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Short Communication

Phosphonoacetic acid inhibits replication of human herpesvirus-6

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Summary

Phosphonoacetic acid (PAA) inhibits the replication of human herpesvirus-6 (HHV-6) in mononuclear cells from cord bloods which are susceptible for natural HHV-6 infection in humans. Nuclear extracts of uninfected or HHV-6-infected mononuclear cells were applied to phosphocellulose column chromatography, and DNA polymerase activity was measured with or without the addition of 100 mM ammonium sulfate. The major DNA polymerase activities eluted at 0.47 M KCl were suppressed in both uninfected and HHV-6 infected cells by the addition of 100 mM ammonium sulfate. DNA polymerase activity eluted at 0.47 M KCl was observed only from HHV-6-infected cells; it was enhanced by 100 mM ammonium sulfate and neutralized with immune serum. DNA polymerase activity eluted at 0.73 M KCl was determined to be HHV-6 specific and had the properties of a typical herpesvirus-induced DNA polymerase. PAA inhibited HHV-6-specific DNA polymerase activity.

Human herpesvirus-6; Phosphonoacetic acid; DNA polymerase

Introduction

A new member of human herpesvirus (Salahuddin et al., 1986; Josephs et al., 1986), human herpesvirus-6 (HHV-6), is found to be an etiologic agent of an in-

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fantile febrile disease, exanthema subitum (Yamanishi et al., 1988), and lymphadenitis in adults (Kirchesch et al., 1988; Niederman et al., 1988; Eizuru et al., 1989). Recently a new DNA polymerase activity induced in HHV-6 infected cells has been reported to share common properties with herpesvirus DNA polymerase activity (Bapat et al., 1989). This study was aimed at examining the susceptibility of HHV-6 and sensitivity of HHV-6-specific DNA polymerase activity to phosphonoacetic acid (PAA) in mononuclear cells from cord bloods culture which are susceptible to natural infection in human.

Materials and Methods

Cell culture and sensitivity of HHV-6 to PAA

Mononuclear cells were separated from cord blood by centrifugation on Ficoll-Paque gradient and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 5 μg/ml phytohemagglutinin (Difco), and 0.1 unit/ml recombinant human interleukin-2 (Takeda Chemical Industries). HHV-6 was isolated from the blood of a patient with exanthema subitum and used for the infection of cultivated mononuclear cells (Yamanishi et al., 1988). The cells were infected with HHV-6 cell-free virus at 0.001 infectious dose/cell for 1 h and washed with medium. Infected cells (10^6 cells/ml) were incubated in the presence of the indicated concentration of PAA (Sigma). A sample of the cells was washed with phosphate buffered saline, dried and fixed with acetone at −20°C for 5 min to examine the number of infected cells. Infected cells were detected by immunofluorescent antibody to HHV-6 (Yamanishi et al., 1988).

DNA polymerase assay

The extraction of DNA polymerase was based on the procedures reported (Shiraki et al., 1986). Uninfected cells or HHV-6-infected cells with extensive cytopathic effect were harvested by light centrifugation and washed three times with Tris-buffered saline [TBS: 25 mM Tris (pH 8.0), 100 mM KCl]. Approximately 10^8 cells were treated with TBS containing 0.5% Nonidet P40 (BDH: England) and centrifuged at 2000 × *g* for 10 min at 4°C. After repeating this cycle the pellets of the nuclear fraction were suspended in 5 ml of the column buffer [25 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM 2-mercaptoethanol, 20% (V/V) glycerol] (Boezi et al., 1974) and sonicated in an ice bath for 3 min. The extracts were centrifuged at 100000 × *g* for 1 h at 4°C and their supernatants were used for phosphocellulose column chromatography. A 10 ml column of base- and acid-washed phosphocellulose (Whatman P-11) was equilibrated with the column buffer and washed with 10 mg of bovine serum albumin (BSA) dissolved in the column buffer. After application of enzyme extracts and washing the column with the column buffer the bound DNA polymerase activity was eluted with 100 ml of a linear gradient of 0.1 to 0.9 M KCl in the column buffer. The concentration of KCl was determined by the measurement of conductivity.

The reaction mixture for the DNA polymerase assay in a final volume of 200 μl contained 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 μg/ml of BSA, 5 mM dithiothreitol, 2.5 μg of poly(dA)p$(dT)_{12-18}$ (Pharmacia), 2.5 μCi of [^{3}H]thymidine triphosphate (dTTP) (78 Ci/mmol, DuPont-NEN) and 100 μl of enzyme extract. Otherwise the reaction mixture in a final volume of 200 μl contained 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 μg/ml of BSA, 50 μg of activated calf thymus DNA, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.01 mM dTTP, 2.5 μCi of [^{3}H]dTTP and 100 μl of enzyme extract. DNA polymerase activity of each fraction from phosphocellulose column chromatography was examined in the presence or absence of 100 mM ammonium sulfate. The reaction was conducted at 37°C for 90 min and terminated by adding trichloroacetic acid. The acid-insoluble material was precipitated onto glass filters (GF/F Whatman) and their radioactivities were determined.

Neutralization of DNA polymerase activity

Neutralization of DNA polymerase activity by immune sera was used to identify DNA polymerase induced by HHV-6. Sera used were a paired serum from acute and convalescent phase of exanthema subitum, and a negative and a high titered human serum to HHV-6, as determined by immunofluorescent antibody to HHV-6 (Yamanishi et al., 1988). Peak fractions of DNA polymerase activity were incubated for at least 3 h at 4°C with half a volume of serum dialysed in TBS, and residual activities were determined.

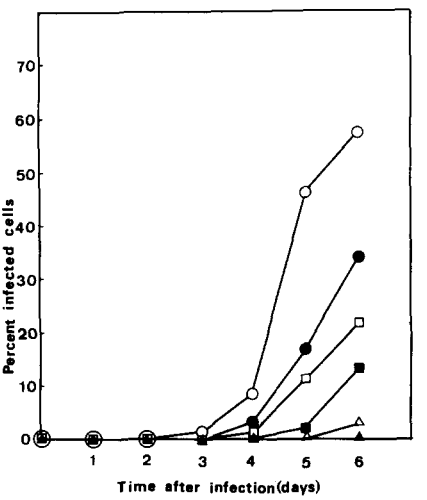

Fig. 1. Effect of PAA on HHV-6 infection in mononuclear cell culture. Mononuclear cells from cord bloods were infected with cell-free virus at 0.001 infectious unit/cell and incubated at the cell concentration of 10^6 cells/ml in the presence of the indicated concentration of PAA. A sample of cells was examined daily for HHV-6-specific immunofluorescence. The number of infected cells was expressed as the percentage of cells examined. PAA concentrations; (○) no PAA; (●) 2 μg/ml (14.3 μM); (□) 5 μg/ml (35.7 μM); (■) 10 μg/ml (71.4 μM); (△) 20 μg/ml (143 μM); (▲) 50 μg/ml (357 μM).

Results

Fig. 1 shows the effect of PAA on HHV-6 infection on mononuclear cells infected with cell-free virus. HHV-6-infected cells were identified by immunofluorescent antibody and the percentage of HHV-6-infected cells was determined by counting 500–1000 cells in two cultures. Although the number of infected cells increased with time in untreated cells, the HHV-6 infection was inhibited by PAA at concentrations of 2 to 50 μg/ml. HHV-6 infection in mononuclear cells from cord blood was sensitive to PAA.

DNA polymerase activity of nuclear extracts from uninfected or HHV-6-infected cells was separated by phosphocellulose. As shown in Fig. 2A, the major activity peak eluted at 0.47 M KCl from uninfected cells was suppressed in the presence of 100 mM ammonium sulfate. This activity peak also appeared in the 0.47 M eluate from infected cells, where it was again suppressed by ammonium sulfate. However, in the eluate of infected cells a new peak activity appeared at 0.73 M KCl, and this activity peak was not suppressed by ammonium sulfate (Fig. 2B). The peak activities eluted at 0.47 and 0.73 M KCl were incubated with sera and their residual activities were determined without or with the addition of 100 mM ammonium sulfate, respectively. Table 1 shows the results of neutralization of DNA polymerase activities eluted at 0.47 and 0.73 M KCl. The activity eluted at 0.47 M KCl was not neutralized with either serum, but that eluted at 0.73 M KCl was neutralized with both immune sera and not with nonimmune sera. Thus three lines of evidence, (i) appearance in the eluate from HHV-6-infected cells only, (ii) enhanced activity in the presence of ammonium sulfate and (iii) specific neutralization by immune sera, suggest that the DNA polymerase activity eluted at 0.73 M KCl is specifically induced by HHV-6. The viral DNA polymerase activity was effectively inhibited by PAA at a concentration of 2 to 10 μg/ml (Table 2).

Discussion

PAA is known to inhibit herpesvirus infection by inhibiting viral DNA polymerase. A greater than 50% inhibition of viral DNA polymerase activity is achieved by PAA at a concentration lower than 5 μg/ml (35.7 μM) for herpes simplex virus, human cytomegalovirus, Epstein-Barr virus, and varicella-zoster virus (Shipkowitz et al., 1973; Mao et al., 1975; Huang, 1975b; Miller and Rapp, 1977; May et al., 1977; Miller et al., 1977). A new herpesvirus, HHV-6, has now been examined for the sensitivity to PAA. PAA at the concentration of 2 to 10 μg/ml effectively inhibited virus infection in mononuclear cells from cord blood. PAA had little effect on the growth of mononuclear cells at these concentrations. To determine the enzymatic basis for PAA sensitivity, viral DNA polymerase was separated by phosphocellulose column chromatography. Cellular DNA polymerase activity at 0.47 M KCl was suppressed by ammonium sulfate but not neutralized by immune sera to HHV-6. This DNA polymerase may correspond to β-DNA polymerase (Weissbach et al., 1975). DNA polymerase activity at 0.73 M KCl was unique for HHV-

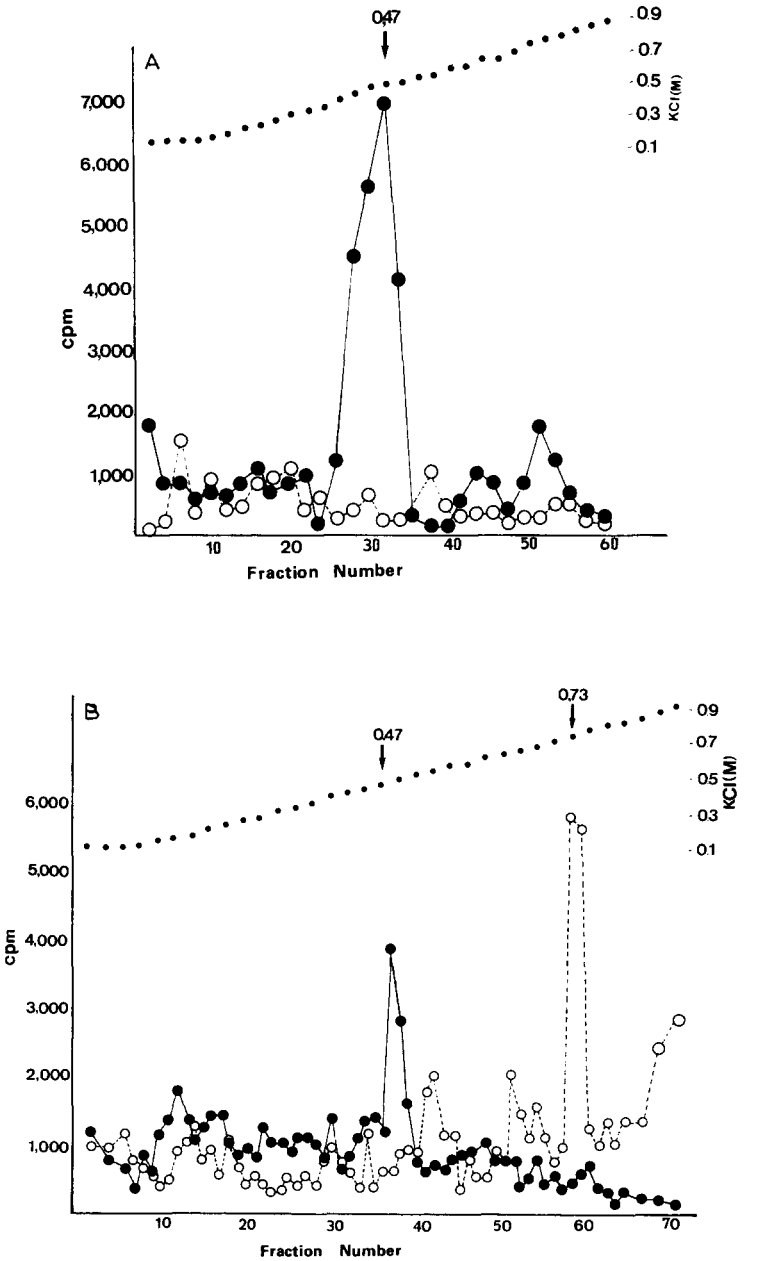

Fig. 2. DNA polymerase activities of uninfected cells (A) and HHV-6-infected cells (B) eluted from phosphocellulose. Nuclear extracts of cells were subjected to phosphocellulose column chromatography and eluted with a linear gradient (0.1–0.9 M KCl). DNA polymerase activity was measured in the absence of ammonium sulfate (●—●) and in the presence of 100 mM ammonium sulfate (○—○) using poly(dA).oligo(dT)$_{12-18}$ as template/primer.

TABLE 1

Neutralization of DNA polymerase activity

Serum	DNA polymerase activity at 0.47 M KCl (cpm)	DNA polymerase activity at 0.73 M KCl (cpm)
Acute phase of E.S. (1:<10 in IFA test)	2950 ± 234 (100)	10196 ± 912 (100)
Convalescent phase (1:512 in IFA test)	3186 ± 317 (107)	2289 ± 248 (22.4)
Non-immune serum (1:<10 in IFA test)	8433 ± 202 (100)	8589 ± 246 (100)
Immune serum (1:>512 in IFA test)	7579 ± 903 (89.9)	2708 ± 106 (31.5)

Sera used were paired sera (acute and convalescent phase) of exanthema subitum (E.S.) from the same patient, and non-immune human serum and immune human serum. The enzyme extracts were preincubated with dialyzed sera, and then DNA polymerase activity at 0.47 M and 0.73 M KCl was measured without and with 100 mM ammonium sulfate. Neutralization with paired sera was done using the different enzyme preparations. Data in parentheses are the percentages of residual DNA polymerase activity after incubation with serum from acute phase or non-immune human serum. IFA: immunofluorescent antibody.

6-infected cells, it was enhanced by ammonium sulfate (Fig. 2A), and neutralized by immune sera to HHV-6 (Table 1). This activity was determined as HHV-6-specific DNA polymerase. PAA at the concentration of 2 to 10 μg/ml effectively inhibited both virus infection (Fig. 1) and viral DNA polymerase activity (Table 2).

HHV-6 DNA polymerase activity was enhanced with ammonium sulfate and inhibited by PAA, which is a common property of DNA polymerases of herpesviruses (Weissbach et al., 1973; Huang, 1975a,b; Miller et al., 1977; Miller and Rapp, 1977; Shiraki et al., 1986). HHV-6 DNA polymerase was eluted from phosphocellulose with 0.73 M KCl. The elution pattern was similar in repeated experiments. This concentration is higher than those required for the elution of DNA polymerases from Marek's disease virus (0.2 M KCl; Boezi et al., 1974), herpes simplex virus type 1 (0.20 M KPO_4; Weissbach et al., 1973; 0.25 M KCl; Mao et al., 1975), human cytomegalovirus (0.26 M NaCl; Huang, 1975a), Epstein-Barr virus (0.29 M KCl; Miller et al., 1977), or varicella-zoster virus (0.33 M KCl; Miller et al., 1977; 0.32 M KCl; Shiraki et al., 1986).

TABLE 2

Effect of phosphonoacetic acid (PAA) on HHV-6 DNA polymerase activity

PAA concentration (μg/ml)	0	2	5	10	50
DNA polymerase activity (cpm)	13136 ± 269	8964± 450	5407 ± 69	5126 ± 123	1610 ± 76
	(100)	(68.3)	(41.1)	(39.0)	(12.2)

Viral polymerase activity was measured in the presence of 100 mM ammonium sulfate. Data in parentheses represent the percentage of DNA polymerase activity.

Recently, Bapat et al. (1989) reported a new DNA polymerase activity in cells infected with human B-lymphotropic virus. They identified a new DNA polymerase activity by the difference in substrate specificity and sensitivity to salt. We have immunologically identified HHV-6 specific DNA polymerase activity obtained from naturally susceptible mononuclear cells, by neutralization of enzymatic activity with paired sera from a patient with exanthema subitum, and a non-immune and immune serum to HHV-6-infected cells. Bapat et al. (1989) assessed the susceptibility of viral DNA polymerase to phosphonoformic acid (PFA) by reduction of total DNA polymerase activity induced in cells treated with PFA. Total DNA polymerase activity in cells treated with 5 μM PFA was suppressed to about 30% of that of untreated cells. The 50% inhibitory doses of PFA and PAA were 0.4 and 2.54 μM, respectively. We examined the susceptibility of HHV-6 to PAA by infecting mononuclear cells from cord blood which are the natural target cells of HHV-6 infection with cell-free virus and identifying the number of infected cells by immunofluorescent antibody. The number of infected cells in the treated culture was 0.03% of that in the untreated culture in the presence of 10 μg/ml (71.4 μM) of PAA.

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