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Antiviral activity of phosphonoformate on rotavirus transcription and replication

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Abstract

The antiviral effect of foscarnet (PFA) on the replication of rotavirus, a member of the *Reoviridae*, was studied. The pyrophosphate analogue is an effective inhibitor of several viral polymerases acting on the enzyme pyrophosphate biding site. Replication of rotavirus in MA104 cells using different u.o.i. was inhibited by PFA in a concentration dependent manner, due to the inhibition of both plus- and minus-strand RNA synthesis. The addition of PFA to infected cells was specific for the inhibition of viral replication since uninfected cell incubated at the same PFA concentrations did not exhibit any cytotoxic effect. The 50% inhibitory effect of PFA on in vitro mRNA synthesis was obtained at a concentration of 150 μM.

Over 80% of the in vitro minus-strand RNA synthesis was inhibited at a concentration of 320 μM, when PFA was assayed using replicase-enriched cell infected fraction. The results suggest that the effect may be due to an interaction of PFA with the viral polymerase, since this protein catalyses both plus- and minus-strand RNA synthesis. The results of experiments using the temperature-sensitive viral polymerase mutant show that the mutant is less sensitive to PFA, suggesting that this polypeptide is the target for PFA.

Keywords: Rotavirus; Transcription-replication; Phosphonoformate

1. Introduction

Rotaviruses, members of the Reoviridae family are the major cause of severe childhood gastroenteritis world wide (Estes, 1990). The prolonged course of the rotaviral

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disease in many infants, is caused by continued replication of virus in superficial epithelial cells of the small intestine or by sequential infection (Kapikian and Chanock, 1990; Spencer et al., 1983). At present, the disease is controlled only by treating the symptoms, replacing the body fluids and electrolytes (Kapikian and Chanock, 1990; Kapikian et al., 1978; Santosham et al., 1982). Several efforts have been made to produce rotaviral vaccines, but up to date they have failed or are in experimental stage of development (Conner et al., 1993; Pérez-Schael et al., 1990).

The viral genome consists of eleven segmented double-stranded RNA's contained in a core made of proteins VP1, VP2, VP3, surrounded by a double-shelled protein capsid. The outer shell contains VP4 and VP7 and the inner shell is made of the major viral polypeptide, VP6 (Estes, 1990; Estes and Cohen, 1989). Viral particles lacking the outer shell, called single-shelled particles, obtained either from infected cells or purified from viral particles have in vitro transcriptional activity capable to synthesize viral MRNA using as template each double-stranded RNA segment (Spencer and Arias, 1981). The non-structural rotavirus proteins NS53, NS35 and NS34 might play an important role in the minus-strand RNA synthesis (Patton, 1993). They might be responsible for the viral mRNA production and also be part of the subparticle that synthesize and encapsidate the genomic RNA (Gallegos and Patton, 1989; Helmberger-Jones and Patton, 1986; Kattoura et al., 1992; Sandino et al., 1988). The minus-strand synthesis is an asymmetrical process where each one of the eleven mRNA segments acts as a template for the synthesis of negative-strand RNA to produce double-stranded RNAs (Patton, 1986).

In previous reports, the antiviral properties of a series of nucleoside derivatives on rotavirus infected cell cultures and on in vitro RNA synthesis were studied (Pizarro et al., 1991; Smee et al., 1982). The results showed that some of these analogues inhibited the virus multiplication by interfering with the plus-RNA (mRNA) synthesis but not the minus-strand RNA synthesis (RNA replication). Other group of analogues such as the 2′,5′-oligoadenylates inhibited transcriptions but not the rotavirus minus-strand RNA synthesis in vitro (Ríos et al., unpublished). These results might be explained because, the synthesis of the minus- and plus-strand RNA is carried out by different rotavirus subparticles, where participate the same viral polypeptides. These particles, differ by the presence of non-structural polypeptides or the inner capsid made of VP6. (Helmberger-Jones and Patton, 1986; Mansell and Patton, 1990; Sandino et al., 1986).

The purpose of the present communication is to describe investigations of the effect of phosphonoformic acid (foscarnet, PFA), a non-nucleoside pyrophosphate analogue inhibitor of viral polymerases, on the in vitro plus- and minus-strand RNA synthesis of simian rotavirus. The effect of PFA on rotavirus replication in infected cells was determined by adding the compound under different conditions to the cell cultures.

2. Materials and methods

2.1. Virus and cells

Simian rotavirus SA-11 was propagated in MA104 cells infected at different multiplicities of infection (m.o.i.), from 1 to 10. Infected cultures were maintained in

minimum essential media (MEM) without serum but containing 2 μg/ml trypsin until all cells displayed a cytopathic effect. The virus was purified from the cell lysate as previously described (Sandino et al., 1988).

2.2. Phosphonoformate effect on rotavirus infection

MA104 cells were seeded on 5-cm^2 culture tubes and incubated at 37°C for 24 hours in MEM containing 5% fetal calf serum. At 2.5 hours before rotavirus infection, the cells were washed with MEM without serum and incubated with different concentrations of phosphonoformate and 20 μg/ml DEAE-Dextran. Then the monolayers were washed and infected with 1 and 10 m.o.i. of rotavirus activated with 10 μg/ml of trypsin for 30 min. After 60 min adsorption, the cells were washed with phosphate-free MEM and incubated in phosphate-free MEM containing 20 μCi/ml [^{32}P]-ortophosphoric acid and the same phosphonoformate concentrations for eight hours. At the end of the incubation of the cells were resuspended in hypotonic buffer, extracted with phenol-chloroform and ethanol-precipitated. After overnight incubation at −20°C, the viral RNA was obtained by centrifugation at 14,900 *g* for 30 minutes and then the double stranded RNA was analyzed by polyacrylamide gel electrophoresis and autoradiography.

2.3. Cytotoxicity assay

The assay using MA104 cells, was carried out as described by Shigeta et al. (1992). The foscarnet effect was determined by counting the viable cell numbers with a hemocytometer. The number of viable cells was determined by trypan blue exclusion after 24 hours of incubation with the compound.

2.4. Preparation of viral particles for in vitro plus- and minus- strand RNA synthesis

The SA-11 rotavirus strains were grown by infection of MA104 cells, using m.o.i. of 1–2, and when 90–100% of cells showed cytopathic effects, the cultures were freeze-thawed three times and centrifugated at 56,800 *g* for 2.5 hours and processed as described elsewhere (Sandino et al., 1988). Intracellular viral particles with minus-strand RNA synthesis activity were obtained from confluent monolayers of MA104 cells infected with virus stock at a m.o.i. of 5–10, under the same conditions as above. At six hours post-infection, the medium was withdrawn and the monolayers treated with 1–2 ml of hypotonic buffer (3 mM Tris-HCl, pH 8.1; 0.5 mM $MgCl_2$; 3 mM NaCl) and the replicative subviral particles were then purified as previously described (Helmberger-Jones and Patton, 1986).

2.5. Assays for in vitro plus- and minus-strand RNA synthesis

Purified rotavirus particles were assayed for RNA polymerase activity for plus-strand synthesis using purified heat-treated double-shelled viral particles incubated in a 25-μl reaction mixture containing 120 mM Tris-HCl (pH 8.5), 14 mM MgCl2, 40 mM NaCl,

0.2 mM *S*-adenosylmethionine, 4 mM each ATP, CTP and GTP and 0.2 mM of α-[^{32}P] UTP (SA: 400 cpm/pmol) or 0.24 mM of [^{3}H] UTP (SA: 100 cpm/pmol). The mixture was then incubated for 30 minutes at 45°C and the reaction products were analyzed by determination of the acid insoluble radioactivity or subjected to electrophoresis in 8 M urea-polyacrylamide gels as described elsewhere (Valenzuela et al., 1991). The effect on the reaction of foscarnet was expressed as the percentage of RNA polymerase activity compared with a control done without the analogue. Minus-strand RNA synthesis was determined by the synthesis of full length double-stranded RNA by the replicase activity associated with subviral particles using a modification of the assay initially described by Patton (1986). The conditions were identical to those described above for plus-strand RNA synthesis except that *S*-adenosylmethionine was eliminated from the mixture and α-[^{32}P] UTP (SA: 3000 cpm/pmol) was used instead of tritium-labelled UTP. The reaction mixture was incubated for 60 minutes at 45°C; at the end of the incubation, the mixture was cooled in ice and incubated for other 10 min at 37°C with 10 μg/ml pancreatic RNase A. The replicase activity was determined by analysis of the reaction products, after phenol extraction and ethanol precipitation, by RNA gel electrophoresis in 7% polyacrylamide gels where the presence of the 11 double-stranded RNA segments was determined according to the migration of genomic RNA obtained from purified virus particles described elsewhere (Vásquez et al., 1993).

3. Results

3.1. Effect of PFA on SA-11 replication in MA104 cells

To explore the antiviral effect of phosphonoformate, MA104 cells were infected with SA-11 in the presence of different concentrations of the compound. The infection was done, using trypsin-activated SA-11 at a m.o.i. of 10 and then PFA was added to the MA104 cells monolayer in the range 0.01 to 1 mM from the beginning of the infection. Under these conditions, PFA did not have any effect on rotavirus growth, measured as both inhibition of CPE and on the detection of viral double stranded RNA synthesized at 20 hours p.i. This result does not seems to be due to a direct effect on the cells, since under these conditions the mock-infected cells did not show any signs of cytotoxicity. Similar results were obtained when cells were infected at a m.o.i of 1 and the effect of PFA was measured as double stranded RNA synthesis at 48 p.i.; in this case the mock-infected cells treated with phosphonoformate did not present any cytotoxic effect as determined by trypan blue cell exclusion.

To overcome the poor cell permeability of phosphonoformate, the cell cultures were preincubated for 2.5 hours with different PFA concentrations, in the range 0–3 mM, in the presence of DEAE-Dextran. Then, the cells were infected with trypsin-activated SA-11 (m.o.i. varying from 1 to 10). After adsorption for 1 hour, the infected cells were further incubated with PFA for an additional 8 hours. The result of this experiment is shown in Fig. 1A. As seen, when cells were infected at 10 p.f.u./cell, after 8 hours of infection the amount of double-stranded RNA present diminished by the addition of increasing amounts of PFA, but the reduction was incomplete (lanes 1–4). However,

when the viral double-stranded RNA present in the incubation medium was analyzed, it was possible to observe an increased effect of PFA over the synthesis of the viral progeny measured as double-stranded RNA present in the cell supernatant. (Fig. 1B, lanes 1–4).

When we analyzed the effect of PFA on infected cells with SA-11 (m.o.i. 1) the inhibition of rotavirus growth is evident at PFA concentrations of 1 mM, and at 2 mM it was almost total (Fig. 1A, lanes 5–7). In these conditions, it was not possible to detect double-stranded RNA in the incubation medium of SA-11 infected MA104 cells (data not shown).

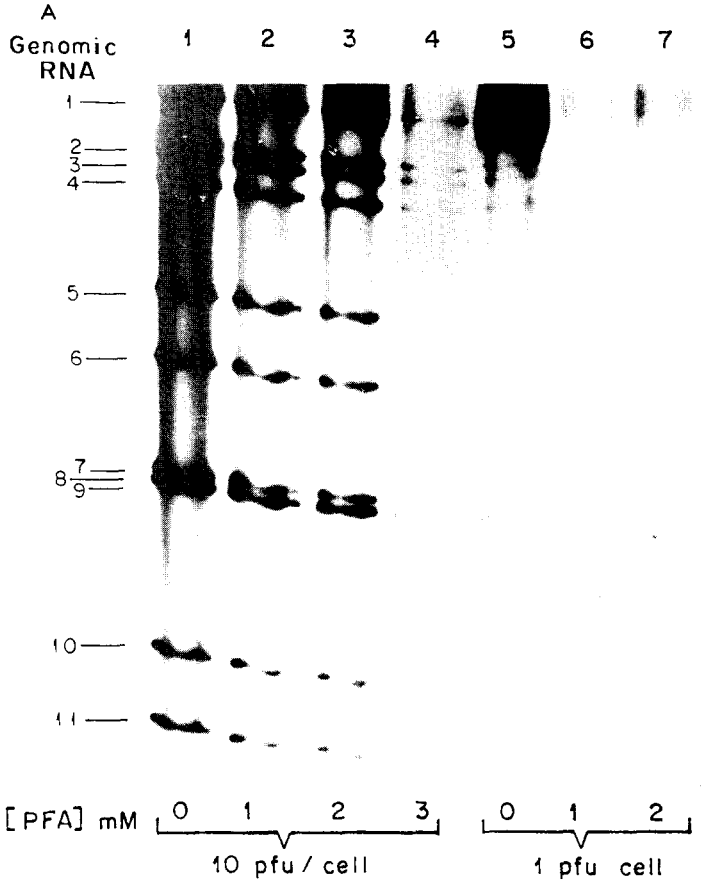

Fig. 1. Effect of foscarnet on SA11 replication in MA104 cells. The effect of foscarnet on rotavirus multiplication was determined by pretreatment of MA104 cells for 2.5 hours with and without the indicated PFA concentrations. Then, control and PFA treated cells were infected with rotavirus SA-11 at a m.o.i. of 10 (Panel A, lanes 1–4), or at a m.o.i. of 1 (Panel A, lanes 5–7). At eight hours post-infection, the cells were harvested and the RNA analyzed, as described in Section 2, by PAGE and autoradiography. The PFA effect on virus released into the incubation medium by infected cells with SA-11 m.o.i. of 10 is shown in Panel B, lanes 1–4.

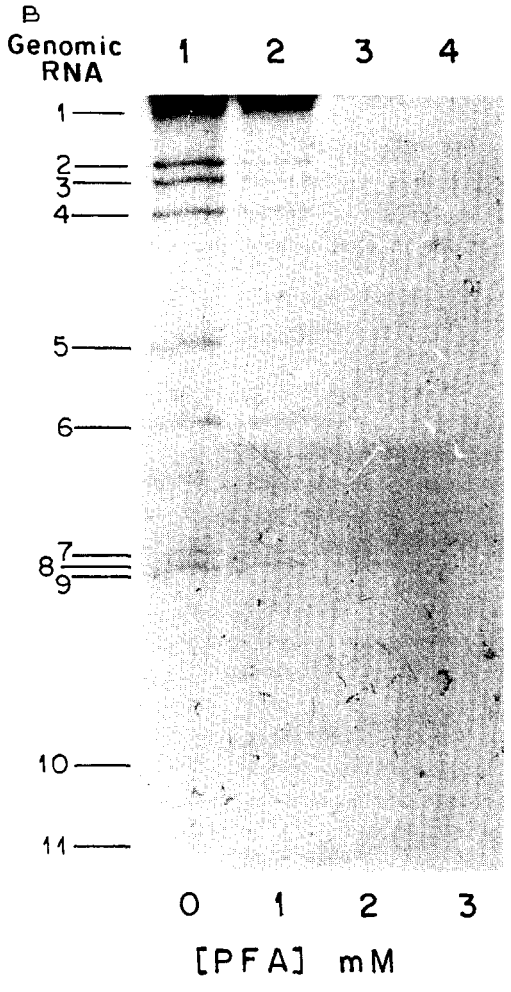

Fig. 1 (continued).

3.2. Effect of PFA on rotavirus in vitro transcription activity

The effect of PFA on in vitro rotavirus transcriptase activity, was tested incubating different concentrations of the pyrophosphate analogue with a standard transcription mixture containing purified heat-activated rotaviral particles. After 30 minutes of incubation at 45°C, the amount of RNA synthesized was determined as acid insoluble radioactivity. The PFA effect on the reaction was expressed as the percentage of RNA polymerase activity compared with a control with no additions. The results of the dose-response curve for the transcriptional reaction assayed at increasing concentrations of PFA are shown in Fig. 2. As seen, the phosphonoformate has a dose-related inhibitory effect on rotavirus transcription, where a 50% inhibition was obtained at PFA concentra-

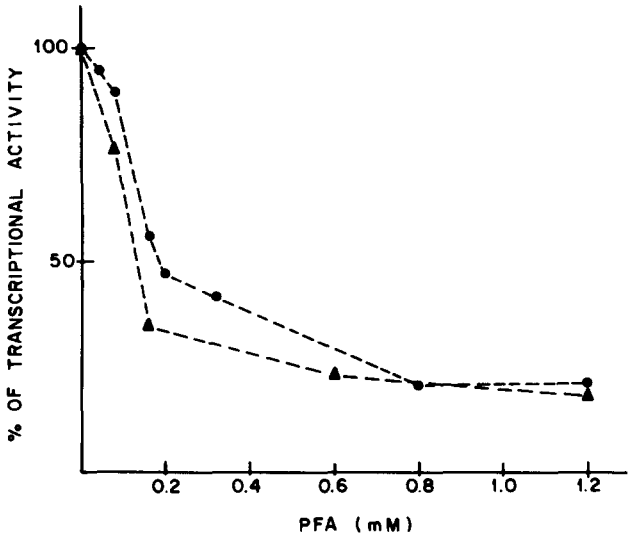

Fig. 2. Inhibition of in vitro rotavirus mRNA synthesis by foscarnet. Rotavirus RNA polymerase activity was assayed using purified heat-activated SA11 particles (●–●) or SA11 single-shelled particles present in infected cell extract isolated six hours post-infection (▲–▲). The activity was determined as incorporation of [^{3}H]-UMP into acid insoluble material in the presence of the indicated PFA concentrations. The results are expressed as percentage of a control reaction carried out without addition of PFA.

tions as low as 150 μM. Addition of 800 μM of PFA decreased the activity of rotavirus RNA polymerase up to 80% and increasing concentrations of PFA did not cause further inhibition of rotavirus mRNA synthesis.

The effect of PFA over in vitro RNA synthesis was also determined using a subviral particles fraction obtained at 6 hours p.i. from SA-11 infected cultures of MA104 cells. Using these subviral particles preparations, which contain viral particles that catalyze both plus- and minus-strand RNA synthesis, an inhibitory effect similar to purified heat-activated rotavirions was obtained (Fig. 2).

The effect of PFA on RNA synthesis products was determined using purified heat-activated SA-11 particles and the same range of concentration of PFA as the experiment shown in Fig. 2, but in this case, the transcriptional products were subjected to an acrylamide-urea gel electrophoresis and could be visualized by autoradiography of the gel. As seen in Fig. 3, a dose-dependent inhibition of the synthesis of all eleven mRNA elements was obtained; the addition of PFA concentrations over 0.8 mM practically abolished all residual transcription.

3.3. Effect of PFA on in vitro rotavirus minus-strand RNA synthesis

To determine the effect of phosphonoformate on the replicase activity associated with the synthesis of the minus-strand RNA segments, different concentrations of the compound were incubated with an in vitro replication mixture as described in Section 2. After 1 hour incubation at 45°C, the different mixtures were treated with 10 μg/ml

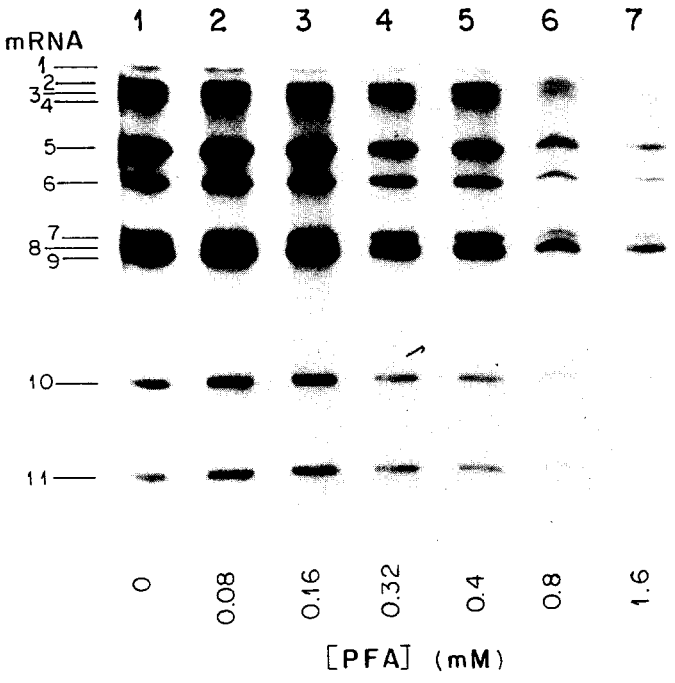

Fig. 3. Effect of foscarnet on in vitro rotavirus transcriptional product synthesis. The effect of different concentrations of PFA on in vitro rotavirus mRNA segments synthesis by the viral RNA polymerase, was determined using purified heat-activated SA-11 rotavirions by incorporation of [^{32}P]-UMP. The visualization of the eleven rotavirus mRNA segments was possible after autoradiography of a polyacrylamide-urea gel electrophoresis of each reaction conditions tested. In the figure, the PFA concentrations and the eleven transcriptional products are indicated.

RNase A for 10 minutes at 37°C, phenol-chloroform extracted and ethanol precipitated. The treatment with RNase allows to eliminate the background product of the single-stranded RNA synthesis. In this case, the activity of rotavirus RNA polymerase was measured by the specific detection of the 11 double-stranded RNA segment when the reaction products were analyzed by RNA gel electrophoresis and autoradiography. As seen in Fig. 4, PFA inhibited the double-stranded RNA synthesis at the same concentrations of those able to inhibit transcription. Furthermore the synthesis of the eleven [^{32}P]-double-stranded RNA segments in the presence of 320 μM PFA was inhibited by $> 80\%$.

3.4. Effect of PFA on in vitro transcriptional activity of rotavirus SA-11 and the temperature sensitive SA-11 mutant tsC

As suggested by the above presented results phosphonoformate is an effective inhibitor of both in vitro transcription and replication of rotavirus. Although the plus- and minus-strand RNA synthesis is carried out by different viral particles, both type of

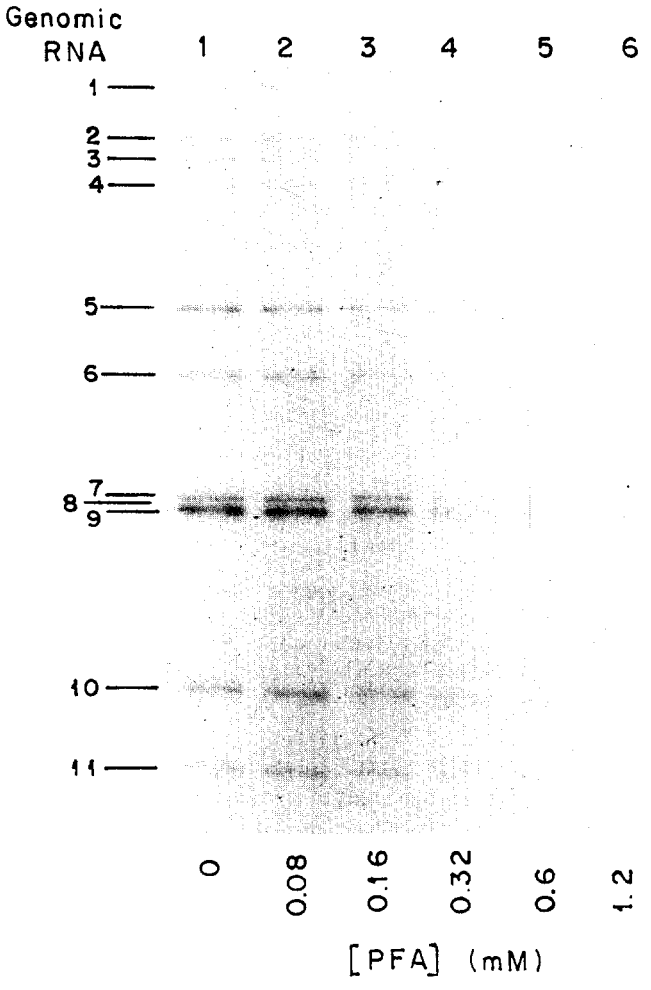

Fig. 4. Effect of foscarnet on in vitro rotavirus minus-strand RNA synthesis. SA-11 subviral particles isolated six hours post-infection were assayed for minus-strand RNA synthesis activity by incubation in a standard reaction mixture that included [^{32}P]-UTP as described in Section 2. The reaction products are the eleven double-stranded RNA genomic segments, as the minus-strand remains associated to the mRNA template, visualized after autoradiography of a polyacrylamide gel electrophoresis of reaction mixtures incubated without or with the indicated PFA concentrations.

particles to share some polypeptides as VP1, VP2 and VP3. Foscarnet might be affecting the activity of VP1, since this protein is the viral RNA-dependent RNA polymerase (Valenzuela et al., 1992). In Fig. 5 is shown the effect of this pyrophosphate analogue on the transcriptional activity of the wild type strain of rotavirus and of a mutant that carries a temperature-sensitive mutation on the gene 1 that codes for VP1 (tsC). When the inhibition of RNA synthesis was compared for both strains, it may be observed that

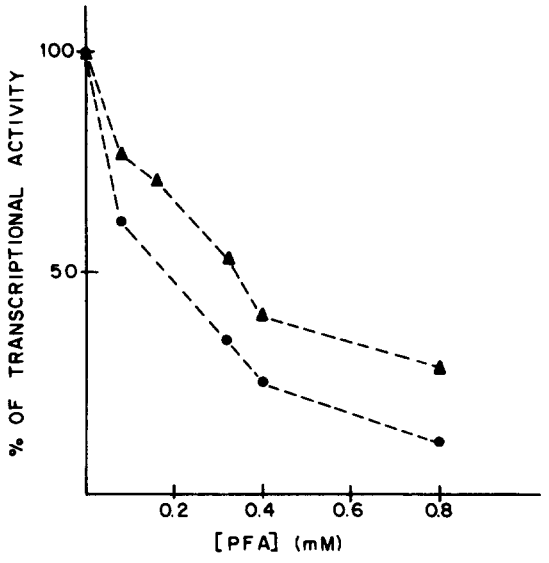

Fig. 5. Effect of foscarnet on in vitro transcriptional activity of rotavirus SA11 and the temperature-sensitive SA11 mutant tsC. The effect of PFA on in vitro mRNA synthesis carried out by SA-11 RNA polymerase, (------) and VP1 termosensitive mutant of SA-11, tsC, (▲–▲), was determined as indicated in Section 2. Results are expressed as percentage transcriptional activity remaining at different doses of PFA.

the mutant showed higher resistance to the inhibition by PFA than SA-11 (Fig. 5). This result might indicate that the foscarnet-blocked pyrophosphate binding site is located on VP1; in this form, PFA may interfere with the exchange of pyrophosphate when the polymerization reaction takes place.

4. Discussion

The results show the inhibitory effect of foscarnet on the replication of rotavirus. This pyrophosphate analogue, also has proved to be an effective inhibitor of transcription and replication of DNA viruses such as different herpes viruses (Crumpacker, 1992; Helgstrand et al., 1978), RNA viruses such as influenza A (Helgstrand et al., 1978; Strid et al., 1989) and retroviruses such as human immunodeficiency virus 1 (HIV-1) (Sandstrom et al., 1985).

The replication of rotavirus in MA104 cells was inhibited by foscarnet, in a concentration dependent manner, but similar to that observed for other viruses (Strid et al., 1989); in this case also it was necessary to preincubate the cells with the pyrophosphate analogue. These findings are in accord with a poor cell permeability to PFA, due to the negative charge present in their phosphonate and carboxylate moieties (Helgstrand et al., 1978). To enhance the cellular permeation to PFA, DEAE-dextran

was added to the incubation medium; when DEAE-dextran (20 μg/ml) was used at 2.5 hours preincubation an effective inhibition of rotavirus replication at different foscarnet concentrations was obtained.

The multiplicity of infection (m.o.i.) was important to obtain a maximal inhibition of rotavirus replication by foscarnet. If the infection of MA104 cells was done with 1 PFU/cell, 1 mM of PFA was required for complete inhibition of rotavirus replication; with a m.o.i. of 10, the amount of double-stranded RNA synthesized at 48 hours post-infection diminished by the addition of 1 to 3 mM foscarnet. In the conditions here described, MA104 cells did not exhibit any signs of cytotoxic effect and previous studies have reported that cellular mRNA synthesis would not be affected by the phosphonoformate concentrations used (Stenberg et al., 1985).

The mechanism of inhibition of rotavirus replication by PFA in infected cell culture, can be explained from the analysis of results of the effect of PFA on the in vitro viral RNA synthesis when a free-cell system was used. Since the results showed that PFA inhibited both rotavirus mRNA synthesis catalyzed by purified rotaviral particles and the double-stranded RNA synthesis catalyzed by subviral particles isolated from infected cells, the effect of this pyrophosphate analogue on rotavirus replication may be related to a direct inhibition of the viral transcriptase involved in both plus- and minus-strand RNA synthesis.

Similar to the effect of foscarnet on the replication of influenza A virus (Strid et al., 1989), in the case of rotavirus the concentration needed to inhibit viral replication is higher than the concentration needed to inhibit viral RNA synthesis in a cell free system. In addition to a poor cell permeability, foscarnet may be inactivated or bound in the cell, thus resulting in a decrease of the effective concentration of the compound to interact with viral enzymes.

Foscarnet has proven to inhibit viral polymerases (Sundquist and Oberg, 1979; Cheng et al., 1981; and this study), by competing with pyrophosphate, indicating that the compound blocks the pyrophosphate binding site on viral polymerase enzymes, interfering with the pyrophosphate exchange. This has been confirmed by findings made with foscarnet-resistant mutants of the HSV polymerase. (Datta and Hood, 1981; Derse et al., 1982). A termosensitive mutant (tsC) of rotavirus, which carries the mutation on the gene that codes for the polypeptide VP1, the viral RNA-dependent RNA polymerase, showed a higher resistance to foscarnet than the wildtype strain SA-11. This observation, might also indicate that the pyrophosphate binding site is located on VP1. Previously, the effect of several nucleotide analogues, including some described as antiviral agents, was analyzed on rotavirus RNA synthesis. In all cases where rotavirus infection was inhibited, the effect was associated with mRNA synthesis but not double-stranded RNA synthesis inhibition.

Thus, rotavirus infection was found susceptible to inhibition by vidarabine (adenine arabinoside) and cordycepin (Pizarro et al., 1991). Vidarabine has been compared to foscarnet for the treatment of acyclovir-resistant herpes simplex infection in patient with AIDS (Safrin et al., 1991), and this study indicated that foscarnet has higher antiviral activity and is less toxic than vidarabine. Our present results indicate that PFA is active against rotavirus and therefore make PFA a possible candidate for clinical trials, specially in the treatment of rotavirus gastroenteritis in immunocompromised patients.

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