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Phosphonoformate Inhibition of Visna Virus Replication

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Phosphonoformate (PFA) inhibits multiplication of visna virus in sheep choroid plexus cells; a 50% reduction of virus yield was obtained by 20 to 80 μM PFA. Morphological changes, such as syncytial formation and cell degeneration, could be reversibly prevented by PFA. Cell growth was not significantly affected at 500 μM PFA, although prolonged treatment with 2 mM PFA did arrest cell growth. Cell-free reverse transcriptase activity primed with various synthetic template-primers was inhibited about 90% in the presence of 100 μM PFA. The results from kinetic experiments suggested that reverse transcriptase was utilized early but not late in the infection cycle. A structurally related substance, phosphonoacetate, did not inhibit visna virus multiplication and had no inhibitory effect on reverse transcriptase activity at a concentration of 500 μM.

Ever since the discovery of reverse transcriptase (1, 14), attempts have been made to find selective inhibitors of this enzyme. Most compounds appeared to be nonspecific or not very active when tested for inhibition of reverse transcriptase (for review, see reference 3). Phosphonoacetate (PAA) has been shown to inhibit the replication of Gross murine leukemia virus in mouse cells at concentrations which caused cell death (10). We have recently found that low concentrations of phosphonoformate (PFA), which is structurally related to PAA, inhibit reverse transcriptase from various retroviruses (B. Sundquist and B. Öberg, submitted for publication), whereas PAA had only a weak inhibitory effect on reverse transcriptase activity. PFA and PAA are effective inhibitors of herpesvirus DNA polymerase (7, 9) at concentrations which do not affect cell proliferation (13). PFA also inhibits influenza virus RNA polymerase (7) and hepatitis B DNA polymerase (E. Nordenfelt, E. Helgstrand, and B. Öberg, Int. Congr. Virol., 4th, Abstr. no. W 28/5, 1978).

In the present report we have used visna virus as a model system to study the inhibitory effect of PFA multiplication of a retrovirus. This virus causes a demyelinating disease of the brain in sheep (11, 12), and it has biochemical properties similar to those of retroviruses (6).

Low concentrations of PFA, but not PAA, inhibited the replication of visna virus in cultures of sheep choroid plexus (SCP) cells. Cytopathic effects (CPE), like syncytial formation and cell degeneration, could be prevented by PFA, and the effects were reversible.

MATERIALS AND METHODS

Cell cultures. Monolayer cell cultures were prepared from SCP cells. The choroid plexus was removed from the brain by dissection, minced, washed twice with phosphate-buffered saline, and treated with 2.5 mg of trypsin per ml at +37°C for 30 min. Dispersed cells were seeded in plastic flasks and incubated at +37°C. SCP cells were propagated in 250- or 800-ml plastic flasks (Nunclon), using Eagle minimal essential medium supplemented with glutamine (30 mg/liter), 10% calf serum, 5% lamb serum, and antibiotics. Virus-infected cells were maintained in medium containing 2% heat-inactivated lamb serum.

Virus. Visna virus, prototype strain K 1514, was kindly provided by G. Pétursson and P. Pàlson, Institute of Experimental Pathology, University of Iceland, Reykjavik.

Infection of cells. Confluent SPC cell monolayers were inoculated with visna virus at a multiplicity of 1 to 2 50% tissue culture infective doses ($TCID_{50}$)/cell. After adsorption for 3 h, medium was added to the cell cultures, and the incubation at 37°C was continued. When distinct CPE appeared, medium was removed and stored at −70°C until analyzed.

Infectivity assay. Confluent SCP cells in roller tube cultures, six to eight per dilution, were washed twice with phosphate-buffered saline, pH 7.2, and 0.1-ml quantities were inoculated with 10-fold dilutions of virus in 1 ml of maintenance medium. The cultures were incubated at +37°C in a roller drum. The cells were examined for CPE on days 10 and 16 of incubation; $TCID_{50}$ were calculated by the method of Reed and Muench (8).

Preparation of radioactive virus. Cell cultures were infected with visna virus. At 4 days postinfection, [^{3}H]uridine (10 μCi/ml) was added to the culture medium, and incubation was continued for 12 h. Radioactive virus was concentrated from the culture

medium as described below.

Virus purification. Tissue culture fluid from virus-infected cells was clarified by low-speed centrifugation at 10,000 × *g* for 15 min. Virus was sedimented from the supernatant at 85,000 × *g* for 90 min, and the pellet was resuspended in 1/10 of the initial volume in TEN buffer (50 mM Tris-hydrochloride [pH 7.5], 0.15 M NaCl, 1 mM EDTA). The concentrates were layered on a preformed 10 to 50% linear sucrose gradient in TEN buffer. After 4 h of centrifugation at 190,000 × *g*, fractions with densities of 1.14 to 1.16 g/cm^3 were pooled and diluted to less than 10% sucrose, and virus was again pelleted at 85,000 × *g* for 90 min. All operations were performed at +4°C. Concentrated virus was dissolved in TEN buffer and stored at −70°C. Virus used for reverse transcriptase assays were dissolved in 50 mM Tris-hydrochloride (pH 8.3)–2 mM dithiothreitol–0.025% Triton X-100–50% glycerol and frozen at −20°C.

Assay for reverse transcriptase. DNA polymerase activity was measured in 100 μl containing 50 mM Tris-hydrochloride (pH 8.3), 80 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.05% Triton X-100, and 2.5 μg of synthetic template-primer. With $(rA)_n \cdot (dT)_{10}$ as template-primer, 10 μM dTTP–0.4 μM [*methyl*-^{3}H]dTTP (60 Ci/mmol) was included in the reaction mixture. With $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ as template-primers, 10 μM dGTP–2 μM [^{3}H]dGTP (9 Ci/mmol) was used.

Two micrograms of detergent-treated virus was added to the reaction mixture as enzyme. The reaction was stopped after 30 min at 30°C by the addition of an equal volume of 20% trichloroacetic acid, containing 0.1 mM sodium pyrophosphate. The precipitates were collected on nitrocellulose membrane filters (Millipore Corp.), washed with 5% trichloroacetic acid and ethanol, dried, and counted in a liquid scintillation spectrometer. The reaction rates were linear for at least 30 min.

Chemicals and template-primers. Trisodium PFA and PAA were gifts from B. Öberg, Astra, Södertälje, Sweden, and were used as the sodium salts in solutions adjusted to pH 7.2. The template-primers $(rA)_n \cdot (dT)_{10}$, $(rC)_n \cdot (dG)_{12-18}$, and $(dC)_n \cdot (dG)_{12-18}$ were obtained from P-L Biochemicals Inc. [*methyl*-^{3}H]-dTTP (60 Ci/mmol), [^{3}H]dGTP (9 Ci/mmol), and [^{3}H]uridine (50 Ci/mmol) were purchased from New England Nuclear. Unlabeled triphosphate nucleotides, dithiothreitol, and Triton X-100 were from Sigma Chemical Co.

RESULTS

Effect of PFA on growth of visna virus. Uninfected cells could be passaged at least five times in the presence of 500 μM PFA without significantly affecting the cell proliferation. At 2 mM PFA the cell generation time increased, and cell death occurred after two to three passages. PAA at the same concentrations as PFA showed a similar effect on cell proliferation.

Monolayer cultures of SCP cells were infected with visna virus at 1 to 2 $TCID_{50}$/cell. Simultaneously with the virus, PFA or PAA was added at different concentrations. When control cells showed typical visna CPE, the culture fluid was withdrawn and titrated for infectious virus particles.

Figure 1 shows the effects of PFA and PAA on visna virus multiplication in SCP cells. A 50% reduction of the virus titer was observed at 20 μM PFA, and the titer decreased from $10^{6.7}$ to $10^{4.6}$ $TCID_{50}$ between 0 and 100 μM PFA. At 200 μM PFA the virus titer decreased 1,000-fold compared with control cells without any drug, but higher concentrations of PFA did not greatly enhance the inhibitory effect.

PAA at a concentration of 500 μM did not show any inhibitory effect on visna virus multiplication.

Syncytial formation and cell degeneration could be seen 6 days after virus infection (Fig. 2B), whereas uninfected cultures showed a regular texture of fibroblasts (Fig. 2A). The addition of 500 μM PFA to infected cells resulted in a total inhibition of the CPE, and the cell monolayer reproducibly had a texture similar to that of uninfected cultures (Fig. 2C), but degenerated cells were observed if the incubation time was prolonged. Figure 2D illustrates that treatment with 500 μM PAA had no effect on development of cytopathic changes.

Reversible inhibitory effect of PFA. SCP cells were infected with visna virus in the presence or absence of 500 μM PFA. After an incubation time of 150 h, the cell monolayers were washed free of PFA, fresh medium was added, and the incubation was continued. Figure 3 demonstrates that control cells produced a virus titer that leveled off at about 75 h postinfection and reached a final titer of $10^{6.5}$ $TCID_{50}$/ml. Incubation in the presence of 500 μM PFA reduced the virus titer by about 3 log units as compared to the titer in control cultures. After removal of

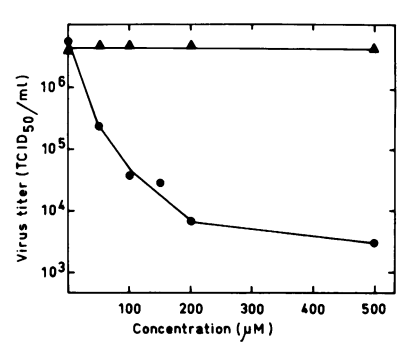

FIG. 1. *Inhibition of visna virus synthesis by PFA and PAA. SCP cell cultures were infected with visna virus, and PFA (●) or PAA (▲) was added at different concentrations. Culture media were assayed for visna virus as described in the text.*

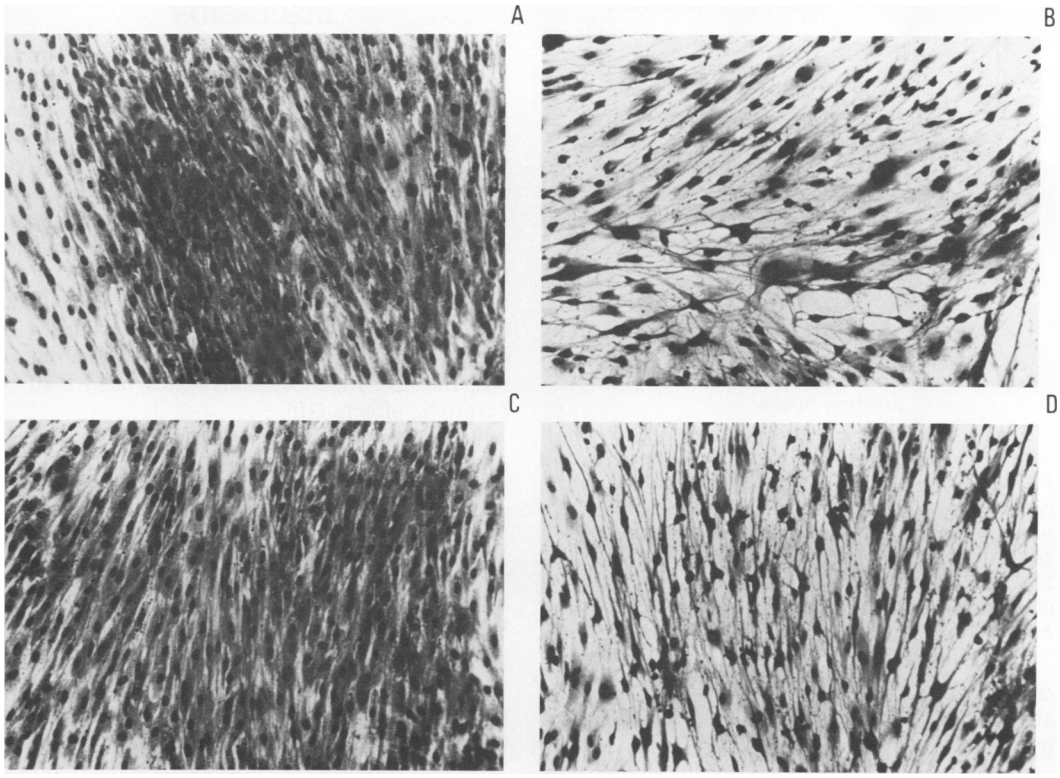

FIG. 2. *CPE in visna virus-infected SCP cells treated with PFA or PAA. SCP cell cultures were infected with visna virus as described in the text. After 4 days of incubation, cells were stained with Giemsa. ×60. (A) Uninfected SCP cells; (B) visna virus-infected SCP cells; (C) visna virus-infected SCP cells plus 500 μM PFA; (D) visna virus-infected SCP cells plus 500 μM PAA.*

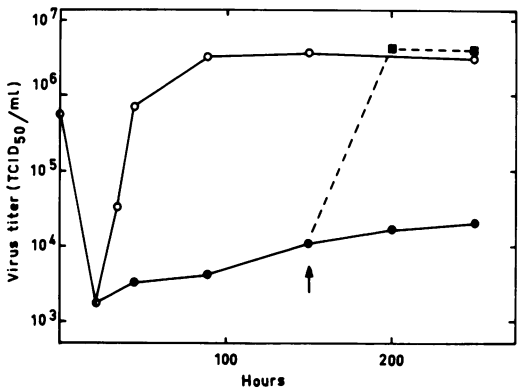

FIG. 3. *Reversibility of PFA inhibition. SCP cell cultures were infected in the presence of 500 μM PFA. After 150 h, one cell culture was washed with fresh culture medium and the incubation was continued without PFA. The culture media were assayed for visna virus. The arrow indicates removal of PFA. Symbols: (○) control cells; (●) cells continuously treated with PFA; (■) cells after removal of PFA.*

PFA the virus titer increased from $10^{3.7}$ $TCID_{50}$ to a final titer of $10^{6.6}$ $TCID_{50}$ within an incubation time of 50 h.

Effect of PFA on formation of visna particles. To study the effect of PFA on the release of visna virus particles, cells were infected and different concentrations (50, 100, and 200 μM) of PFA were added to the cell medium. Four days after infection, [^{3}H]uridine was added to the medium, and the incubation was continued for 12 h. Virus was concentrated from the culture fluid and analyzed in preformed 10 to 50% sucrose gradients.

Figure 4 shows the profile of radioactive material separated on sucrose gradients. Panel A shows the material from infected, untreated cells. The main peak of radioactivity had a density of 1.14 to 1.16 g/cm^3, and this material contained a RNA species that had a sedimentation coefficient of about 70S in sucrose. Furthermore, it cosedimented with an RNA-dependent DNA polymerase (reverse transcriptase) which was absent in gradients with materials from uninfected cells. It was concluded that the peak material corresponding to a density of 1.14 to 1.16 g/cm^3 represented intact virus particles. Panels B, C, and D show that increasing concentrations of PFA decreased the release of intact virus particles into the culture medium. A

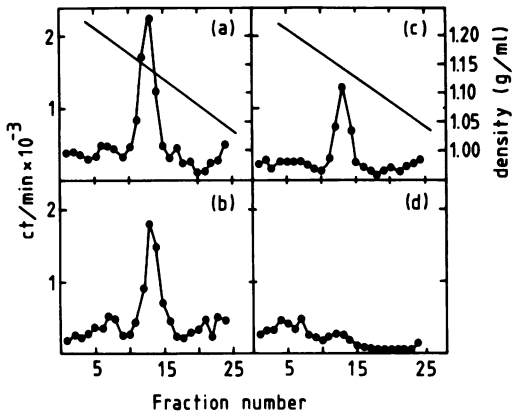

FIG. 4. *Inhibition of visna virus release by PFA. SCP cell cultures were infected with visna virus, and PFA at different concentrations was added to the culture medium. Virus particles labeled with [^{3}H]-uridine for 24 h were concentrated and analyzed on 10 to 50% sucrose gradients as described in the text. Shown is the profile of total radioactivity per fraction of material prepared from cell cultures treated with PFA at: (a) 0 μM; (b) 50 μM; (c) 100 μM; (d) 200 μM.*

50% reduction in radioactivity was observed at about 80 μM PFA, and 200 μM PFA inhibited the release of virus particles by about 90% as estimated from the radioactivity in the fractions representing intact virus particles.

Kinetics of PFA inhibition. SCP cells were infected with visna virus at a multiplicity of 5 $TCID_{50}$/cell. PFA, at a concentration of 500 μM, was added to the cell medium at different times after infection. The cells were harvested 4 days postinfection, and the virus titer was determined. At this time the one-step growth curve of the virus had leveled off (Fig. 3). The data presented in Fig. 5 show that PFA was an effective inhibitor from zero time and up to about 15 h after infection, whereas it had no inhibitory effect when added later than 24 h after infection.

PFA inhibition of reverse transcriptase activity. Visna virus particles were concentrated from culture medium, and the reverse transcriptase was activated by detergent treatment of the particles. The inhibitory effect of PFA and PAA on reverse transcriptase activity was tested by using two synthetic RNA/DNA hybrids, $(rA)_n \cdot (dT)_{10}$ and $(rC)_n \cdot (dG)_{12-18}$, and one DNA/DNA hybrid, $(dC)_n \cdot (dG)_{12-18}$ (Table 1). Addition of 100 μM PFA inhibited the enzyme activity by 90% with all three template-primer combinations. PAA at a concentration of 100 μM did not show any inhibitory effect on the enzyme activity, whereas 500 μM PAA inhibited the reactions primed with $(rA)_n \cdot (dT)_{10}$ and $(dC)_n \cdot (dG)_{12-18}$ by 30% but that primed with $(rC)_n \cdot (dG)_{12-18}$ by only 12%.

DISCUSSION

Viruses belonging to the retrovirus family, both mammalian and avian oncornaviruses, have for a long time been known to produce tumors in virus-infected animals (5). Attempts to find a specific and nontoxic inhibitor of reverse transcriptase (1, 14) have met with little success (for review, see reference 3). Allaudeen and Bertino found that 400 μM PAA could inhibit by 62% the release of type C particles from a lymphoid leukemia cell line, L 1210 (Fed. Proc. **36**:2430, 1977). PAA has also been claimed to inhibit replication of Gross murine leukemia virus, but the high concentrations used lead to cytotoxic effects (10).

We have recently shown that PFA is a general inhibitor of reverse transcriptase (Sundquist and Öberg, submitted). In the present study we describe the effects of PFA on replication of visna virus. The cytotoxicity tests with PFA or PAA on SCP cells are in agreement with those of Stenberg and Larsson (13), who reported that 500 μM PFA did not significantly affect cell proliferation of human lung cells and HeLa cells.

PFA is clearly a potent inhibitor of visna virus multiplication; the CPE caused by visna virus replication in SCP cells could be inhibited by

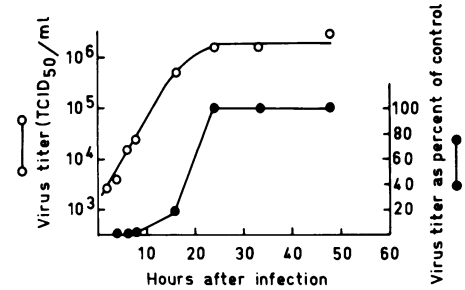

FIG. 5. *Time dependence for inhibition by PFA. SCP cell cultures were infected with visna virus as described in the text. A concentration of 500 μM PFA was added at different times after infection. On day 4 postinfection the cell culture media were assayed for visna virus.*

TABLE 1. *PFA and PAA inhibition of visna virus reverse transcriptase activity, using various template-primer combinations*

Template-primer	Inhibition (%)[a]			
	100 μM PFA	500 μM PFA	100 μM PAA	500 μM PAA
$(rA)_n \cdot (dT)_{10}$	90	>99	4	31
$(rC)_n \cdot (dG)_{12-18}$	92	94	−8	12
$(dC)_n \cdot (dG)_{12-18}$	93	>99	−5	28

[a] Percent inhibition is the inhibition by PFA in comparison with a control without drug. Negative values indicate stimulation over the control values.

50% by the addition of 20 μM PFA, whereas PAA had no effect on virus replication (Fig. 1 and 2). The end point titration method that we have used is based on the degree of CPE in infected cells, i.e., the formation of degenerated cells which are shown in Fig. 2B. It has been shown that virus particles are released by budding from cells, which are seen as stellate degenerated cells late in the infection cycle (4). However, it could not be ruled out that the CPE observed depended upon other factors not necessarily associated with virus synthesis. The experiment shown in Fig. 4 demonstrated that the release of intact virus particles from infected cells was inhibited by PFA; it was calculated that 80 μM PFA was required to inhibit release of virus particles by 50%. The discrepancy between the two values (80 and 20 μM) necessary for 50% inhibition could be explained by the fluctuations known to be involved in the end point titration method.

The inhibitory effect of PFA could be reversed (Fig. 3). The lag period from the removal of PFA and until cells produced normal virus titers was about 50 h compared with a viral multiplication cycle of about 75 h. Thus, cells may have been arrested in an early event in the lytic cycle in the presence of PFA. This idea is strengthened by the results obtained from kinetics of PFA inhibition (see below). The increasing virus titer in the presence of PFA (Fig. 3) and the appearance of polykaryocytes in infected cells after prolonged treatment with 500 μM PFA may be due to selection of resistant virus. However, so far we have not been able to isolate such a PFA-resistant visna virus mutant.

Some event in the first part of the lytic cycle, from 0 to 15 h postinfection, appeared to be the target for PFA (Fig. 5). The most likely event is the transcription of virus RNA into a DNA by the virus-coded reverse transcriptase. The first part of the lytic cycle (from 0.5 to 10 h) corresponds to the phase of proviral DNA synthesis, and it has been suggested that proviral DNA is detected approximately 3 h after infection (1). Cell-free visna virus reverse transcriptase activity was effectively inhibited by 100 μM PFA, whereas PAA had no significant inhibitory effect (Table 1). These results are in agreement with the above-presented results on replication of visna virus in SCP cells (Fig. 1). The second part of the lytic cycle (between 10 and 72 h postinfection), which is characterized by proviral DNA transcription into virus RNA (1), does not seem to be affected by PFA (Fig. 5).

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