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Effect of Arabinofuranosylthymine on the Replication of Epstein-Barr Virus and Relationship with a New Induced Thymidine Kinase Activity

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1-β-D-Arabinofuranosylthymine (araT) is a selective inhibitor of Epstein-Barr virus replication induced in both thymidine kinase (TK)-negative (TK^-) and TK^+ variants of the lymphoid cell line P3HR-I. This analog has no effect on the growth of noninduced cells (T. Ooka and A. Calender, Virology **104**:219–223, 1980). The synthesis of early antigens is not affected by the analog, whereas that of late viral capsid antigens is completely inhibited, as demonstrated by the indirect immunofluorescence technique; kinetic reassociation experiments have also shown that araT strongly inhibits replication of viral DNA. Phosphorylation of the tritiated form of the analog ([^{3}H]araT) was analyzed by thin-layer chromatography in cultures of control and induced cells, and the results demonstrated that only induced cells can convert the analog to the triphosphate form. These results indicate that the selective effect of araT in induced cells is probably related to a new virally induced TK activity. Preliminary characterization of this new activity has shown that it is able to phosphorylate the analog specifically, whereas cellular TKs cannot. araTTP, a final phosphorylation product of araT, is a potent inhibitor of Epstein-Barr virus-specific DNA polymerase, suggesting a possible inhibitory action of this product on Epstein-Barr virus replication.

Epstein-Barr Virus (EBV), one of the most common and most widely disseminated human viruses, is the cause of infectious mononucleosis and has been implicated in the etiology of three human cancers: nasopharyngeal carcinoma, Burkitt's lymphoma, and immunoblastic lymphoma. Biochemical studies of the virus are a necessary prerequisite for the understanding of its relationship with these cancers.

The identification of virus-specific polypeptides involved in DNA synthesis, particularly those with enzyme activity, is an important step towards understanding their role in viral replication.

Most herpesviruses are capable of inducing DNA polymerase, DNase, and thymidine kinase (TK) in virus-infected cells (4, 14–16, 22). However, in the case of EBV, the virus can induce DNA polymerase (8, 11, 25) and DNase (3, 5), but there is no evidence of the induction of a deoxypyrimidine kinase, such as that induced in herpes simplex virus (HSV)-infected cells (2, 6, 7, 17).

We recently reported that treatment of the EBV genome-carrying lymphoid cell line P3HR-I TK^- variant, which lacks cellular TK, with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and sodium butyrate induced the synthesis of early and late EBV antigens and high [^{3}H]thymidine incorporation into viral DNA (23). 1-β-D-Arabinofuranosylthymine (araT), which has no effect on normal cell growth, completely inhibited both late antigen expression and viral DNA synthesis in activated cells. Since inhibition by araT of HSV replication in vitro is mediated by HSV-induced deoxypyrimidine kinase, which specifically phosphorylates the analog (1, 20), our previous results raised the question of the existence of an EBV-induced deoxypyrimidine kinase activity similar to that induced in the HSV system which specifically catalyzes the phosphorylation of araT and thus inhibits EBV replication.

In the present report, we have tried to determine whether viral deoxypyrimidine kinase activity can be induced in EBV-carrying lymphoid cells after induction of the viral cycle and is related to the inhibitory process of EBV replication by araT. Our results revealed the existence of a deoxypyrimidine kinase activity, similar to that observed in HSV-infected cells, in chemically induced P3HR-1 TK^- cells. This new enzyme activity phosphorylates araT with high efficiency, whereas the cellular fetal deoxythy-

midine kinase does not possess this ability. araTTP seems to be a potent inhibitor of EBV-specific DNA polymerase activity.

MATERIALS AND METHODS

Cell cultures. Cells from EBV producer (P3HR-1 TK^+ and TK^- variants) and nonproducer (Raji TK^+ and TK^- variants) lines as well as those from EBV-negative BJAB and Ramos TK^- lines were seeded at 5 $\times$ 10^5 cells per ml and grown in TPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 250 μg of streptomycin per ml at 37°C. The P3HR-1 and Raji TK^- variants were maintained in the same medium in the presence of bromodeoxyuridine (100 μg/ml): in this condition, the reversion rate is low, as demonstrated by inoculation in HAT medium (hypoxanthine-aminopterine-deoxythymidine [12]).

Cell growth studies. Growth curves were performed with several lymphoid B-cell lines: P3HR-1 TK^+ and TK^- variants and BJAB. The cells were grown at 37°C for 4 days in the absence or in the presence of araT at the indicated concentrations. Samples were counted with a hemacytometer, and the mortality rate of the cells was measured in the presence of trypan blue.

Induction with TPA-sodium butyrate. TPA-sodium butyrate induction of the viral cycle was performed as previously described (23). Briefly, all cells were grown to a density of at least 2 $\times$ 10^6 cells per ml and diluted to a concentration of 5 $\times$ 10^5 cells per ml. TPA and sodium butyrate were added at final concentrations of 2 mM and 20 ng/ml, respectively. After 3 days, the cells were harvested by centrifugation.

Evaluation of the percentage of early antigen (EA)-positive cells was carried out on smears, using the indirect immunofluorescence test as described by Henle and Henle (13). For this purpose, samples of cells were recovered and centrifuged at 600 $\times$ *g* for 2 min. The cells were then washed twice in phosphate-buffered saline, seeded on glass cover slips, dried, and fixed in cold acetone for 10 min.

Intracellular phosphorylation of [^{3}H]araT. Samples (P3HR-1 TK^+ or TK^-) containing 2 $\times$ 10^6 cells in 4 ml were incubated with 2 mM butyrate, 20 ng of TPA per ml, and 30 μl (1 μl = 0.25 $\times$ 10^6 cpm) of [^{3}H]araT (final concentration, 2.5 μM) for 3 days and then washed with phosphate-buffered saline. Cold 5% trichloroacetic acid was added, and insoluble materials were removed by centrifugation at 4,000 rpm for 10 min. The supernatants were twice extracted with an equal volume of cold ether to remove the trichloroacetic acid (24), and the trichloroacetic acid-soluble phosphorylated products of araT were then examined by thin-layer chromatography. A portion (50 μl) of trichloroacetic acid-soluble material was spotted onto MN-polygram cell 300 polyethyleneimine/UV 254 plates and then eluted with the solvents described below. The slip was cut into 0.5-cm segments, suspended in a scintillator, and counted in a spectrometer. The solvent systems used for thin-layer chromatography were: solvent 1—1.1 M NaCl–1.4 M NaCl; solvent 2—isobutyric acid–NH_3 (26). R_f values of compounds with solvent 1 were: thymidine, 0.82; dTMP, 0.73; dTTP, 0.12; araT, 0.79; araTMP, 0.67; araTTP, 0.09. With solvent 2 they were: thymidine, 0.76; dTMP, 0.58; dTTP, 0.1; araT, 0.66; araTMP, 0.48; araTTP, 0.08.

Determination of TK and EBV-specific DNA polymerase activities. (i) Preparation of cell extract. All manipulations were carried out at 0 to 4°C. Cells in suspension were centrifuged at 600 $\times$ *g* for 6 min and washed twice in phosphate-buffered saline. After resuspension at a concentration of 10^8 cells per ml (3.5 mg of protein per ml) in TKMAD buffer (50 mM Tris [pH 8], 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 50 mM ε-aminocaproic acid, 5% [vol/vol] glycerol), the cells were sonically disrupted for four 30-s periods at a high setting (4°C) in an MSE sonicator. The sonically disrupted suspension was centrifuged at 105,000 $\times$ *g* for 60 min in a Spinco-L2 centrifuge (50 Ti rotor). Portions of the supernatant fluid were assayed for enzyme activity and protein content and were also analyzed by disk polyacrylamide gel electrophoresis.

(ii) TK assays. The enzyme extract was incubated at 37°C with an equal volume of the reaction mixture containing 150 mM phosphate (pH 7.5), 20 mM ATP, 40 mM KCl, 1 mM dithiothreitol, 10 mM NaF, 20 mM $MgCl_2$, 2.5% (vol/vol) glycerol, and 100 μM [^{3}H]thymidine or [^{3}H]araT (0.5 Ci/mmol). The reaction was stopped at 0 to 4°C, and 40-μl samples of the reaction mixture were spotted onto Whatman DE81 chromatography paper disks. The amounts of [^{3}H]dTMP or [^{3}H]araTMP formed were determined by batch washing of the disks four times in 1 mM ammonium formate–95% ethanol, followed by liquid scintillation counting of the dried disks for radioactivity.

DNA polymerase assays. DNA polymerase assays have been described previously (25). In brief, activated calf thymus DNA was prepared by digested with DNase I. A total volume of 125 μl of the reaction mixture comprised 100 mM Tris-hydrochloride buffer (pH 8.5), 4 mM $MgCl_2$, 34 μM activated DNA, 50 μM each dTTP, dGTP, dCTP, and dATP containing 0.5 μCi of [^{3}H]dTTP (specific activity, 30 Ci/mmol), 50 μg of bovine serum albumin per ml, and DNA polymerase. The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by cooling in ice; the reaction mixtures were then transferred onto Whatman GF/C glass fiber disks. DNA was precipitated by means of a 5% cold trichloroacetic acid solution, and the disks were dried with ethanol and ether and then counted.

Disk polyacrylamide gel electrophoresis of TK activity. The procedure used for polyacrylamide gel electrophoresis was that previously described by Kit et al. (18) except that the polyacrylamide-concentrating gel was replaced by a solution of 50% (vol/vol) glycerol. Electrophoresis was performed in glass tubes (0.6 by 10 cm) at 3 mA per gel for 4 h at 4°C. At the end of the electrophoresis run, the gels were cut at 4°C into 2-mm sections, which were incubated overnight at 38°C in the wells of microtitration plates containing 100 μl of a solution composed of 1 volume of extraction buffer and 1 volume of TK reaction mixture (see TK assay conditions).

DNA-DNA renaturation kinetics analysis. Whole-cell DNA of control and induced P3HR-1 TK^- cell lines was purified by two cycles of isopycnic neutral cesium chloride gradients after extraction of the total nucleic acid by phenol-chloroform-sodium dodecyl sulfate. P3HR-1 virus DNA was purified from viral particles

contained in culture supernatants after 13 days of activation by TPA (20 ng/ml) (29). The purification procedure used was that described by Dolyniuk et al. (9). Viral DNA was labeled in vitro by nick translation, as described by Rigby et al. (27). Briefly, 0.1 to 1 μg of the P3HR-1 DNA, in 145 μl of a solution containing 10^{-11} to 10^{-10} g of activated DNase, 1 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM Tris-hydrochloride (pH 7.0), 20 μM dATP, 20 μM dGTP, 50 to 100 pmol each of ^{32}P-labeled dCTP and dTTP (250 Ci/nmol; The Radiochemical Centre, Amersham, U.K.), and 20 U of *Escherichia coli* DNA polymerase, was incubated at 16°C for 2 to 3 h. The labeled DNA was separated from unincorporated nucleotides on a Sephadex G-50 column in 10 mM Tris (pH 7.0)–1 mM EDTA–0.1% Sarkosyl. The specific activity of the viral DNA ranged between 1×10^7 and 5×10^7 cpm/μg. Reassociation experiments were used to detect the viral sequences present in the bulk of whole-cell DNA. The viral and cell DNAs were dialyzed overnight against hybridization buffer, and the cell DNA was sonicated at 0°C for 8 min (Branson Sonifier; λ = 2 μm). The average size of the fragments ranged from 400 to 600 base pairs, as measured by electrophoresis on 1.2% horizontal agarose gels, in relation to 580- and 540-base pair fragments of DNA *Hin*dIII and *Sal*I digests. The hybridization mixture consisted of 1 mg of sonicated cell DNA and approximately 10^5 cpm of labeled viral DNA per ml in a solution of 10 mM Tris (pH 7.4) and 1 mM EDTA. The nucleic acid was denatured by heating at 100°C; NaCl was added at a final concentration of 1.5 M, and the reaction mixture was dispensed into 10-μl samples in Corning microtubes. All samples for the same experiment were analyzed at the same time. The fraction of viral DNA hybridized was determined by using single-strand-specific nuclease S1 after dilution of each sample to 1/50 in a solution containing 50 μg of native and 50 μg of denatured calf thymus DNA per ml, 1 mM $ZnCl_2$, 100 mM NaCl, and 25 mM potassium acetate, pH 4.5. The extent of self-hybridization of labeled viral DNA was controlled by experiments in which cell DNA was substituted by calf thymus DNA. The conditions of S1 nuclease digestion were studied on nick-translated viral DNA and were found to be sufficient to digest >90% of the single-strand DNA and <7% of the native molecules. The zero-time reassociation kinetics was obtained by diluting a 10-μl sample in an excess of hybridization buffer immediately after denaturation.

Reagents. araT from Calbiochem was tritiated by C.E.A. (Paris, France) and purified on polyethyleneimine-cellulose chromatography (Macherey-Nagel. Düren, West Germany). Other radioisotopes were purchased from The Radiochemical Centre. All unlabeled nucleosides and nucleoside triphosphates, *E. coli* DNA polymerase I, and nuclease S1 were obtained frm Boehringer Mannheim (Mannheim, West Germany). araTTP was generously supplied by G. A. Gentry, Mississippi Medical Center, Jackson, Miss. Calf thymus DNA was obtained from Sigma Chemical Co. (St. Louis, Mo.). All other common chemicals were reagent grade.

RESULTS

Effect of araT on EBV antigen synthesis. The inhibitory action of araT on the growth of lymphoid cell lines was examined on the EBV genome-carrying P3HR-1 TK^+ and TK^- lines and the EBV-negative BJAB line. In all cases, araT at concentrations up to 100 μg/ml had no effect on the growth of these noninduced cells.

Since araT specifically inhibits the synthesis of late viral capsid antigen but not that of early protein (EA) (23), we studied the dose-response effect of this analog on the synthesis of EBV-specific antigens in the producer cell lines P3HR-1 TK^+ and TK^- after chemical induction by TPA alone or by TPA in combination with sodium butyrate (Fig. 1). In both cases, the induction of EAs was not affected by increasing concentrations of the analog, whereas the production of viral capsid antigen, which is dependent on the replication of viral DNA, was completely inhibited by araT at concentrations >10 μg/ml. Similar results have been obtained after superinfection of nonproductive Raji TK^- and TK^+ cells by the P3HR-1 strain of EBV (data not shown). Noninduced P3HR-1 TK^+ cells, which spontaneously express EBV antigens (7% EA and 5% viral capsid antigen), lost EA and viral capsid antigen expression at 14 and 7 days, respectively, after araT continuous treatment. In these experiments, cell growth was not affected for several weeks. The inhibition of EA expression in the araT-treated culture can be explained by an effect of araT on the induction process itself, but more probably by a retroactive effect of the analog after continuous treatment.

EBV DNA synthesis in the presence of araT in a producer cell line. araT inhibits the late stage of the viral cycle, probably by interrupting viral DNA replication. In fact, as demonstrated by our previous experiments (23) on CsCl gradient analysis, the high stimulation of [^{3}H]thymidine incorporation into viral DNA P3HR-1 TK^- activated by chemical inducers was entirely inhibited by araT. The specific inhibition of viral DNA replication by araT is confirmed by kinetic reassociation experiments (Fig. 2). Viral DNA on the P3HR-1 strain was labeled by nick translation and hybridized to an excess of cellular DNA. In induced cells, the reassociation rate increased, whereas with the DNA from araT-treated cells it was similar to that of control cells. These results demonstrate that araT acts by inhibition of EBV replication during the period of initiation of viral DNA replication.

Intracellular phosphorylation of [^{3}H]araT. The experiments of Aswell et al. (1) and Miller et al. (20) showed that the specific phosphorylation of araT by a viral deoxypyrimidine kinase caused the selective inhibition of HSV DNA synthesis. It therefore remained to be seen whether or not this inhibitory action on EBV replication was related to the appearance of a new deoxypyrimi-

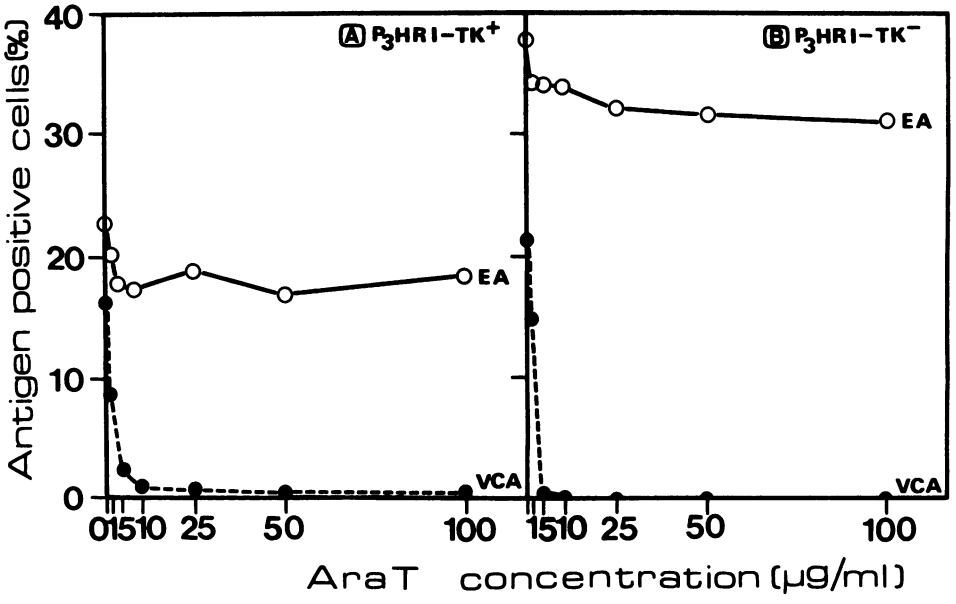

FIG. 1. Dose-response effect of araT on EB viral antigen production in P3HR-I TK$^+$ and TK$^-$ cells. The viral antigens were induced by TPA alone for P3HR-I TK$^+$ cells and by TPA in combination with sodium butyrate for P3HR-I TK$^-$ cells. After 3 days, the cells were harvested by centrifugation. In every experiment, a total of 10,000 cells was examined for each concentration of the drug. VCA, Viral capsid antigen.

dine kinase activity in the induced cells. To examine this possibility, the intracellular phosphorylation of tritium-labeled araT was analyzed by ascending thin-layer chromatography (Fig. 3). The activation of the viral cycle by chemical inducers led to the phosphorylation of araT to monophosphate and triphosphate derivatives, whereas the intermediate diphosphate form was never observed. In these cells, 60 and 35% of the total intracellular radioactivity derived from [^{3}H]araT were found in the mono- and triphosphate forms for P3HR-1 TK$^+$ and TK$^-$ cells, respectively. A high production of monophosphate in induced P3HR-1 TK$^+$ cells (50% of total intracellular radioactivity) may be due to a higher EA induction (48%) in this culture than in that (29%) of activated P3HR-1 TK$^-$ cells. In contrast, no phosphorylation of the analog was observed in the noninduced cells and BJAB lymphoid cells, which lack the EBV genome (data not shown). Slight phosphorylation to the monophosphate in P3HR-1 TK$^+$ control cells is probably because about 7% of cells spontaneously express EA, whereas the P3HR-1 TK$^-$ control cells express almost no viral antigens (<0.04%).

TK and DNA polymerase activities in induced or control cell extracts. The ability of induced and control cell extracts to phosphorylate the analog was tested in vitro; Table 1 shows that the total TK activity, tested with [^{3}H]thymidine as substrate, decreased after chemical induction in both Raji TK$^+$ and P3HR-1 TK$^+$ cells. This decrease, which was not observed in the EBV-negative BJAB cell line, is probably due to the inhibition of cellular metabolism occurring after induction of the viral cycle. Total TK activity increased significantly after induction of the TK$^-$ cell lines, indicating the appearance of a new, induced enzyme activity. This increase was not observed in the EBV-negative Ramos TK$^-$ line, suggesting that this new, induced enzyme activity may not be of mitochondrial TK origin. The synthesis rate of this new activity is probably too low to compensate for the partial inhibition of cellular TK activity.

A new deoxypyrimidine kinase activity, which appeared after the induction of TK$^+$ and TK$^-$ variants of the P3HR-1 and Raji cell lines, has the ability to phosphorylate araT, whereas cellular enzymes are unable to do so to any significant degree. In the case of P3HR-1 TK$^-$ cells, the new deoxypyrimidine kinase activity decreases if the induction is carried out in the presence of araT (Table 1); this is probably due to a dilution effect of the tritiated analog by the addition of unlabeled araT. In fact, in this case we observed a 20% increase of [^{3}H]araT phosphorylation when the cellular extracts were dialyzed against TKMD buffer.

Since this new activity appeared in induced Raji cells, which lack the ability to synthesize late viral protein, it is clearly correlated to the appearance of EAs and therefore to the early

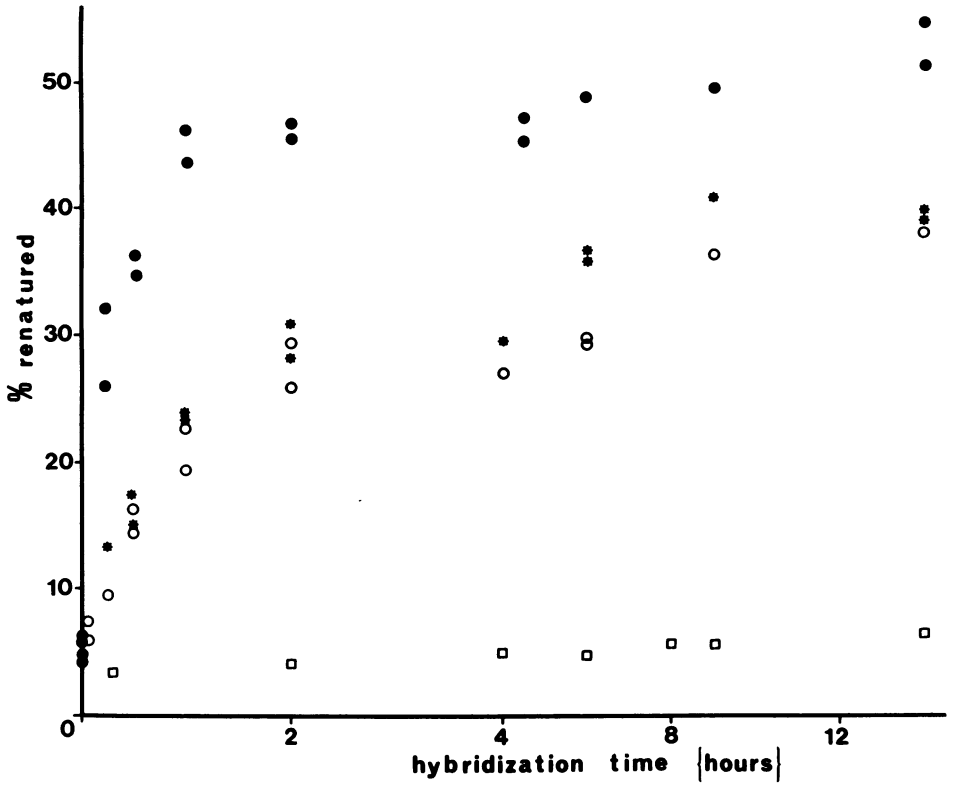

FIG. 2. Hybridization of unlabeled DNAs extracted from control and induced P3HR-I TK$^-$ cell lines with denatured EBV (HR-I) DNA labeled by nick translation. The hybridization conditions were described in the text. Symbols: ●, DNA from induced P3HR-I TK$^-$ cells; ⁎, DNA from induced P3HR-I TK$^-$ cells treated with araT (50 μg/ml); ○, DNA from control P3HR-I TK$^-$ cells; □, calf thymus DNA.

stage of the viral cycle. In a second experiment, the EBV-specific DNA polymerase activity was determined in the presence of 150 mM ammonium sulfate, a concentration at which cellular DNA polymerase activity is completely inhibited (25). EBV DNA polymerase was produced after chemical induction, at which time the cells expressed EAs; the production of this enzyme was not significantly affected by extracellular addition of araT.

We tried to characterize induced deoxypyrimidine kinase activity by examining cell extracts prepared from control and induced P3HR-1 TK$^-$ and Raji TK$^-$ cells, and from the control of Raji TK$^+$, on 5% acrylamide gel electrophoresis. The [^{3}H]araT phosphorylating activity has been localized on the top of the gel in aggregated material, which does not enter the gel. This is probably due to a particular aggregation process not observed in other herpesvirus systems. The reason for this process will be further studied in relation to the characterization of this new enzyme activity. The fetal TK activity of Raji TK$^+$, with an R_f of 0.2, efficiently phosphorylated [^{3}H]thymidine, but failed to phosphorylate the analog.

Effects of araTTP on EBV-specific DNA polymerase activity. One possible explanation of the inhibitory action of araT on EBV DNA synthesis is that TK, induced by herpesvirus infection, phosphorylates araT, and the resulting product would be expected to be inhibitory to EBV and cellular DNA polymerases if converted into araTTP. The experiments of Matsukage et al. (19) demonstrated, in fact, an effective inhibition by araTTP of the activity of cellular DNA polymerases α, β, and γ, as well as that of reverse transcriptase from Rauscher leukemia virus.

We therefore examined the effect of araTTP on viral and cellular DNA polymerase activities. Figure 4 shows the response of partially purified DNA polymerases to increasing concentrations of araTTP. The concentration giving 50% inhibition was estimated by extrapolation; EBV DNA polymerase was found to be the most sensitive enzyme (50% inhibitory concentration = 0.015 mM). Similar inhibition patterns have been obtained with DNA polymerase α (50% inhibitory

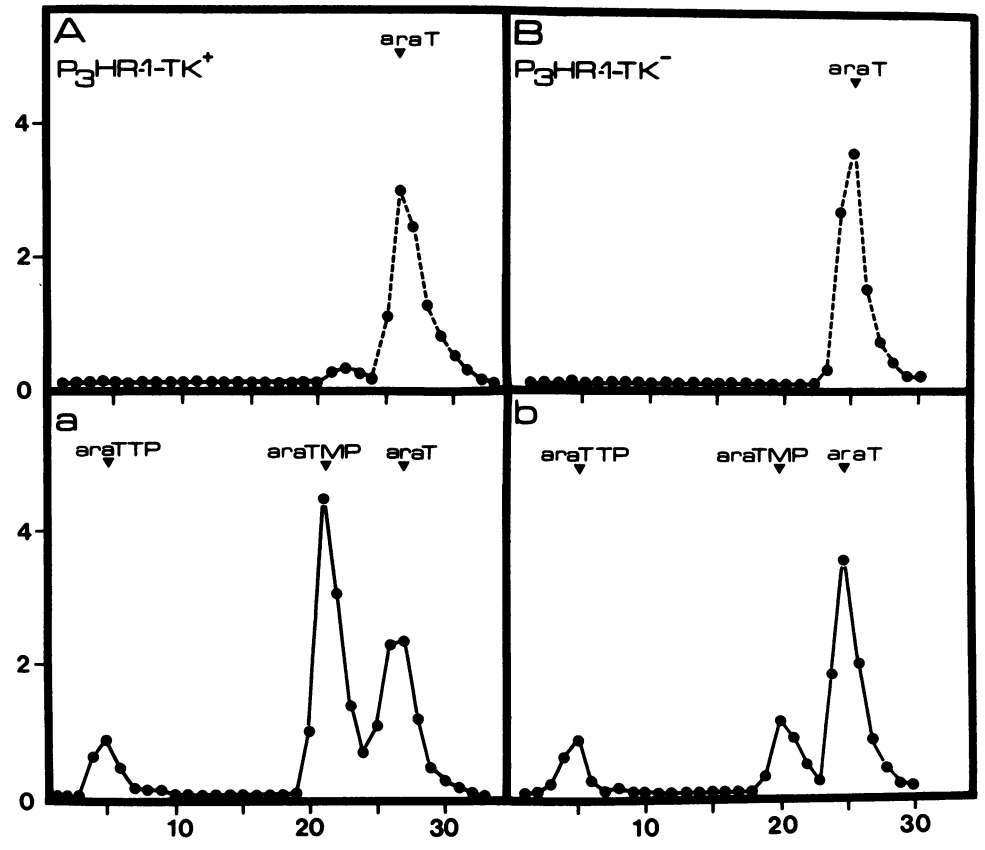

FIG. 3. Phosphorylation of [^{3}H]araT in P3HR-I TK$^+$ and TK$^-$ cells treated with TPA and sodium butyrate. A 2.5 μM concentration of [^{3}H]araT was added in 4 ml of culture (0.5 × 10^6 cells per ml), and the cultures were incubated for 3 days before nucleotide analysis by thin-layer chromatography. (A) Control P3HR-I TK$^+$ cells; (a) induced P3HR-I TK$^+$ cells; (B) control P3HR-I TK$^-$ cells; (b) induced P3HR-I TK$^-$ cells.

concentration = 0.05 mM); DNA polymerase β is inhibited to a lesser extent with lower concentrations of araTTP. However, the activities of all polymerases were inhibited by almost 90% with 0.5 mM araTTP.

DISCUSSION

Hampar et al. (12) and Glaser et al. (10) observed an increase in TK activity in Burkitt lymphoid cells and epithelial-Burkitt hybrid cells, respectively, which poorly expressed EA. This induced activity was not detectable, however, on polyacrylamide gels (17). In 1978, Chen et al. reported the possible existence of an EBV-specific TK, distinguishable from cellular TK, in Raji cells superinfected by P3HR-1 virus (2). In spite of this finding, the results of these authors were not sufficiently reproducible and they concluded the absence of any detectable viral TK activity (6, 7).

The ambiguous situation with regard to the existence of EBV-induced TK seems to stem from (i) the use of cells expressing low percentages of EA, (ii) the use of cellular TK-positive cell lines in which the detection of a virus-specific TK activity can be difficult, and (iii) the restricted study on substrate specificity of this enzyme, with respect to the analogs of deoxythymidine.

With regard to the above-mentioned results, one possible approach to demonstrate the existence of EBV-specific TK was to study the effect of araT on EBV replication produced in EBV-carrying cells lacking cellular TK. The present studies demonstrated that araT appears to be a very potent compound, which is active against EBV, inhibiting the synthesis of both viral DNA and viral capsid antigens during the productive viral cycle induced in TK$^-$ cells treated with TPA-sodium butyrate. The analog does not affect the growth of control TK$^+$ and TK$^-$ cells, and these results demonstrate that the inhibition of EBV replication by this compound follows a process similar to that reported in HSV (1) and varicella-zoster virus (20) systems with the same analog, suggesting the possi-

TABLE 1. Effect of chemical induction on expression of EBV antigens and activity of TK and DNA polymerase in human lymphoblastoid cell lines[a]

Cell line	Chemical induction	% of EA-or VCA-positive cells		TK activity (pmol of dT or araT phosphorylated/mg of protein per h)		DNA polymerase activity (pmol of dTTP incorporated/mg of protein per 30 min)	
		EA	VCA	[^{3}H]thymidine	[^{3}H]araT	Total activity	EBV-specific DNA polymerase activity[b]
RaJI TK$^+$	Control	0	0	18,471	171	4,680	1
	TPA + SB	19.5	0	10,247	714	10,115	862
RaJI TK$^-$	Control	0	0	706	73	2,181	8
	TPA + SB	35.8	0	7,741	1,341	11,651	1,115
P3HR1 TK$^+$	Control	7	4	16,741	439	3,198	5
	TPA + SB	42	31.7	5,000	1,342	4,433	581
P3HR1 TK$^-$	Control	<0.1	0	1,377	317	2,793	3
	TPA + SB	34.6	13.9	5,153	1,220	5,403	714
	TPA + SB + araT[c]	33.4	0	3,671	781	5,123	570
BJAB TK$^+$	Control	0	0	19,000	298	3,523	4
	TPA + SB	0	0	18,470	289	4,077	0
Ramos TK$^-$	Control	0	0	1,091	297	4,665	1
	TPA + SB	0	0	637	136	1,614	0

[a] The lymphoblastoid cell lines were grown at 37°C without or with TPA and sodium butyrate (SB) for 3 days. The evaluation of the percentage of EA- and viral capsid antigen (VCA)-positive cells was carried out on smears, using an indirect immunofluorescence test. The assays were performed under the conditions described in the text.

[b] EBV-specific DNA polymerase activity was measured in the presence of 150 mM $(NH_4)_2SO_4$ in enzyme assays.

[c] 50 μg of araT per ml was added in culture medium.

ble induction of a deoxypyrimidine kinase activity which can specifically phosphorylate the analog and thus inhibit viral replication. In fact, the activation of the EBV genome by chemical inducers led to the appearance of a new TK activity in P3HR-1 and Raji TK$^-$ cells which was capable of phosphorylating araT to araTTP in vivo, whereas TK-negative and -positive control cells such as P3HR-1, Raji, and BJAB failed to phosphorylate the analog. The cellular extract prepared from activated EBV-carrying cells catalyzed the phosphorylation of both araT and thymidine, and in this respect this activity has the same properties as that induced in HSV-infected cells. Since control TK-positive cells, such as BJAB, P3HR-1, and Raji, grow normally in the presence of araT and their cellular extracts do not phosphorylate araT, this activity differs from cellular TK. Polyacrylamide gel analysis confirmed that this activity possesses specific migration properties distinct from those of fetal TK activity which have an R_f value of 0.2 and, as already reported by Kit et al. (17, 18), have no affinity to the analog. This again supports the conclusion that there is a clear difference between the new induced TK and cellular TK. Our recent experiments demonstrated that this aggregate form could be dissociated by enzymes, such as hyaluronidase and lipase (M. Turenne et al., manuscript in preparation).

To rule out the possibility that the new activity is related to the activation of mitochondrial TK or modified cellular TK by chemical inducers, we propose the following observations: cellular extracts prepared from both BJAB (EBV-free cellular TK$^+$ cells) and Ramos (EBV-free TK$^-$ cells, in which only mitochondrial TK activity is expressed) cells did not succeed in phosphorylating the analog; thus, the induction of araT kinase activity is dependent on the presence of the EBV genome.

To explain a possible mechanism of the inhibition of HSV replication by this analog, several investigators have suggested that this specific inhibition may be due to phosphorylation of araT to the triphosphate form which inhibits virus-specific DNA polymerase activity (19, 21). In the case of EBV, we observed that only activated cells were capable of converting araT to araTTP in vivo and, moreover, that araTTP had a high affinity towards EBV-specific DNA polymerase. These observations favor the conclusions of previous authors.

It must be pointed out that Colby et al. (6) recently demonstrated the specific inhibition of EBV replication by acyclovir [9-(2-hydroxyethoxymethyl)guanine], one of the active antiviral compounds which is specifically phosphorylated by HSV-specific deoxypyrimidine kinase. In this case, acyclovir was phosphorylated to acyclo-GTP only in EBV genome-activated cells, and these authors conclude that the formation of

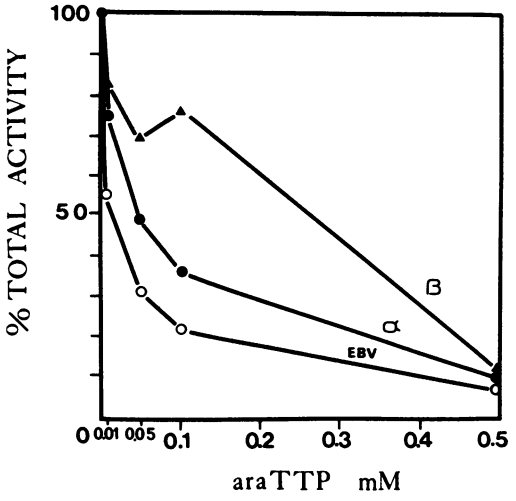

FIG. 4. Effect of araTTP on various DNA polymerase activities in vitro. α, β, and the EBV-specific DNA polymerases were tested in DNA polymerase assays with the indicated concentrations of araTTP. Cellular DNA polymerases α and β were prepared from direct chromatography on phosphocellulose, and EBV-specific DNA polymerase was prepared by sequential column chromatography on Sepharose 6B, DEAE-cellulose, phosphocellulose, and an affinity column of poly(dC)-oligo$(dG)_{12}$. 100% values were as follows: α, 15,660 cpm; β, 6,050 cpm; EBV specific, 4,220 cpm.

acyclovir phosphates in lymphoblastoid cells may be related to the induction of host enzymatic activity during replication of EBV.

It would be interesting to examine whether the araT phosphorylating activity induced in EBV-carrying TK^- cells can phosphorylate acyclovir.

Another possible explanation of inhibition by araT is that araTTP may be incorporated into DNA chains to block their elongation, as suggested by Müller et al. (21). We could not, however, succeed in demonstrating this possibility, due to the low specific activity of the [^{3}H]araT used in this experiment, which renders the detection of radioactivity in DNA after CsCl gradient centrifugation difficult.

In any event, we believe that the phosphorylation of araT is an essential first step in the induction of the inhibition of EBV DNA synthesis. One important question that could arise from these observations is whether this new activity is of viral origin. The activity reported here seems to be a new viral enzyme, based on the following observations: the activity can be induced in EBV-carrying TK-negative cells after the induction of the viral cycle; its broad substrate specificity is similar to that of other herpesvirus-induced enzymes; and, finally, it is induced only in EA-positive cells. However, final proof that the enzyme is virally coded would result from the purification and characterization of this new enzyme activity or from the conversion of TK-negative cells to positive cells by transfection experiments with EBV DNA or from both.

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