Phosphonoacetic Acid Inhibition of Frog Virus 3 Replication

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Phosphonoacetic acid (PAA) at concentrations above 200 μg/ml inhibited the replication of frog virus 3 in BHK cells. The inhibition of viral DNA replication observed in these cells was reversible and correlated with the inhibition of the virus-induced DNA polymerase activity in an in vitro assay. The synthesis of frog virus 3-induced late or γ polypeptides was also inhibited by phosphonoacetic acid, although the early (α and β) polypeptides were unaffected.

Phosphonoacetic acid (PAA) is a potent inhibitor of the replication of a number of herpesviruses in tissue culture (9, 19, 20). It affects a virus-induced DNA polymerase (9), and the drug significantly reduces the severity of herpesvirus infections in model animal systems (4, 12, 15, 19, 20). This compound has little effect on other animal DNA viruses, with the exception of vaccinia virus (2, 9).

Frog virus 3 (FV3) is a large, icosahedral deoxyribovirus which apparently replicates in the cytoplasm of infected cells (7, 11) but has a requirement for the cell nucleus (5, 6). A virus-induced DNA polymerase activity has been described in FV3-infected cells (14, 17). Certain features of the replication of FV3 are reminiscent of herpesvirus replication and McAuslan and Armentrout (16) have suggested that FV3 represents a "missing link" between herpesviruses and poxviruses. We therefore thought it pertinent to study the effect of PAA on FV3 replication, especially since Honess and Watson (9) have suggested that PAA sensitivity may be a herpesvirus group defining characteristic. In this paper we demonstrate that PAA inhibits FV3 replication in a reversible manner by blocking FV3 DNA synthesis at the level of the induced DNA polymerase and report on the effect of PAA on the synthesis of polypeptides in FV3-infected cells.

The disodium salt of PAA was a gift from Abbott Laboratories Inc., Chicago, Ill. The following radioisotopes were purchased from the Radiochemical Centre, Amersham, England: [methyl-3H]thymidine (20 Ci/mmole), [methyl-3H]thymidine 5′-triphosphate (41 Ci/mmole), and L-[35S]methionine (840 Ci/mmole). Unlabeled deoxyribonucleoside triphosphates, calf thymus DNA, and salmon sperm DNA were obtained from Sigma Chemical Co., London, England. The maintenance of baby hamster kidney (BHK) cells and the plaque assay of FV3 in fathead minnow (FHM) cells are described in another paper.

To test the effect of PAA on the growth of FV3, monolayers of BHK cells (10⁶ cells) were infected with about 10 PFU of FV3 per cell for 1 h at room temperature and washed with medium, and incubation was continued at 28°C in the presence of different concentrations of PAA. After 24 h the cells were scraped into the medium, sonicated to release cell-associated virus, and assayed for infectious virus by plaqueing in FHM cells.

The effect of PAA on FV3 DNA replication was tested by using petri dishes containing 2 × 10⁶ BHK cells which were infected with 10 PFU of virus per cell, as described above. After adsorption, the cultures were incubated in medium without PAA or with 300 μg of PAA per ml present. To determine the reversibility of PAA, some cultures were washed five times with minimal essential medium, and incubation was continued in the absence of PAA. At various times after infection, the medium was replaced by minimal essential medium containing 30 μCi of [3H]thymidine per ml. Monolayers incubated with PAA were also labeled in the presence of the drug. After a 1-h pulse, the label was removed, the monolayers were washed with ice-cold phosphate-buffered saline, and the cells were scraped into cold 5% trichloroacetic acid. Trichloroacetic acid-precipitable material was collected by filtration, washed with trichloroacetic acid and ethanol, and counted in a liquid scintillation analyzer. Analysis of DNA in infected cells with CsCl gradients shows that over 95% of the cellular synthesis is viral 3 h after infection (14; Elliott and Kelly, unpublished data).

DNA polymerase was assayed in cells extracts as follows. Mock-infected or FV3-infected (multiplicity of infection, 1 PFU/cell) BHK cells were...
harvested 24 h after infection by washing in cold phosphate-buffered saline scraping into phosphate-buffered saline, and pelleting by centrifugation. The pellet (approximately $5 \times 10^6$ cells) was resuspended in 2 ml of sterile distilled water and sonicated for 10 min in a sonicating water bath. Samples of these lysates were stored at $-20^\circ C$ until use. The DNA polymerase reaction mixture contained, in a volume of 280 $\mu$l, 17.9 mM Tris-hydrochloride, pH 7.8, 250 mM KCl, 7.1 mM MgCl$_2$, 0.36 mM EDTA, 3.6 mM dTTP, 1.8 mM each unlabeled dATP, dGTP, and dCTP, 1.8 $\mu$M dTTP, 1.8 $\mu$Ci of $[^3H]$dTTP, 70 $\mu$g of nicked salmon sperm DNA, and 50 $\mu$l of cell extract containing about 150 $\mu$g of protein. The reaction mixture was incubated for 1 h at 28°C and terminated by chilling and adding 10 ml of 205 mM perchloric acid containing 1 mM sodium pyrophosphate, 0.41 mM thymidine, and 100 $\mu$g of heat-denatured calf thymus DNA carrier. After incubation at 4°C overnight, the precipitate was collected by filtration and washed with perchloric acid and ethanol, and the radioactivity was counted.

Radiolabeling and analysis of polypeptides synthesized in infected cells were performed as described in an accompanying paper (3).

Figure 1A is a dose-response curve showing the effect of treating infected cells with various concentrations of PAA on the yield of infectious FV3. A concentration of 25 $\mu$g of PAA per ml resulted in a 50% decrease in the yield of FV3, and 200 $\mu$g/ml inhibited replication 100-fold.

To examine the effect of PAA on FV3 DNA replication, we studied the uptake of $[^3H]$thymidine by FV3-infected cells in the presence of an excess (300 $\mu$g/ml) of the drug. Figure 1B shows that in untreated FV3-infected monolayers there was a marked increase in incorporation of label into trichloroacetic acid-precipitable material between 5 and 8 h after infection. In PAA-treated cells, DNA synthesis was less than 10% of the synthesis in untreated infected controls (as assessed by $[^3H]$thymidine uptake), and thus we concluded that PAA inhibits FV3 DNA replication. Figure 1B also shows that this inhibition is reversible if the drug is removed by extensive washing. After 2 and 4 h of exposure to the drug, there was a delay of 5 h (2-h treatment) or 6 h (4-h treatment) after removal of the PAA before DNA synthesis occurred. This presence of 300 $\mu$g of PAA per ml (○). Some PAA-treated cultures were extensively washed (□), and incubation was continued from 2 h (□) or 4 h (□) in the absence of the drug. (C) Effect in PAA on DNA polymerase activity in FV3-infected or uninfected BHK cells. Symbols: ○, incorporation of $[^3H]$dTTP directed from FV3-infected cell extracts 24 h after infection; □, incorporation directed by uninfected cell extracts.

![Figure 1](http://jvi.asm.org/)

**Fig. 1.** (A) Effect of concentration of PAA on yield of FV3 in BHK cells 24 h after infection. The yields were calculated as percentages of the yield in the absence of the drug. The insert shows the structure of PAA. (B) Incorporation of $[^3H]$thymidine into trichloroacetic acid-insoluble material in FV3-infected BHK cells in the absence of PAA (○) or in the presence of 300 $\mu$g of PAA per ml (□). Some PAA-treated cultures were extensively washed (□), and incubation was continued from 2 h (□) or 4 h (□) in the absence of the drug. (C) Effect in PAA on DNA polymerase activity in FV3-infected or uninfected BHK cells. Symbols: ○, incorporation of $[^3H]$dTTP directed from FV3-infected cell extracts 24 h after infection; □, incorporation directed by uninfected cell extracts.
corresponded to the time taken for viral DNA synthesis to occur in untreated cultures.

Figure 1C shows the effect of PAA concentration on DNA polymerase activity in FV3-infected and mock-infected cells. A concentration of 20 μg of PAA per ml reduced the activity of the FV3-induced enzyme by 50%; about 80% inhibition of the enzyme was observed at 100 μg of PAA per ml. In contrast, the activity of the DNA polymerase in uninfected BHK cells was not affected by concentrations of PAA up to 100 μg/ml.

Three phases of polypeptide synthesis have been observed in FV3-infected cells; these phases are designated α, β, and γ (4). The synthesis of γ polypeptides ("late" polypeptides) is dependent on the replication of viral DNA (3), and experiments were designed to examine the effects of PAA on FV3-induced polypeptides.

In the experiment shown in Fig. 2 FV3-infected cells maintained in the presence or absence of 300 μg of PAA per ml were pulse-labeled at various times after infection. There was a marked overall decrease in the incorporation of [35S]methionine in the PAA-treated cultures, but the time of appearance of the α and β polypeptides was unaffected. By 9 h after infection (by which time γ polypeptide synthesis was clearly observed in untreated infected cells) no γ polypeptides were seen in the PAA-treated cells. However, after 24 h of incubation in PAA, some γ polypeptides were synthesized (e.g., infected cell-specific polypeptide 55), but the synthesis of others (e.g., infected cell-specific polypeptide 23) was still inhibited. The synthesis of cellular polypeptides in mock-infected cells was not affected by PAA.

In this communication we have demonstrated that PAA inhibits the replication of FV3 under conditions where uninfected cells remain unaffected. The drug inhibited the replication of FV3 DNA (as judged by radioactive thymidine uptake), but its effects could be reversed by extensive washing. Consequently, the synthesis of γ or late polypeptides, which is dependent on viral DNA replication, was inhibited by PAA, but the synthesis of prereplicative or α and β polypeptides was unaffected. These data suggested that an early virus function was inhibited, and this conclusion was supported by the in vitro inhibition of the virus-induced DNA polymerase (which by definition must be an early polypeptide). Thus, our findings are consistent with the observation that the inhibition of FV3 is via the induced DNA polymerase (9).

The concentrations of PAA required to inhibit FV3 replication are higher than those reported for the herpesviruses, and this suggests either an inherent reduced sensitivity to the drug or a high proportion of PAA-resistant mutants; this could prove useful in genetic studies with FV3, as PAA-resistant mutants can be isolated with relative ease among herpesviruses (9, 12).

While this manuscript was in preparation, Moreno et al. (18) reported the inhibition by PAA of another icosahedral cytoplasmic deoxyribovirus, African swine fever virus (9); this suggested that sensitivity to PAA may be a characteristic of the icosahedral cytoplasmic deoxyribovirus group.

Our preliminary observations indicate that the replication of another icosahedral cytoplasmic deoxyribovirus, iridescent virus type 22 (1), is not affected by PAA; however, the possibility that this was due to poor uptake of the drug by the insect cell line used for the experiment cannot be ruled out.

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LITERATURE CITED


