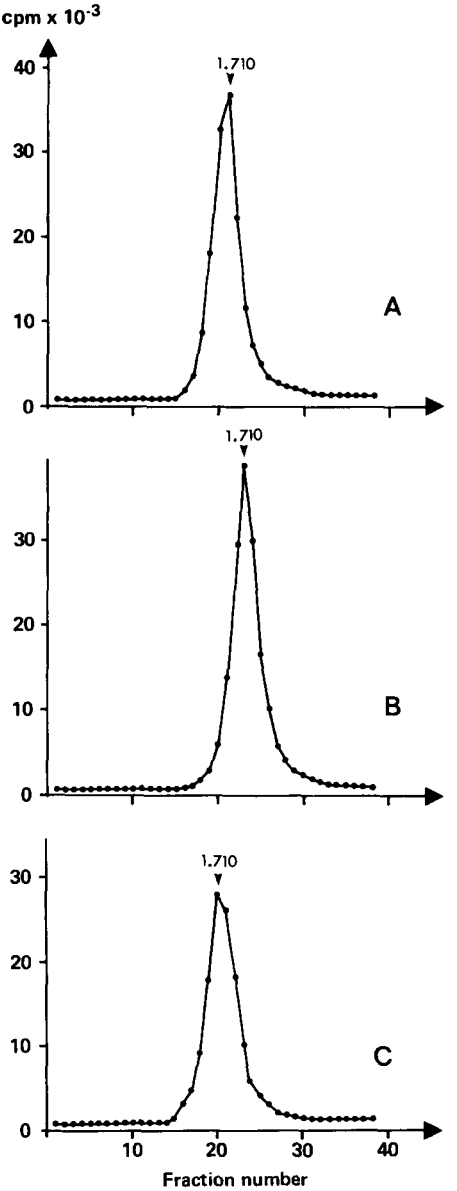

Fig. 1. Effect of PFA on the synthesis of cellular DNA. GMK cells were treated with different concentrations of PFA and isopycnic centrifugation of ortho[^{32}P]phosphate-labelled DNA was as described in Materials and Methods. The density of the peak is given at the arrow. A) Control cells, no PFA added. B) 100 μM PFA. C) 500 μM PFA.

TABLE 2

Effect of PFA on DNA synthesis in uninfected GMK and Vero cells

PFA, μM	DNA synthesis % inhibition of control	
	GMK cells	Vero cells
100	-6	-6
500	14	12

The amount of DNA synthesized was determined after isopycnic contrifugation in CsCl gradients and compared to the amount made without PFA.
Ortho[^{32}P]phosphate was used to label the DNA as described in Materials and Methods.

poration of ortho[^{32}P]phosphate into DNA. The same result was obtained using [^{3}H]-thymidine as label and excluding the KOH treatment (not shown). Density determination showed that the cellular DNA banded at a density of 1.710 g/cm^3 both without PFA treatment and with 500 μM PFA in the medium.

Selective inhibition of HSV-1 DNA synthesis by PFA

In HSV-1 infected GMK cells 50 μM PFA inhibited viral DNA synthesis by about 50% and at 500 μM all viral DNA synthesis was stopped (Fig. 2). Cellular DNA synthesis in the HSV-1-infected cells was not affected by 50 μM PFA and inhibited only 11% at 500 μM PFA (Fig. 2). The same pattern of selective inhibition was seen in Vero cells as shown in Table 3. In Vero cells 250 μM PFA completely blocked HSV-1 DNA synthesis without any significant effect on cellular DNA synthesis. The density of the viral DNA was determined to be 1.726 g/cm^3 and of the cellular DNA as 1.710 g/cm^3. These densities were the same for DNA from control and PFA treated cells. The same inhibition was observed with ortho[^{32}P]phosphate and [^{3}H]thymidine as labelled precursor to DNA (not shown). The plaque reduction caused by PFA on HSV-1 strain C42 is shown in Table 3. A 50% inhibition was seen at about 50 μM PFA.

DISCUSSION

The difference in inhibition of viral and cellular DNA synthesis by antiviral drugs has earlier been determined by isopycnic banding of [^{3}H]thymidine-labelled DNA in CsCl gradients [2]. The use of [^{3}H]thymidine incorporation as a measure of DNA synthesis could be misleading if the tested drug interferes with the phosphorylation of thymidine or competes with thymidine incorporation into DNA. To avoid this possibility ortho-[^{32}P]phosphate incorporation was used to measure DNA synthesis and was compared to the incorporation of [^{3}H]thymidine. In infected cells the relative incorporation of ortho[^{32}P]phosphate into cellular and viral DNA was similar to the incorporation of

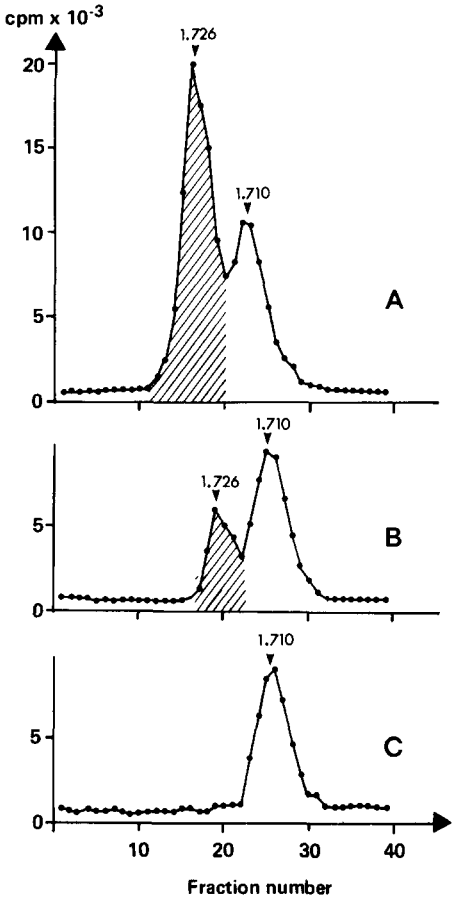

Fig. 2. Selective inhibition of HSV-1 DNA synthesis by PFA. HSV-1 infected GMK cells were labelled with ortho[^{32}P]phosphate at different PFA concentrations. The DNA was separated on CsCl gradients as described in Materials and Methods. The striped area shows the viral DNA. The density of the peaks are given at the arrows. A) Control infected cells, no PFA added. B) Infected cells, 100 μM PFA. C) Infected cells, 500 μM PFA.

[^{3}H]thymidine (Table 1). It was also found that the ^{32}P-labelled material banding as DNA was degraded by DNase I. Since HSV DNA has a lower thymidine content than cellular DNA, a lower ratio of ^{3}H/^{32}P could have been expected for labelling viral DNA. The reason for the present finding is not clear. The effect of PFA on [^{3}H]thymidine and ortho[^{32}P]phosphate incorporation into cellular and HSV-1 DNA were identical for the two isotopes. This agrees with the findings that PFA in a cell-free DNA polymerase assay is a non-competitive inhibitor of nucleoside triphosphates [3] and does not affect the phosphorylation of thymidine in a cell-free assay (A. Larsson, unpublished observation). A 50% inhibition of HSV-1 DNA synthesis was obtained at about 50 μM PFA in both GMK and Vero cells. This pattern is different from that obtained with acycloguanosine

TABLE 3

Inhibition by PFA of DNA synthesis and plaque formation in HSV-1 infected GMK and Vero cells (% inhibition of control)

PFA (μM)	GMK cells			Vero cells		
	Cellular DNA synthesis	HSV-1 DNA synthesis	Plaque formation	Cellular DNA synthesis	HSV-1 DNA synthesis	Plaque formation
50	2	54	55	-6	58	30
100	4	74	>90	-1	73	76
250	–	–	>90	6	100	>90
500	11	100	–	–	–	–

The amount of DNA synthesized at different PFA concentrations was determined as the incorporation of ortho[^{32}P]phosphate into the different types of DNA separated by CsCl gradient centrifugation. Cell cultures without PFA were used as controls. Plaque assays were as described in Materials and Methods.

(aciclovir) in these two cell lines where the inhibition of HSV-1 is significantly more effective in Vero cells than in GMK cells [6]. Since the concentration of nucleosides and nucleotides could influence the effect of nucleoside analogues [5], it is likely that their inhibitory effects will be more dependent on cell type and condition than the inhibitory effect of PFA [6].

The 50% inhibition of HSV-1 DNA synthesis at 50 μM PFA corresponds to the 50% plaque reduction observed with this HSV-1 strain (Table 3). This concentration is considerably higher than that necessary to cause a 50% inhibition of the DNA polymerase induced by the C42 HSV-1 strain, 0.4 μM [4]. The intracellular concentration of PFA remains, however, to be determined. The weak (10–14%) inhibition of cellular DNA synthesis by 500 μM PFA was the same for infected and uninfected cells. This corresponds well with results from actively growing cells where an about 20% inhibition of thymidine incorporation at 500 μM of PFA and a 50% inhibition of thymidine incorporation and cell proliferation at 1000 μM of PFA were seen [12].

Phosphonoacetate, which is structurally similar to PFA, has earlier been reported to selectively inhibit the incorporation of [^{3}H]thymidine into HSV-1 DNA [2, 9]. The present results with PFA correspond closely to those obtained with phosphonoacetate which at 100 μg/ml caused a total inhibition of HSV DNA synthesis, determined as [^{3}H]thymidine incorporation, while not inhibiting cellular DNA synthesis [2].

As pointed out by Drach and Shipman [2], isopycnic centrifugation of HSV and cellular DNA is a convenient method to determine the selectivity of antiviral drugs. In some instances, the method makes it possible to observe density shifts caused by incorporation of drugs like idoxuridine [1]. To make an unambiguous determination of DNA synthesis, ortho[^{32}P]phosphate incorporation is preferable to the incorporation of [^{3}H]thymidine, especially when the effect of nucleoside analogues is determined.

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Note: Repeated experiments with [^{3}H]thymidine and ortho[^{32}P]phosphate as double label in the same HSV-1-infected cell cultures resulted in a ratio of $^{3}H/^{32}P$ label in cellular DNA which was 1.7 times higher than this ratio in HSV-1 DNA and close to the expected value.