JOURNAL OF VIROLOGY, Apr. 1976, p. 151-155

Vol. 18, No. 1
Printed in U.S.A.

Inhibition of Epstein-Barr Virus DNA Synthesis and Late Gene Expression by Phosphonoacetic Acid

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Received for publication 30 September 1975

Growth of lymphoblastoid cells (B95-8, Raji) is not inhibited by the presence of 0.4 mM phosphonoacetic acid. The synthesis of Epstein-Barr virus (EBV) in the producer line B95-8 is completely inhibited, as shown by the total inhibition of viral capsid antigen synthesis. Early viral antigens are made normally in the presence of phosphonoacetic acid, but EBV DNA synthesis is blocked in cells entering the productive cycle. Nonproducer cells in the population replicate the resident EBV DNA by a mechanism that is resistant to phosphonoacetic acid. These results are consistant with the hypotheses that EBV DNA is replicated by two mechanisms, one in the noninduced cell and a different mechanism in the producer cell, and that prior replication of EBV DNA, probably by the second mode, is a prerequisite for late gene expression.

Epstein-Barr virus (EBV) is a herpesvirus which is known to exist in a partially repressed state in human continuous lymphoblastoid cell lines (8). Some of these cells produce infectious virus particles spontaneously or, in some cases, upon treatment with the halogenated pyrimidines iododeoxyuridine and bromodeoxyuridine. The sequence of events in the induction of virus synthesis has been deduced from the appearance of certain virus-specific functions after induction and in the presence of certain inhibitory compounds (4, 5). A general outline of the EBV lytic cycle is as follows. EBV DNA-containing cells synthesize the EBV-specific nuclear antigen (EBNA), whereas those cells that begin virus synthesis first synthesize those proteins recognized as early antigens (EA). Viral DNA synthesis then takes place, followed by synthesis of viral capsid antigens (VCA). Assembly, maturation, and release of virus occurs in a certain fraction of cells. Studies with cytosine arabinoside show that in the absence of any DNA synthesis (cell and virus) the late antigens (VCA) are not synthesized. The extents to which the other steps of the virus growth cycle are interrelated are not yet known.

The recent demonstration of phosphonoacetic acid (PAA) as a specific inhibitor of DNA polymerase that is active against several herpestype viruses (9, 13) but inactive against mammalian cells suggested an experimental test of the hypothesis that the EBV VCA is dependent specifically upon viral DNA synthesis rather than host DNA synthesis.

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MATERIALS AND METHODS

Cell lines. The B95-8 line is a marmoset cell line derived by treatment with EBV from a human cell line established from a patient with acute infectious mononucleosis believed to be induced after blood transfusion (10). This line releases infectious virus particles. The Raji cell line was derived from a tumor biopsy from a patient with Burkitt's lymphoma (14). This cell line harbors EBV DNA sequences (11) and expresses EBNA but does not produce other viral products (EA, VCA, virions) either spontaneously or upon induction with iododeoxyuridine.

Cells were grown exponentially in medium RPMI 1640 with 5% calf serum and were judged mycoplasma free by culture (6). These sublines were derived from cultures treated with multiple antibiotics by F. Wiener.

Immunological methods. Direct immunofluorescence was used to detect EA and VCA as previously described (4). The serum M'rumuri was EA^-,VCA^+; the serum Tamani was EA^+,VCA^+. Each serum was used at a 20- or 40-fold dilution of the fluorescein-conjugated immunoglobulin G fraction. EBNA was assayed by anticomplement immunofluorescence using the method of Reedman and Klein (15). A total of 10^3 cells or enough total cells to enumerate 20 EA- or VCA-positive cells were counted, whichever was smaller. Positive and negative control cell smears were included to test the specificity of the staining.

DNA preparation. About 10^7 cells were washed in saline and suspended in 0.2 ml of 10^{-2} M Tris-chloride (pH 7.8)-10^{-3} M EDTA, and sodium dodecyl sulfate (SDS) was added to 1% and Pronase to 1 mg/ml. The sample was incubated at 37 C for 2 h, cooled, and extracted once with a mixture of chloroform and *n*-butanol (3:1, vol/vol). The aqueous phase was made 0.12 M in NaOH and treated at 100 C for 25 min. Each sample was neutralized to pH 7.5 with HCl, and then 2 volumes of 95% ethanol was added. After 16 h at −20 C the denatured DNA precipitate was

removed by centrifugation at 7,000 × *g* for 10 min. The DNA was suspended in 100 μl of 10^{-2} M Tris-chloride (pH 7.8)-10^{-3} M EDTA, and 10 μl was removed and assayed by Burton's modification of the Dische reaction (3) for the DNA content. Phage T7 DNA that had been measured spectrophotometrically (50 μg/ml = 1.0 $OD^{1\,cm}_{260\,nm}$ was used as a standard.

DNA renaturation rate analysis. ^{3}H-labeled EBV DNA (labeled by nick translation [7] with unlabeled, purified B95-8 EBV DNA, [^{3}H]dTTP, and *Escherichia coli* DNA polymerase I) and unlabeled EBV DNA were the generous gifts of Bill Sugden. The specific activity of the labeled DNA was 160 counts/min per ng. The cellular DNA was tested for the presence of EBV DNA by its ability to increase the renaturation rate of a standard amount of ^{3}H-labeled EBV DNA. The procedure of Sugden (personal communication) was followed to measure the EBV DNA content of the cellular DNA preparations. Briefly, 5,000 counts/min of ^{3}H-labeled EBV DNA was added to 100 μl of cellular DNA (50 to 150 μg) in 0.28 M sodium phosphate (pH 6.8)-0.1% SDS. Aliquots of 10 μl were sealed in capillaries, and the sealed capillaries were heated to 125 C and then incubated at 60 C. At various times samples were removed, cooled, and diluted in 1 ml 0.14 M sodium phosphate. Each sample was chromatographed on hydroxylapatite in 0.14 M sodium phosphate (pH 6.8) with 0.4% SDS at 60 C to elute the single-stranded DNA and then eluted with 0.4 M sodium phosphate in 0.4% SDS to recover the renatured DNA. These two fractions were then counted in a liquid scintillation spectrometer. Standards included 1 mg of calf thymus DNA per ml and 0, 3, 6, or 9 ng of unlabeled EBV DNA in the 100-μl renaturation mixture. From plots of the reciprocal of the fraction of the single-stranded DNA versus the time of incubation, the rate of renaturation of the ^{3}H-labeled probe could be calculated. Comparison of this rate with the rates for the standards allowed a sensitive determination of the EBV DNA content of the cellular DNA samples.

Miscellaneous. PAA was the kind gift of Abbott Laboratories. It was dissolved in medium at 0.5 M, sterilized by filtration (0.22-μm pore size) and stored at −20 C. After thawing, it was used in appropriate dilutions.

RESULTS

We first determined the range of PAA concentrations which showed progressive cytotoxicity. Exponentially growing cells were diluted to 4 × 10^4 cells/ml in various concentrations of PAA. The cell growth was then monitored by periodic cell counting. Figure 1 shows the result of one of several such experiments. The growth inhibitory range for B95-8 as well as Raji cells was greater than 0.4 mM PAA, and it took 36 to 48 h to observe any diminution of growth rate. Nearly normal growth in 0.1 to 0.4 mM PAA could be maintained for several weeks.

The expression of the EBV genome in B95-8 cells in the presence of PAA concentrations that permitted continual cell proliferation was determined by measuring the fraction of cells that were EA$^+$ and/or VCA$^+$, and those that were VCA$^+$ at various times after addition of PAA. Figure 2 shows the cell growth as well as the fraction of EA$^+$ and/or VCA$^+$ and VCA$^+$ cells. The cells continued to grow at nearly the control rate, but the VCA$^+$ cells rapidly disappeared from the PAA-treated culture. In contrast, staining with EA$^+$,VCA$^+$ serum revealed no significant change after PAA treatment, which is attributable to the presence of EA$^+$ cells in the treated culture. The decrease in VCA$^+$ cells started in about 24 h and no BCA cells could be detected after 48 to 72 h. If PAA was removed on day 4 and the cells were incubated further, VCA expression was detectable at 2 days and returned to normal within 3 days after removal of the drug.

The concentration of PAA that was necessary to block VCA synthesis was determined by enumerating the VCA-positive cells after 4 days in various concentrations of PAA. Figure 3 shows the results of one such experiment. The effective concentration for inhibition of VCA synthesis was between 0.2 and 0.4 mM, which is two- to threefold lower than the concentrations that are even slightly cytotoxic. This particular experiment was unusual in that inhibition was slow and some VCA-positive cells were still observed at day 4, but several other experiments carried out at single concentrations in this range all showed absolutely no VCA$^+$ cells by day 4.

To determine the effect of PAA on the synthe-

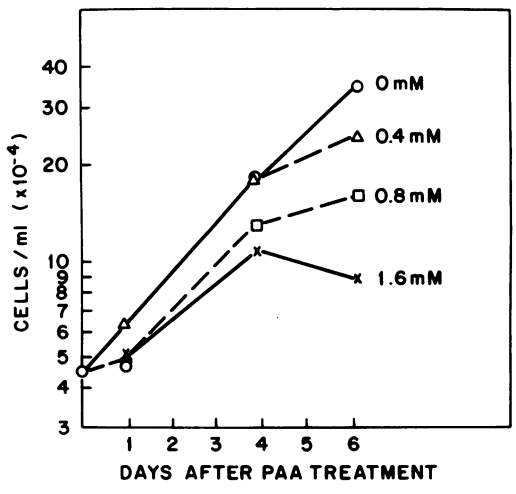

FIG. 1. *Growth of B95-8 cells in various concentrations of PAA. The drug was present in the culture for the entire experiment.*

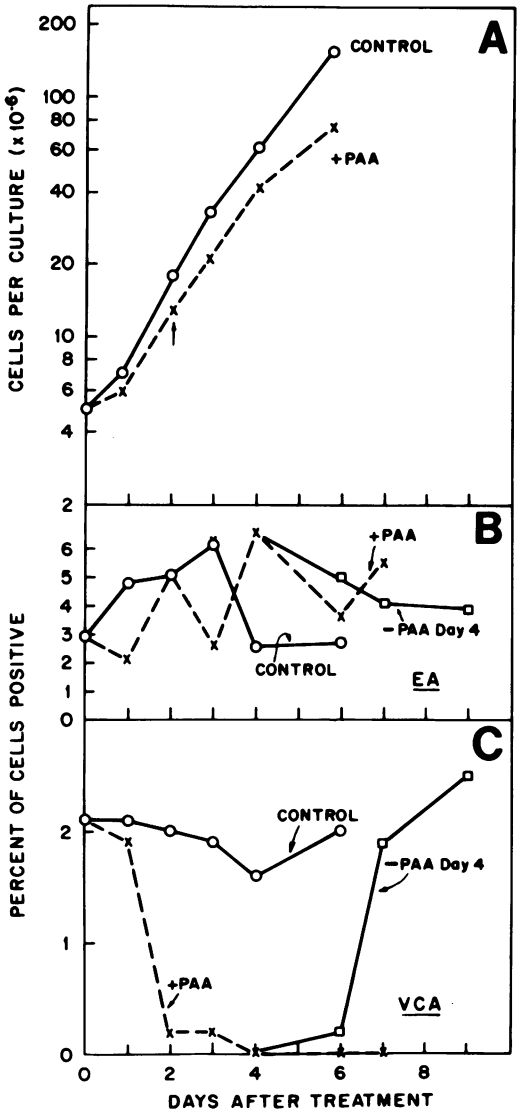

FIG. 2. *Effect of PAA on B95-8 cells. Growth (A), EA (B), and VCA (C) were monitored. At day 2 the cultures were diluted into fresh medium containing PAA (arrow in A). The growth curves have been corrected for this dilution. On day 4 a portion of the treated culture was harvested and suspended in drug-free medium (−PAA, □). Control cultures (○) had no PAA; treated cultures (×) contained 0.4 mM PAA.*

sis of EBV DNA, the cellular content of EBV DNA was determined before and after treatment with PAA. Two different cell lines were used, B95-8 and Raji. Figure 4 shows the EBV DNA content of each cell line before and 4 days after treatment with 1 mM PAA. The B95-8 line which is spontaneously synthesizing virus contained about 150 EBV genome equivalents per cell prior to treatment with PAA. This dropped to about 40 EBV genome equivalents after PAA treatment. The Raji cell line contained about 65 EBV genome equivalents per cell before and after PAA treatment.

The kinetics of the decrease in EBV DNA content of the B95-8 cell line was determined by serial determinations of EBV DNA content (Fig. 4). By day 3 after adding PAA, the EBV DNA content had reached a plateau value of about 20 to 25% of the untreated cell line. Removal of the drug at day 4 allowed recovery to control levels by day 9.

Both the Raji and the B95-8 cells were stained for EBNA before and after PAA treatment (1 mM) for 4 and 7 days. In all cases greater than 95% of the cells were positive for EBNA.

DISCUSSION

The results of this investigation show that PAA is relatively nontoxic to lymphoblastoid cell lines even with prolonged exposure to the drug. The expression of the resident EBV genome in these cells, however, is strongly affected by this compound. Early antigen synthesis continues normally in the presence of PAA so that the fraction of EA-positive cells is con-

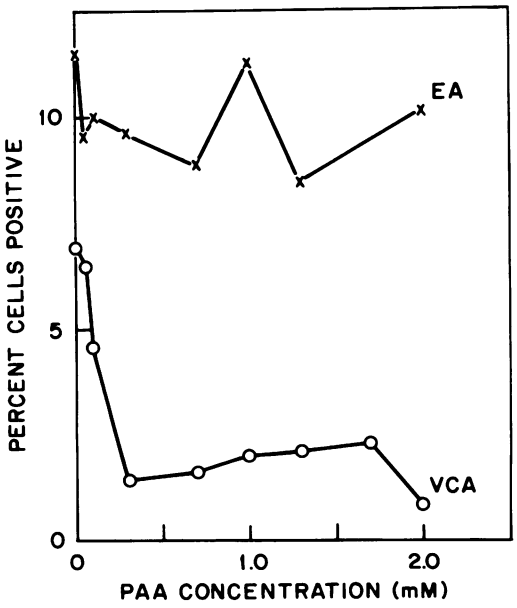

FIG. 3. *Effect of various concentrations of PAA on EA and VCA synthesis in B95-8 cells. Measurements of the fraction of cells stained by immunofluorescent sera were made on day 4. As noted, the time course of inhibition was somewhat slower than usual in this experiment so that a few VCA-positive cells were seen even with high doses of PAA.*

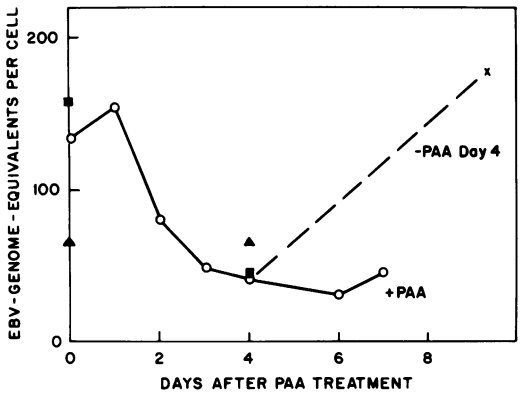

FIG. 4. *Effect of PAA on the average EBV DNA content per cell at different times after beginning treatment. EBV DNA was measured by renaturation rate analysis as described. These determinations were carried out on the same cultures of B95-8 shown in Fig. 2 (○). At day 4 the PAA was removed from one culture, and after 5 days of growth in drug-free medium the sample was analyzed (−PAA, ×). A similar experiment was carried out with 1 mM PAA on B95-8 (■) and Raji (▲) cells with samples taken at day 0 and day 4.*

stant, since the cells continue to grow. VCA-positive cells disappear from the population within 2 days. This could be the result of either inhibition of synthesis of VCA or increase in the rate of loss of VCA from the cells. We prefer the former explanation because it is consistent with previous studies in which late gene expression (VCA) was shown to be dependent on prior DNA synthesis using the cytosine arabinoside blocking method (4, 5). In addition, the inability to find any (less than 1 cell in 10,000) VCA-positive cells 4 days after PAA treatment suggests that synthesis is blocked rather than lysis of VCA-positive cells by PAA, since we would expect to see a few VCA-positive cells, except in the unlikely instance where lysis is essentially instantaneous.

Analysis of the content of EBV DNA in the producer cell line B95-8 shows that after treatment with PAA the average number of EBV genome equivalents per cell decreases from about 150 to about 40. Since the population consists of virogenic but nonproducing cells and a few cells (about 2 to 5%) that are actively making virus (at least VCA), and hence probably have a great many copies of the EBV genome, any inhibition of the net synthesis of viral DNA in the productive cells should decrease the amount of EBV DNA per cell. If we assume that the VCA-negative cells in the population have the same amount of EBV DNA as the average cell in the PAA-treated culture, which is entirely VCA negative, the average EBV DNA content of the VCA-positive cells in the untreated population can be calculated to be about 5,000 EBV genome equivalents. This is clearly an upper limit since the calculation neglects the likely possibility that some cells have synthesized EBV DNA and not yet made enough late proteins to become VCA positive. Since the average EBV DNA content plateaus at 40 genome equivalents, rather than being reduced to zero, it is likely that the cells which are not in the productive viral cycle have about 40 genome equivalents of the viral DNA associated in some stable way with the host cell. The results with the nonproducer cell line Raji suggest that this interpretation is correct. The Raji line has no cells in the productive virus cycle, and the average EBV DNA content per cell is not affected by PAA. This shows that there are two classes of EBV DNA in cells. The resident EBV DNA that replicates in concert with the host DNA is not sensitive to PAA, but the EBV DNA being made during the lytic or productive mode of virus growth is inhibited by PAA.

The most direct explanation of our results is that the EBV DNA synthesis associated with the productive cycle of virus growth is mediated by an EBV-specific DNA polymerase that is analogous to polymerases of other herpesviruses in its sensitivity to PAA. Furthermore, the late class of EBV genes, i.e., VCA genes, is not expressed in the absence of viral DNA synthesis mediated by the viral DNA polymerase. The observation that a substantial amount of the EBV DNA in the cell replicates even in the presence of PAA suggests that these EBV DNA molecules (about 40 genome equivalents in B95-8 cells) are under cellular rather than viral control. They might be integrated into the cellular DNA (2) and replicate along with cellular sequences, or such EBV DNA might exist as some form of nonintegrated plasmid (1, 12) yet be replicated by cellular enzymatic functions.

The kinetics of loss of VCA-positive cells, together with the loss of EBV DNA from the cells, allows some tentative conclusions as to the duration of the late phase of the EBV lytic cycle. If we assume that PAA acts rapidly to block further EBV DNA synthesis, it follows that existing EBV DNA destined to enter mature virus takes 1 to 2 days after synthesis to begin to exit from the cell (Fig. 4). Since VCA content stays constant for about 24 h and then declines rapidly (Fig. 2), VCA synthesis may continue from pre-existing mRNA, or late mRNA synthesis can continue for awhile on previously replicated viral DNA. An alternative hypothesis is that although VCA synthesis stops, VCA must remain in the cell for 24 h while it participates in virus assembly and mat-

uration, at which time cell lysis occurs and the VCA-positive cells begin to disappear from the population.

The present study shows that EBV is similar to other herpesviruses in its sensitivity to PAA, that late gene expression is dependent in some way on prior viral DNA synthesis, and that the repressed viral genomes in nonproducer cells are under cellular rather than viral control. Since PAA is relatively nontoxic to mammalian cells, and since EBV virus synthesis is effectively blocked by this drug, PAA might have interesting applications in the laboratory and even in the clinic.

ACKNOWLEDGMENTS

We are grateful to Abbott Laboratories for supplying us with PAA and to Bill Sugden for samples of labeled and unlabeled EBV DNA as well as much helpful advice and many stimulating discussions.

W. C. S. is a Faculty Research Awardee of the American Cancer Society (PRA-97). This work was supported by Public Health Service contract no. NOI CP 33316 within The Virus Cancer Program of the National Cancer Institute, the Swedish Cancer Society, and King Gustaf V's Jubilee Fund.

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