Human Cytomegalovirus

IV. Specific Inhibition of Virus-Induced DNA Polymerase Activity and Viral DNA Replication by Phosphonoacetic Acid

ENG-SHANG HUANG

Cancer Center Program and Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Phosphonoacetic acid specifically inhibited human cytomegalovirus DNA synthesis in virus-infected human fibroblasts as detected by virus-specific nucleic acid hybridization. Inhibition was reversible; viral DNA synthesis resumed upon the removal of the drug. The compound partially inhibited DNA synthesis of host cells in the log phase of growth but had little effect on confluent cells. Studies of partially purified enzymes indicated that phosphonoacetic acid specifically inhibited virus-induced DNA polymerase and had only a slight effect on normal host cell enzymes. The drug was shown to interact directly with virus-induced enzyme but not with the template-primers.

There has been considerable effort in the search for chemical compounds which inhibit virus replication without affecting the normal host cell functions. Among these compounds several nucleoside analogues, e.g., 5-iodo-2'-deoxyuridine, 5-trifluoro-methyl-2'-deoxyuridine, cytosine arabinoside, and adenosine arabinoside, have been shown to possess anti-herpesvirus activity, but each compound is also cytotoxic (1, 2, 5, 10).

In random screening of chemical compounds, Shipkowitz et al. (12) showed that phosphonoacetic acid [HO—P—CH$_2$C—OH] appeared to be very active against herpes dermatitis in mice and herpes keratitis in rabbits. In addition they have shown that replication of herpes simplex virus and viral DNA is impeded by phosphonoacetic acid and the drug seems to have little effect on host cell function (8, 9). The suggestion has been made that inhibition involves the specific recognition of herpes simplex virus-induced DNA polymerase (8).
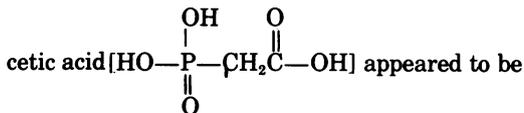
Infection of WI-38 human fibroblasts with human cytomegalovirus (CMV) led to the stimulation of host cell DNA polymerase synthesis and induction of a new virus-specific DNA polymerase (6). This new CMV-induced DNA polymerase has been purified and separated from host cell enzymes by DEAE-cellulose and phosphocellulose · chromatography. Based on the availability of the partially purified CMV-induced and host cell DNA polymerases (6), and the availability of CMV-specific [3H]-labeled complementary RNA for quantitation of viral DNA (7), we have been able to observe in detail the effect of phosphonoacetic acid on human CMV replication and demonstrate specific inhibition of viral DNA replication and virus-induced DNA polymerase activity.

MATERIALS AND METHODS

Cells and virus. WI-38 human fibroblasts (Hayflick, passage 21–28), obtained from HEM (Rockville, Md.), and human CMV strain AD-169 (7, 11) were used throughout. The cultivation and maintenance of cells and virus have been described (6, 7). Cell monolayers in roller bottles (surface area 1,000 cm²) or in Falcon tissue culture flasks (75 cm²) were infected with CMV as described previously (6, 7).

Phosphonoacetic acid (PAA) solution. The stock solution of PAA (Bodman Chemical, Richmond) was prepared in distilled water at a concentration of 20 mg/ml, adjusted to pH 7.4 with 1 N NaOH, and sterilized by filtration through membrane filters (Millipore Corp.).

WI-38 cell DNA synthesis. Cellular DNA synthesis was measured in terms of the incorporation of [3H]thymidine into acid-insoluble material. WI-38 cells in Falcon petri dishes (60 by 15 mm) were pulse-labeled with [3H]thymidine (10 µCi/ml; specific activity, 45 Ci/mM; Nuclear Dynamics) for 60 min at various times after subculture. After each 60-min labeling period, the cell cultures were washed three times with cold TBS (0.05 M Tris-hydrochloride, pH 7.4, 0.15 M NaCl) and lysed with 3 ml of 1% sodium dodecyl sulfate buffer solution (1% sodium lauryl sulfate, 0.01 M Tris-hydrochloride, pH 7.8, and 0.01 M EDTA). The lysates were collected and precipitated with ice-cold trichloroacetic acid (final concentration of 5%) and passed through membrane filters. After extensive washing with 5% trichloroacetic acid, the filters were dried and counted in a liquid scintillation counter.

DNA extraction. The virus-infected or mock-infected WI-38 cells were lysed with 1% sodium dodecyl sulfate buffer solution and digested with Pronase (preheated at 80 C for 15 min; final concentration 1 mg/ml) for 2 h at 37 C. DNA was extracted from the cells with phenol, precipitated with alcohol, and treated with pancreatic RNase to remove contaminating RNA. The RNase was removed from the DNA solution by one more cycle of phenol extraction and alcohol precipitation. The DNA was finally dissolved in 0.1× SSC (0.15 M NaCl plus 0.015 M sodium citrate).

^{3}H-labeled cRNA-DNA membrane hybridization. The infected or mock-infected cell DNA was denatured in 0.5 N NaOH at 37 C for 2 h and then neutralized with 1.1 N HCl in 0.2 M Tris. The solution was adjusted to 6× SSC and immobilized on nitrocellulose membranes (B-6, Schleicher and Schuell). The tritiated human CMV cRNA with specific activity of 10^7 counts/min per μg was synthesized in vitro from purified viral DNA template (strain AD-169) as described previously (7). The hybridization of ^{3}H-labeled cRNA to the immobilized denatured DNA was by the procedure of Gillespie and Spiegelman (4) and as described before (7).

Purification of human CMV-induced and host-cell DNA polymerases. The virus-induced and host-cell (WI-38) DNA polymerases were purified from cytoplasmic or nuclear extract (6) by DEAE-cellulose (DE52, Pharmacia) and phosphocellulose (P 11, Pharmacia) column chromatographies as described previously (6). The virus-induced enzyme isolated from infected cytoplasm was designated CyD2P3 (cytoplasmic fraction, DEAE-cellulose column peak 2, phosphocellulose column peak 3; see reference 6). Similarly, the viral enzyme isolated from nuclei was designated as NuD2P3. Four other host cell DNA polymerases were used in this study for comparison; these are NuD1, CyD1, NuD2P1, and CyD2P2. NuD1 and CyD1 are low-molecular-weight enzymes eluted by 0.05 M NaCl from a DEAE-cellulose column. NuD2P1 and CyD2P2 are high-molecular-weight enzymes obtained from peak 1 and peak 2 of phosphocellulose chromatography of nuclear and cytoplasmic extracts, respectively (6). All the enzymes used in this experiment were kept at −20 C in buffer D (0.05 M Tris-hydrochloride, pH 7.8, 0.001 M EDTA, 0.001 M dithiothreitol, and 5% glycerol) with 50% glycerol.

DNA polymerase assay. The DNA polymerase assays are based on measurement of the incorporation of [^{3}H]thymidine triphosphate into the acid-precipitable product as previously described (6). In system A, the stock reaction mixture (2.5× concentration) contained: 0.1 M Tris-hydrochloride, pH 7.8, 0.025 M MgCl$_2$, 0.001 M dithiothreitol, 1.25 mg of bovine serum albumin per ml, and 0.25 mM each of dATP, dCTP, dGTP, and 0.025 mM TTP. One-tenth milliliter of the stock reaction mixture, 0.1 ml of activated calf thymus DNA (200 μg/ml), 0.05 ml of enzyme, 1 μCi of [^{3}H]TTP (specific activity 48 Ci/mM; New England Nuclear), and different concentrations of PAA (final concentration from 0 to 200 μg/ml) were mixed and incubated at 37 C for 30 min. The reaction was terminated by trichloroacetic acid

precipitation. The precipitate was washed and counted as described previously (6). In system B, the reaction mixture contained the same ingredients as in system A, except dATP, dCTP, and dGTP were omitted and the synthetic template-primer poly(dA)·oligo(dT)$_{12-18}$ in the amount of 2 μg per reaction was used instead of calf thymus DNA. Based on their template-primer preference (6), activated calf thymus DNA was used as template-primer (system A) to assay two host-cell enzymes (NuD2P1 and CyD2P2) and poly(dA)·oligo(dT)$_{12-18}$ (system B) was used to assay viral enzymes and a host cell enzyme (CyD1 or NuD1).

RESULTS

Inhibition of viral DNA synthesis by PAA in virus-infected cells. The amount of viral DNA synthesis in virus-infected cells was monitored by nucleic acid membrane hybridization with virus-specific ^{3}H-labeled cRNA as a probe. Increasing amounts of viral DNA were detected in the virus-infected cells for at least 48 h after infection (Fig. 1). In contrast, viral DNA synthesis was completely inhibited by the presence of as little as 50 μg of PAA per ml; partial inhibition of the viral DNA synthesis was observed
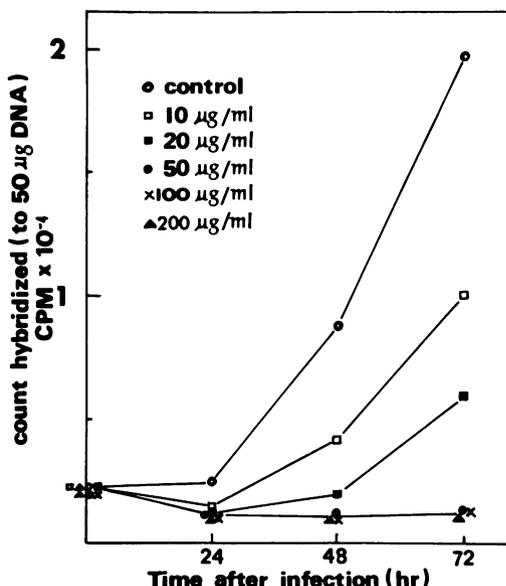
FIG. 1. Influence of viral DNA synthesis in virus-infected cells by PAA. PAA was added to the virus-infected cell cultures immediately after virus adsorption at a final concentration of 0 to 200 μg/ml. At various times after infection, the total DNA was extracted from the cell culture for virus genome quantitation by ^{3}H-labeled cRNA-DNA membrane hybridization. Amounts of DNA and CMV-specific ^{3}H-labeled cRNA applied to each filter were 50 μg and 1.2 × 10^5 counts/min, respectively. The hybridization was done in 6× SSC at 66 C for 20 h as described (4, 7).

using 10 and 20 μg of PAA per ml.

PAA cured the early cytotoxicity due to CMV infection. Typical cytomegalovirus cytopathic effect (CPE) was found 24 h after infection. More severe development of CPE was demonstrated 72 h after infection. In the presence of PAA (50 to 500 μg/ml), the CPE was also demonstrated 24 h after infection. However, this CPE was overcome and cured by 48 to 72 h after infection.

Viral DNA synthesis was also measured after the removal of the drug. The PAA was removed from the infected cultures by replacing with fresh maintenance media at various times after infection (Fig. 2, indicated by arrowhead). Viral DNA synthesis was measured 4 to 12 days thereafter by the nucleic acid membrane hybridization technique. Inhibition of viral DNA synthesis by PAA was reversible; there was resumption of viral DNA synthesis upon the removal of the drug. Nevertheless, as the period of treatment with PAA was extended, the amount of virus DNA synthesized after its removal was reduced, and the time taken for CPE to reappear was increased. It is speculated that some of the uncoated viral genome might persist in the infected cells and not be degraded by the host cell restriction enzyme system for a period of time; persistence of viral DNA would then be dependent on its resistance to host cell restriction nuclease digestion.

Influence of PAA on the DNA synthesis of normal WI-38 cells. No obvious morphological alteration or damage was observed in the confluent cells in the presence of 100 to 1,000 μg of PAA. Cultures that had been divided from one to three usually took 4 days to regain confluence; in the presence of 100 μg of PAA per ml, they took 6 to 7 days. The effect of PAA on WI-38 cellular DNA synthesis was measured by pulse labeling with tritiated thymidine in the presence of PAA (Table 1). Partial inhibition of WI-38 DNA synthesis was observed with concentrations of PAA of 100 μg up to 1,000 μg; these levels inhibit viral DNA synthesis totally. A dose response effect was observed. At the maximal dose used (1,000 μg/ml) cell DNA synthesis was still 15 to 40% of control. The compound apparently has less effect on the DNA synthesis of confluent or nearly confluent cells than on cells in the log phase of growth.

Effect of PAA on host cell and virus-induced DNA polymerase activities. The effect of PAA on the partially purified host cell (Fig. 3a) and virus-induced DNA polymerase activities (Fig. 3b) was determined. A remarkable inhibition of virus-induced DNA polymerase (80 to 95%) was observed when the drug was added at a concentration of 10 μg/ml. More

complete inhibition was obtained when the concentration of the drug reached the level of 50 μg/ml. The host cell enzymes were relatively resistant to PAA; at a drug concentration of 200 μg/ml, there was less than 15% inhibition of NuD1, CyD1, and NuD2P1. One of the host cell enzymes, CyD2P2, appeared somewhat sensitive to PAA, but only 40% of the enzyme activity was inhibited when 200 μg of PAA per ml was used. Evidently there is selective inhibition of virus-induced DNA polymerase by PAA.

Failure to overcome inhibition of activity by increasing template concentration indicated that the PAA directly interacts with the virus-induced enzyme and not with the template-primer (Table 2). The template-primer at a concentration of 6 μg/ml almost saturated the constant amount of enzymes used in this experiment. By comparison with the control without PAA, partial inhibition of virus-induced enzymes in the presence of a low concentration of PAA (5 μg/ml) was observed. Increase of the template-primer concentration to 75 μg/ml did
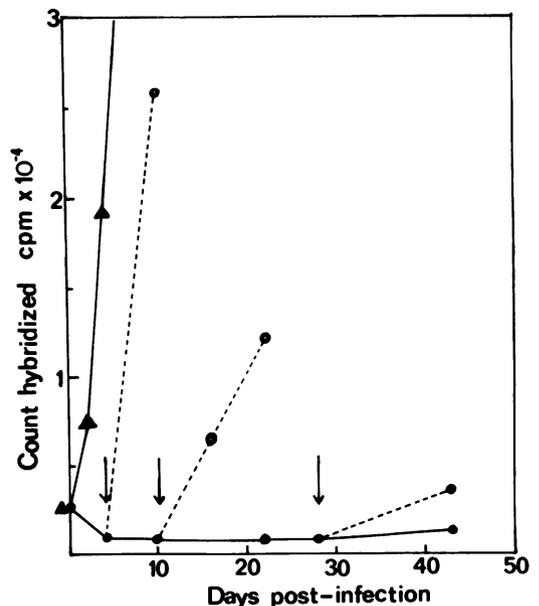
FIG. 2. *Effect of PAA on CMV DNA synthesis in virus-infected WI-38 cells. PAA was added to the virus-infected cell cultures at a final concentration of 100 μg immediately after virus absorption. At various times after infection, the drug was removed from the infected cultures and replaced with fresh maintenance media (as shown by arrowhead). Viral DNA synthesis was detected by ³H-labeled cRNA-DNA membrane hybridization (as shown in Fig. 1). Symbols:* ▲, *viral DNA content of the cultures without PAA treatment;* ●, *indicates the viral DNA content in the presence of PAA;* ○, *indicates the viral DNA content after the removal of the drug.*

TABLE 1. *Incorporation of tritiated thymidine in WI-38 human fibroblasts treated with PAA*

Pulse label period	Phosphonoacetate concn (µg/ml)[a]				
	0	100	200	500	1,000
60 min (48 to 49 h after subculture)[b]	55,828[c]	54,862	35,162	26,332	9,312
	(100)	(98.4)	(63.0)	(47.2)	(16.7)
60 min (72 to 73 h)[b]	14,118	10,565	10,120	8,023	5,574
	(100)	(74.8)	(71.7)	(56.8)	(39.5)
60 min (93 to 94 h)[b]	5,312	4,550	4,514	2,258	2,014
	(100)	(85.7)	(85.8)	(42.5)	(37.9)
46 h (48 to 94 h)[d]	300,086	252,603	233,815	167,224	67,396
	(100)	(84.2)	(77.9)	(55.7)	(22.5)

[a] PAA solution (pH adjusted to 7.4) was added to WI-38 cells 47 h after subculture when the cells reached 50 to 60% confluence.

[b] [^{3}H]thymidine concentration is 10 µCi/ml.

[c] Counts per minute incorporated. Counts per minute obtained in absence of PAA was used as 100% control. Parentheses show percentage.

[d] [^{3}H]thymidine concentration is 3 µCi/ml.
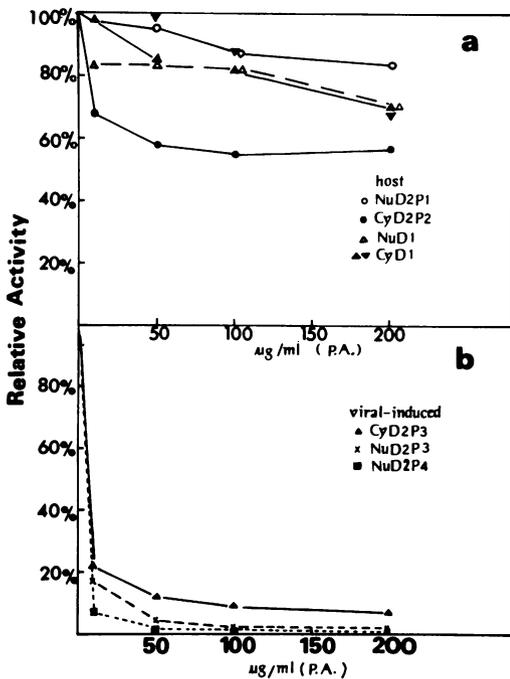
FIG. 3. *Effect of PAA on the partially purified host cell and virus-induced DNA polymerase activities. PAA was added to the reaction mixture at a final concentration of up to 200 µg/ml. The incubation was carried out at 37 C for 30 min, terminated, and washed with trichloroacetic acid as described in the text. The polymerization activity obtained without PAA was used as the 100% control. The upper panel shows the results with host cell enzymes; the bottom panel indicates results with virus-induced enzyme. Activated calf thymus DNA was used as template-primer for NuD2P1 and CyD2P2 (system A). Poly(dA)·oligo(dT)$_{12-18}$ was used for NuD1, CyD1, and virus-induced enzymes (system B).*

not overcome the PAA inhibition; the activity still remained around 55 and 25% for CyD2P3 and NuD2P3, respectively.

DISCUSSION

PAA specifically inhibits herpes simplex virus DNA replication in tissue culture (9). The inhibition directly involved the specific recognition of the herpesvirus-induced DNA polymerase (8). A similar specific inhibition of CMV DNA replication and CMV-induced DNA polymerase activity by this compound has been shown here. Viral DNA replication was completely inhibited by PAA at a concentration of 50 µg/ml in CMV-infected WI-38 human fibroblasts. This inhibition appeared to be a virostatic reversible type of inhibition; viral DNA synthesis resumed upon the removal of the inhibitor.

The specific inhibition of viral DNA synthesis by PAA in the CMV system was attributed to its specific inhibition on virus-induced DNA polymerase activity. As little as 10 µg of the compound per ml inhibited up to 95% of virus-induced enzyme activity, whereas a relatively low level of inhibition of host cell enzymes was observed. There was no great increase in the inhibition of host cell enzymes as the drug concentration was increased from 10 to 200 µg per ml. Additional evidence that PAA acts on the polymerase was provided by the fact that increasing the template-primer concentration did not overcome inhibition in the presence of a constant amount of virus-induced enzyme.

Inhibition of viral DNA synthesis by PAA seems to be universal among herpesviruses. Our preliminary observations indicate that mouse CMV, herpesvirus saimiri, herpes sim-

TABLE 2. *Influence of template-primer concentration on the inhibition of virus-induced enzyme activity by PAA*

Enzymes	Poly(dA)·oligo(dT)$_{12-18}$ template-primer concn (μg/ml)	[^{3}H]TMP incorporated		Activity[a] (%)
		With PAA (5 μg/ml)	Without PAA	
Virus-induced				
CyD2P3	0	342		
	0.6	1,892	4,971	38
	6	2,954	6,992	42
	15	4,040	6,783	59
	30	4,380	7,897	56
	75	3,818	7,389	52
NuD2P3	0	247		
	0.6	2,326	7,539	31
	6	4,027	11,614	35
	15	4,571	18,359	25
	30	4,626	17,750	26
	75	4,307	19,398	22
Host cell				
CyD1	0	268		
	0.6	7,814	8,579	91
	6	37,307	42,262	88
	15	39,296	47,693	82
	40	46,025	48,845	94
	100	57,338	59,542	96

[a] Activities obtained in absence of PAA were used as 100% control.

ple, and Epstein-Barr virus DNAs syntheses are also inhibited by PAA in tissue culture (Symp. Antivirals Clin. Potential., Stanford University, Abstr. B11, 1975). It is possible that the above herpesviruses are all capable of inducing their own specific DNA polymerases which are sensitive to PAA; at least this would appear to be the case in herpes simplex and human CMV systems.

It is worth mentioning that the early CPE in CMV infection is induced by early transcription and translation product (3); viral DNA replication is not essential for the early cell damage. The inhibition of viral DNA synthesis by PAA did not prevent the early CPE. The continuation of the CPE in control, nontreated infected cells and the curing of the CPE by PAA suggested that viral DNA replication is essential for the persistence of CPE; the newly synthesized virus DNA might contribute to the continuing transcription and translation of certain viral gene functions which are also responsible for the continuation of CPE.

PAA is a simple compound with a highly charged moiety. Its mode of interaction with virus-induced enzyme and its mode of penetration through biological membranes still remain a mystery and require further examination. The results of our preliminary study of the relation of chemical structure and biological activity indicate that the modification of phos-

phonate and carboxylate moieties totally abrogate the inhibitory effects (our unpublished results). Synthesis of PAA analogues, e.g., lengthening the carbon chain between the phosphonate and carboxylate moieties, introducing bulky groups, introducing hydrophobic moiety and electron-donating and withdrawing groups, is underway to elucidate the mechanism of specific inhibition and understand the topography of virus-induced DNA polymerase.

LITERATURE CITED

1. Chow, A. W., A. Ronald, M. Fiala, W. Hryniuk, M. L. Weil, J. St. Greme, Jr., and L. B. Guze. 1973. Cytosine arabinoside therapy for herpes simplex encephalitis—clinical experience with six patients. Antimicrob. Agents Chemother. 3:412–417.
2. Fiala, M., A. W. Chow, and L. B. Guze. 1972. Susceptibility of herpes viruses to cytosine arabinoside; standardization of susceptibility test procedure and relative resistance to herpes simplex type 2 strains. Antimicrob. Agents Chemother. 1:354–357.
3. Furukawa, T., A. Fioretti, and S. Plotkin. 1973. Growth characteristics of cytomegalovirus in human fibroblasts with demonstration of protein synthesis early in viral replication. J. Virol. 11:991–997.

4. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12:829–842.
5. Goz, B., and W. H. Prusoff. 1970. Pharmacology of viruses. Annu. Rev. Pharmacol. 10:143–170.
6. Huang, E.-S. 1975. Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. 16:298–310.
7. Huang, E.-S., S.-T. Chen, and J. S. Pagano. 1973. Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. 12:1473–1481.
8. Mao, J. C.-H., E. E. Robishaw, and L. R. Overby. 1975. Inhibition of DNA polymerase from herpes simplex virus-infected WI-38 cells by phosphonoacetic acid. J. Virol. 15:1281–1283.
9. Overby, L. R., E. E. Robishaw, J. B. Schleicher, A. Rueter, N. L. Shipkowitz, and J. C.-H. Mao. 1974.

Inhibition of herpes simplex virus replication by phosphonoacetic acid. Antimicrob. Agents Chemother. 6:360–365.
10. Prusoff, W. H., and B. Goz. 1973. Chemotherapy—molecular aspects, p. 641–685. In A. S. Kaplan (ed.), The herpesviruses. Academic Press Inc., New York.
11. Rowe, W. P., J. W. Hartley, S. Waterman, H. C. Turner, and R. J. Huebner. 1956. Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. Exp. Biol. Med. 92:418–424.
12. Shipkowitz, N. L., R. R. Bower, R. N. Appell, C. W. Nordeen, L. R. Overby, W. R. Roderick, J. B. Schleicher, and A. M. Von Esch. 1973. Suppression of herpes simplex virus infection by phosphonoacetic acid. Appl. Microbiol. 26:264–267.