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Differential effect of phosphonoacetic acid on the expression of Epstein–Barr viral antigens and virus production

(cell growth/DNA synthesis/5-iodo-2′-deoxyuridine induction/lymphocyte transformation)

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ABSTRACT **The effects of phosphonoacetic acid on cell growth, expression of Epstein–Barr virus antigens, and virus production in human and marmoset lymphoblastoid cell lines have been studied. The drug had no significant effect at concentrations up to 100 μg/ml on cell growth or total cell DNA synthesis. Higher doses induced not only a drastic decrease in DNA synthesis and cell growth, but also a dramatic cell enlargement. Immunofluorescence studies showed that ≥30 μg/ml of phosphonoacetic acid inhibited viral capsid antigen synthesis without affecting the expression of the nuclear antigen or the spontaneous and 5-iodo-2′-deoxyuridine-induced early antigens. Production of transforming Epstein–Barr virus was also blocked. Inhibition of viral capsid antigen expression and of virus production at low doses was reversible. The transforming virus produced after the removal of the drug from the B95-8 marmoset cell line transformed peripheral blood lymphocytes as effectively as virus prepared from untreated cells. It is known that the expression of viral capsid antigen but not early antigen requires viral DNA synthesis. Therefore, these results suggest that phosphonoacetic acid interferes specifically with viral DNA synthesis at concentrations that do not affect cellular DNA synthesis.**

The recent discovery of the anti-viral activity of phosphonoacetic acid (PAA) in a random screening test of compounds has aroused intense interest in this chemical. The antiviral activity was demonstrated in herpes simplex virus types 1 and 2 infection of rabbits (1, 2) and in the infection of cultured WI-38 (human fibroblast) cells (3). PAA inhibits herpes simplex virus replication by interfering with the DNA polymerase activity (4). It has also been found to inhibit the replication of several other herpes viruses, including cytomegalovirus (5), Marek's disease virus (6), and equine abortus virus (7), and a non-herpes virus, vaccinia virus (8). In this paper we present data which show that PAA inhibits the synthesis of Epstein–Barr (EBV) viral capsid antigens (VCA) and of virus itself at concentrations which have no detectable effect on the synthesis of nuclear antigen (EBNA) and early antigens (EA), or on cellular DNA synthesis and cell proliferation. PAA also has no detectable effect on 5-iodo-2′-deoxyuridine- (IdUrd) or superinfection-induced EA synthesis in the nonproducer Raji cell line.

MATERIALS AND METHODS

Cell Cultures. Lymphoblastoid cell lines B95-8, P3HR-1, and Raji used in this investigation were obtained from Dr. George Klein and from Pfizer, Inc. The B95-8 cell line is an EBV-transformed marmoset lymphocyte line which produces a lymphocyte-transforming virus (9). P3HR-1 and Raji are Burkitt's lymphoma-derived lines which produce nontransforming cytopathic EBV (10) and no virus (11), respectively. Cells were routinely maintained in RPMI-1640 medium supplemented with 100 μg/ml of streptomycin, 100 units/ml of penicillin, 300 μg/ml of glutamine, and fetal calf serum. They were fed every 3–4 days and the concentration was adjusted to 2 to 5×10^5 cells per ml at each feeding. All cultures were kept at 37° in a humidified 5% CO_2 incubator.

Reagents. Phosphonoacetic acid was obtained from Abbott Laboratories, Chicago, Ill. Anti-EBV sera were gifts of Drs. George Klein and Werner Henle. Fluorescein-isothiocyanate-conjugated goat anti-human IgG was obtained from Hyland Laboratories, California, and Grand Island Biological Company, N.Y.

Cell Growth and DNA Synthesis. For measurement of DNA synthesis, B95-8 cells were seeded in duplicate at 5×10^5 per ml in a total of 3 ml for 48 hr and pulse labeled with 4 μCi/ml of[^{3}H]dThd (New England Nuclear, specific activity: 13 Ci/mmol) for 1 hr, and radioactivity in the trichloroacetic-acid-precipitable material was assayed in a Beckman liquid scintillation counter. For measurement of growth, cells were seeded in duplicate at 5×10^5 per ml and cultivated for various times. Viable cells were determined by the trypan blue exclusion technique. The effect of PAA on chromosomes was studied in exponentially growing B95-8 cells treated with colchicine (0.05 μg/ml for 2–3 hr). Metaphase chromosomes (12) were stained with Giemsa (13) or quinacrine mustard (14).

Viral Antigens. B95-8 and P3HR-1 cultures were prepared in duplicate, incubated for 4 days with or without PAA, harvested, smeared, acetone-fixed, and stained for VCA and EA by the indirect (15) or direct method (16). Anti-EBV sera, including some VCA+EA- discordant sera, were generously supplied by Drs. George Klein and Werner Henle, and were used at 1:20 and 1:40 dilutions. The fluorescein-isothiocyanate-conjugated goat anti-human IgG was used at a 1:20 dilution. EA was induced in duplicate cultures of Raji cells by treatment with 50 μg/ml of IdUrd in the presence of various concentrations of PAA for about 72 hr, at which time the cells were harvested and processed for EA staining.

Cells were stained for EBNA by the anticomplement immunofluorescence method (17) using the three-step procedure (18). In preparing cells for staining, it is essential that the smears be dried almost instantly. To facilitate this, washed cells were resuspended at a final concentration of about 5×10^7 cells per ml. The smear was made with a pasteur pipette, dried by passing the slides over a flame or by blowing a stream of warm air on them, and fixed with a chilled 50:50 acetone:methanol mixture for 10 min. Serum from an EBV-seropositive normal person (R.Y. or P.A.) was used as the anti-EBNA serum at 1:40. An EBV-seronegative serum (D.B.) was used as the source of complement at 1:10

Abbreviations: PAA, phosphonoacetic acid; EBV, Epstein–Barr virus; EBNA, Epstein–Barr virus nucleus-associated antigen; EA, early antigen; VCA, viral capsid antigen.

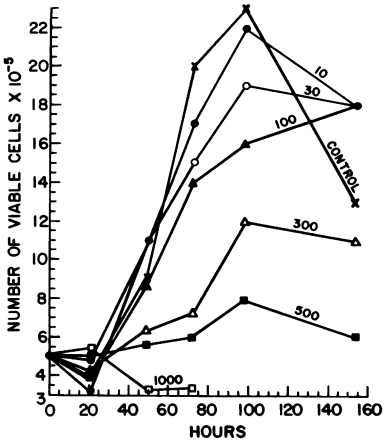

FIG. 1. The effect of PAA on B95-8 cell growth. Duplicate cultures were prepared and incubated with different concentrations of PAA (μg/ml as indicated).

and fluorescein-isothiocyanate-conjugated goat anti-human β1C/β1A (Hyland Laboratories) was the anti-complement serum used at 1:20 dilution.

Long-Term Effect of PAA on Viral Activities. Two duplicate sets of B95-8 cultures were prepared in either 30 μg/ml or 100 μg/ml of PAA, together with controls. Cell smears were prepared from each culture every 24–48 hr and stained for EA and VCA. To see whether inhibited cells can regain their ability to synthesize VCA, we split the PAA-treated cultures into two portions, one of which we continued to treat with PAA while the other was washed free of PAA and subsequently maintained in PAA-free media. Smears were prepared and stained for VCA and EA as before.

Lymphocyte Transformation. The effect of PAA on the production of transforming EBV in B95-8 cells was studied by the lymphocyte transformation assay (19) with some modification. Aliquots of the cultures used for studying the long-term effect of PAA on EBV (see above) were utilized for virus preparation. Six days after the initiation of PAA treatment, cells treated with 100 μg/ml of the drug together with controls were washed three times with regular growth medium. The experimental cultures were split into two portions, one of which was fed with medium containing 100 μg/ml of PAA while the other was fed with regular growth medium. After 7 days at 37° without feeding virus was prepared by pelleting the suspension at 1000 rpm (Sorvall GLC-2) for 5 min, clarifying the resulting supernatant at 2000 rpm for 10 min, dividing it into aliquots, and freezing it at −70° until needed. Purified bone-marrow-derived (B) lymphocytes from peripheral blood were transformed with a 1:20 dilution of the virus preparation at 37° in microtiter plates (Falcon Plastics). PAA was added to the virus prepared from PAA-free cultures in order to compensate for the

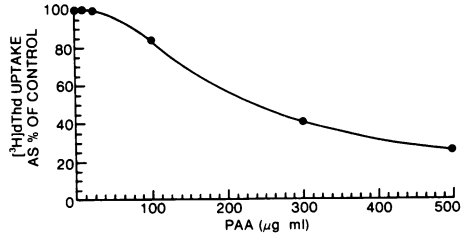

FIG. 2. The effect of PAA on [³H]dThd uptake by B95-8 cells.

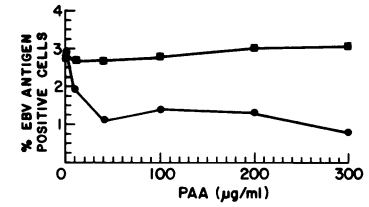

FIG. 3. The effect of PAA on EA (■) and VCA (●) syntheses in P3HR-1 cells. Smears for immunofluorescence tests were prepared from duplicate cultures on the fourth day. The fluorescence tests were read without the readers' knowing what the sample was and at least 500 cells were counted for each smear.

effect of residual PAA present in preparations from PAA-treated cultures. After 7 days, EBV-induced blast transformation was measured by pulse-labeling the cultures with 0.4 μCi/ml of [³H]dThd (New England Nuclear, specific activity: 2 Ci/mmol) for 16 hr, and counting radioactivity in the acid-insoluble materials.

RESULTS

Effect of PAA on Cell Growth and DNA Synthesis. Growth of the marmoset B95-8 cells in various PAA concentrations over a 154 hr period was measured (Fig. 1). Concentrations less than 100 μg/ml had no significant effect on cell growth, while concentrations greater than 500 μg/ml completely inhibited cell growth. These effects on cell growth were paralleled by effects on total cell DNA synthesis. Treatment of B95-8 cells with 20–30 μg/ml of PAA had no significant effect on DNA synthesis as measured by [³H]dThd incorporation into acid-insoluble materials. About 240 μg/ml caused a 50% reduction and 500 μg/ml caused about a 75% reduction in DNA synthesis (Fig. 2). Similar results were obtained with a number of other cell lines.

In addition, concentrations of PAA greater than 200 μg/ml induced a dramatic increase in cell size. The dose-dependent reciprocal increase in cell volume and decrease in cell proliferation led to the question of whether or not PAA was interfering with some cellular function which was essential for proper cell division. Examination of B95-8 cells treated with various concentrations of PAA revealed none of the abnormalities usually associated with improper cell division, such as nondisjunction, multinucleation, and polyploidization. The only observable difference between enlarged and normal size cells was the very low mitotic index found in cells treated with >100 μg/ml of PAA. They appeared to be arrested at interphase. The few metaphase plates found among enlarged cells appeared normal with respect to the number, type, and morphology of chromosomes. While interphase cells were clearly mononuclear, none showed any sign of partial cytokinesis or lack of nuclear envelope. These observations suggest that although a high dose of PAA inhibits cellular DNA synthesis, PAA does not seem to interfere

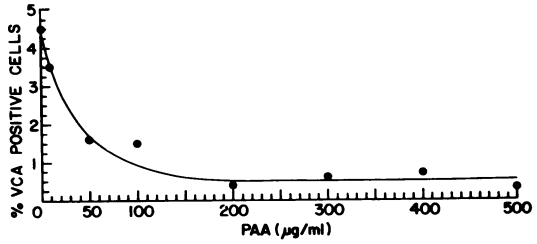

FIG. 4. The effect of PAA on VCA synthesis in B95-8 cells.

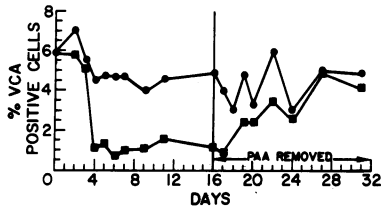

FIG. 5. Time course of the effect of PAA on VCA synthesis in B95-8 cells. The test was carried out as described in Fig. 3. On day 16, PAA-treated and control cultures were washed three times with regular growth medium and were subsequently kept in PAA-free medium. Some of the PAA-treated cells were continuously kept in PAA. (●), Control and (■), 30 μg/ml of PAA.

with any of the cellular processes which usually follow chromosomal DNA replication.

Differential Effect of PAA on EBV Antigen Expression. In P3HR-1 cells (a human lymphoblastoid line which is the commonly used producer of infectious EBV), PAA inhibited expression of VCA but not EA (Fig. 3). VCA synthesis was markedly inhibited by 30 μg/ml of PAA, whereas even 100 μg/ml had little effect on cell growth. EA synthesis was not affected at even 500 μg/ml. Similarly, VCA synthesis in B95-8 cells (a marmoset lymphoblastoid line which produces a transforming strain of EBV) was markedly sensitive to PAA, while EA synthesis was unaffected (Figs. 4 and 5).

The inhibitory effect of PAA on VCA synthesis was first observed at about 4 days (Fig. 5). One possible explanation for the lag is that VCA seen between day 0 and day 3 is not newly synthesized antigen but rather is that already synthesized or in the process of being synthesized when drug treatment was initiated. This VCA would mask the effect of PAA on new VCA until it is lost after cell lysis. A lag in drug penetration could also account for part of this result.

The effect of PAA on VCA expression was independent of the duration of treatment. VCA synthesis in cells that were continuously treated with PAA for 3 months was still inhibited as at day 4. The level of EA remained consistently unaffected. When PAA was removed from the growth medium, VCA synthesis was restored whether the cells had been treated with 30 or with 100 μg/ml and even after 7–10 weeks of treatment (Table 1). Upon removal of PAA several days were required before the VCA level equaled that of untreated cells. The time for reappearance of VCA was the same whether the cells had been treated for 1 week or for 7 weeks with PAA. PAA treatment had no detectable effect on EBNA expression in either producer or non-producer lines (Tables 1 and 2) regardless of the time of exposure or the concentration used. About 90% of both treated and control cells synthesized EBNA. This finding agrees well with the observation that when inhibited cells were released, they resumed virus production (see below) and that inhibited cells continued to synthesize early antigens. All of these observations suggested that PAA inhibition did not cure cells of their EBV infection.

Table 1. The effect of PAA on expression of EBNA and other antigens in B95-8 marmoset cell line

	Exp. 1			Exp. 2		
	A	B	C	A	B	C
PAA (μg/ml)	0	30	30	0	100	100
Time (days)	0	42	24*	0	71	67*
EBNA†	+	+	+	+	+	+
% EA+ cells	4.4	4.9	4.1	3.1	3.5	4.0
% VCA+ cells	3.8	0.5	3.4	3.0	0.2	2.0

* PAA removed after times indicated.
† + = >90% positive cells.

Table 2. Effect of PAA on induction of EA by IdUrd in Raji cells

	Treatment (μg/ml)			
Culture*	IdUrd	PAA	% EA†	EBNA
1	0	0	0	+
2	50	0	1.6	+
3	50	50	1.7	+
4	50	100	1.4	+
5	50	200	1.7	+
6	50	400	1.8	+

* Each culture was prepared in duplicate.
† The results shown here are those of the diffuse form of early antigen, EA(D). The results for the restricted form, EA(R), were evaluated qualitatively only, but they were about the same as those for EA(D). EA(R)+ and EA(D)+ sera were obtained from Dr. Werner Henle.

Effect of PAA on Production of Transforming Virus by B95-8 Cells. Supernatant solutions from cultures of B95-8 cells were assayed by blast transformation of normal peripheral blood lymphocytes. After exposure to PAA for 20 days there was virtually no transforming EBV produced (Fig. 6). However, a culture released from PAA inhibition for 2 weeks produced almost as much transforming EBV as the untreated culture. Thus, the effect of PAA on virus production was reversible, and the progeny virus was not altered by exposure to PAA, at least as measured by its ability to transform peripheral blood lymphocytes.

Effect of PAA on Induction of EA in Raji Cells by Treatment with IdUrd or Superinfection with P3HR-1 Virus. Superinfection of nonproducer Raji cells with P3HR-1 virus or treatment of these cells with IdUrd usually results in an abortive viral cycle in which the cells make only early antigens (20). PAA had no effect on either of these processes (Table 2). This observation is in contrast to the finding of Klein and Dombos that a DNA inhibitor such as cytosine arabinonucleoside (Ara-C) completely inhibited IdUrd induction.

DISCUSSION

PAA at concentrations >100 μg/ml had no significant effect on the growth of several lymphoblastoid cell lines, including

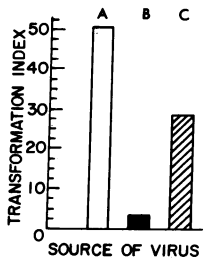

FIG. 6. Histogram showing the effect of PAA on the production of transforming EBV in B95-8 cells. Each histogram represents the average of three samples. Stimulation index is the ratio of virus-induced transformation to transformation without virus. A. (□), Control; B. (■), 20 days in 100 μg/ml of PAA; C. (▨) 6 days in 100 μg/ml of PAA followed by 14 days without PAA.

marmoset and human lines. The effect of PAA on cell growth was dose-dependent, in agreement with what had been observed in WI-38, a human fibroblast line (2). However, concentrations which completely inhibited cell growth did not kill cells but merely arrested them at interphase. Cells so arrested became larger than untreated ones, but this effect was reversible. Possibly, PAA at high concentrations might inhibit some specific functions such as the initiation of DNA synthesis by competing with factors which normally occur in the cell.

PAA specifically inhibits some but not all EBV functions. None of the concentrations tested (up to 500 μg/ml) had any detectable effect on EBNA or EA, whereas 30 μg/ml inhibited VCA synthesis. Furthermore, PAA did not inhibit the expression of EA after induction of the endogenous virus by IdUrd or superinfection with P3HR-1. These observations agree with those of other investigators that DNA synthesis is not required for the expression of early EBV functions such as EA and membrane antigen (MA) (21). The lack of effect of PAA on induction and superinfection also suggests that the mechanisms involved in these two phenomena do not require a PAA-sensitive DNA synthesis. In contrast, cytosine arabinonucleoside, which completely inhibits induction but not superinfection, presumably does so by preventing both cellular and viral DNA synthesis (20), whereas PAA fails to do so because it has no effect on cellular DNA synthesis. In order to explain their results, Klein and Dombos (20) suggested that incorporation of IdUrd into cellular DNA may be required for induction, and Hampar *et al.* (22) actually showed that incorporation of IdUrd into DNA in early S phase is a prerequisite for induction. It is interesting that PAA inhibition of late EBV functions is completely reversible. It suggests that PAA inhibition of virus replication is unlikely to involve alterations of the genetic information of the virus or the host cell.

During the course of our investigation, we learned that similar results of the effect of PAA on EBV replication were obtained by Summers and Klein*. They have also made the interesting observation that the average number of EBV genomes per cell in the B95-8 marmoset cell line is reduced from about 150 to 40. This result is compatible with the observation reported here that production of transforming virus by these cells is inhibited by PAA. Only about 5% of the cell population is EA- and VCA-positive and thus, if the stable number of viral genomes per cell in the absence of viral production is 40, the number of genomes in virus-producing cells must be about 2000. If PAA prevents virus production by the same mechanism as it does in other herpes viruses, i.e., inhibition of virus-specific DNA polymerase, then it is likely that replicating cells continue to maintain about 40 copies of EBV genomes per cell, utilizing the host DNA replication apparatus. This is also suggested by the observation that the non-producer line, Raji, maintains a stable number of viral genomes at 65 copies per cell with or without PAA treatment (26).

Clearly, genes for EBNA and EA, but not VCA, can be expressed by the remaining 40 EBV genomes in PAA-treated B95-8 cells. The failure of VCA synthesis in these cells could suggest either a gene dosage effect (since VCA-synthesizing cells may contain about 50 times more viral genomes than non-VCA-synthesizing cells), or a requirement for independently replicating viral DNA in the expression of this gene. A similar phenomenon has been observed for expression of some of the late genes in bacteriophage T4 (23).

The selective inhibition of EBV replication by PAA may facilitate the selection of mutant EBV strains similar to the recently reported isolation of PAA-resistant strains of herpes simplex virus (24, 25). Furthermore, the reversibility of PAA inhibition might facilitate the study of EBV–cell regulatory mechanisms in somatic cell hybrids. That is, it might be possible by controlling virus production with PAA to isolate some types of hybrids which would otherwise fail to survive.

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