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Epstein-Barr Virus Transformation of Human B Lymphocytes Despite Inhibition of Viral Polymerase

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Epstein-Barr virus transformed human lymphocytes despite the presence of up to 500 μM acyclovir [9-(2-hydroxyethoxymethyl)guanine], a viral DNA polymerase inhibitor. The transformed cells contained multiple Epstein-Barr virus genome copy numbers. Functional viral DNA polymerase is probably not required for cell transformation and the initial amplification of the viral genome.

Epstein-Barr virus (EBV) is a transforming herpesvirus which, on infection of human B lymphocytes in vitro, is capable of producing permanent lymphoblastoid cell lines. This effect mirrors the consequence of infection of B lymphocytes in vivo (14). In its latent state, the viral genome confers on the host cell the potential for unlimited cell growth. A critical step in EBV-induced cell transformation probably involves early EBV replication within the infected target cell. Human umbilical cord lymphocytes that have been transformed by EBV generally contain several copies of the EBV genome in its episomal form but do not freely synthesize viral DNA or produce virus (1, 12). A related question, therefore, is whether EBV-induced DNA polymerase is needed for transformation. These basic questions are also important because of the interest in applying antiviral drugs in the treatment of the EBV-induced polyclonal lymphoproliferative diseases that occur in immunocompromised persons (13, 18).

In vitro use of the viral DNA polymerase inhibitor phosphonoacetic acid (PAA) as a probe of early transformational events has produced results with conflicting interpretations. Thorley-Lawson and Strominger (19, 20) and Lemon et al. (8) have reported the absence of cell outgrowth in lymphocytes infected with EBV and maintained on PAA. However, Lemon et al. did note increased cellular nucleic acid synthesis consistent with beginning transformation (8). Moreover, the EBV nuclear antigen (EBNA), a marker of EBV-induced cell transformation, was detected in some PAA-treated cells (20). Rickinson and Epstein have described not only the establishment of permanent lymphoblastoid cell lines after comparable exposures of cells to PAA, but also apparent amplification of the incoming viral genome (16). These discrepancies may be due in part to dose-dependent inhibition by PAA not only of viral (10) but also of cellular DNA polymerase (7, 10). To eliminate any ambiguity introduced in these results by the lack of specificity of PAA, we have used the acyclic nucleoside analog acyclovir [9-(2-hydroxyethoxymethyl)guanine] (ACV) as a highly selective inhibitor of EBV-specific DNA polymerase to investigate EBV-induced transformation of human umbilical cord lymphocytes.

ACV was a gift of the Burroughs Wellcome Co. Clinical isolates of EBV from saliva of persons with acute infectious mononucleosis were obtained by throat washings with phosphate-buffered saline solution. Filtered samples capable of producing permanent, EBNA-positive cell lines from umbilical cord lymphocytes were selected for use in the assays described below. Mixed populations of umbilical cord lymphocytes were prepared by centrifugation of heparinized cord blood on Ficoll-Isopaque (Pharmacia Fine Chemicals) (2). B-cell enrichment of adult peripheral blood lymphocytes was accomplished by erythrocyte rosetting (21). EBV-induced cell transformation was confirmed by detection of EBNA (15). Numbers of EBV genome equivalents in duplicate lines from ACV- and mock-treated groups were determined by nucleic acid hybridization as previously described (11).

A transformation centers assay similar to that described by Henderson et al. (6) was performed to study the effects of ACV on the induction of lymphocyte transformation by EBV isolates (Table 1). We obtained cell outgrowth and establishment of permanent EBNA-positive lymphoblastoid cell lines with two different preparations of umbilical cord lymphocytes after exposure to EBV in the presence of up to 500 μM ACV, a concentration at least five times that required for complete inhibition of EBV DNA synthesis in superinfected Raji cells (4, 9). Efficiency of transformation in ACV-treated cells was identical to that in control cultures at levels of up to 250 μM, 25 times the 90% effective dose of the drug for EBV (4, 9). There was no significant delay in establishing transformation (Table 1). Only at 500 μM did transformation efficiency decrease, presumably due to drug toxicity.

Drug-induced enhancement of transformation did not occur. This provides evidence against a cytolytic stage in a subset of lymphocytes shortly after infection which, if inhibited by the drug, would increase the chances for viral latency, cell survival, and eventual transformation (8). This point is worth noting given the possibility that the clinical isolates used might contain both lytic and transforming strains of virus (17). None of the 48 wells containing umbilical cord lymphocytes maintained in 100 μM ACV spontaneously transformed in the absence of EBV exposure.

ACV requires phosphorylation by either viral or cellular enzymes to be effective against EBV-specific DNA polymerase (3). EBV-positive and -negative lymphoid cell lines and fresh peripheral lymphocyte preparations from both EBV-seropositive and -seronegative adult donors are capable of phosphorylating drug in vitro (3). To ensure that our results were not a consequence of inadequate phosphorylation of

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ACV in umbilical cord lymphocytes, we also studied a B lymphocyte-enriched preparation from the peripheral blood of an EBV-seronegative adult whose uninfected cells were previously shown to be capable of ACV phosphorylation in vitro (3). EBV-induced cell transformation in the presence of ACV occurred in all cases regardless of the source of the lymphocytes and with all three viral isolates used.

Previous determinations have established that most cell lines produced by in vitro EBV infection of umbilical cord blood cells contain 10 to 15 copies of the EBV genome per cell (12). Because we achieved cell transformation at ACV concentrations capable of completely inhibiting viral polymerase-directed EBV replication, we measured the copy number of EBV genomes in cell lines established in ACV-containing and control medium (Table 2). EBV DNA genome equivalents were estimated from levels of hybridization to total cellular DNA with a tritiated cRNA probe derived from the cloned *Bam*HI V fragment of EBV DNA. The probe was first calibrated by hybridization with known amounts of purified viral DNA from Raji cells (11). Umbilical cord lymphocytes transformed in ACV-free medium contained an average of seven genome equivalents per cell, whereas cells transformed in the presence of 100 μM ACV contained an average of six.

TABLE 1. Transformation of human umbilical cord lymphocytes by EBV in the presence of the viral DNA polymerase inhibitor ACV[a]

ACV (μM)	No. of EBV-exposed cells per well	Wells transformed/ total wells[b]	No. of transformants per 10^5 cells	Mean time to transformation (days)
0	10^5	10/12	0.8	46
	10^4	4/12		53
	10^3	0/12		
10	10^5	10/12	0.8	46
	10^4	3/12		43
	10^3	0/12		
100	10^5	10/12	0.8	58
	10^4	0/12		
	10^3	1/12		66
250	10^5	10/12	0.8	50
	10^4	0/12		
	10^3	0/12		
500	10^5	5/12	0.4	63
	10^4	1/12		66
	10^3	0/12		

[a] Human umbilical cord lymphocytes (2×10^6) were incubated for 1 h at 37°C with 0.2 ml of filtered throat washings from patients with infectious mononucleosis. Control or ACV-containing medium (RPMI 1640, 20% fetal calf serum) was added to cells, and cultures were incubated for 24 h at 37°C in 5% CO_2. The medium was removed, and the cells were incubated for 1 h in human serum containing EBV-neutralizing antibodies (diluted 1:10 in control or ACV medium) to eliminate all extracellular virus. Cells were washed twice and diluted to concentrations of 10^6, 10^5, and 10^4 lymphocytes per ml. One-tenth milliliter of each lymphocyte dilution was added to 12 replicate wells of a microassay plate containing feeder cells prepared from autochthonous, noninfected lymphocytes to give a final cell number in all wells of 2×10^5 cells. The cultures were incubated at 37°C in 5% CO_2 and fed twice weekly with control or ACV medium by replacing half of the medium. Controls consisted of mock-infested umbilical cord lymphocytes and lymphocytes exposed to freeze-thawed extracts and washings of EBV-exposed cells after treatment with EBV neutralizing antibody.

[b] Transformation was judged microscopically on the basis of obvious cell growth and clumping. Only wells producing permanent EBNA-positive cell lines on passage were called transformed. Final results were scored at 10 weeks. Efficiency of transformation was calculated as (number of transformed wells)/[(total number of cells per well) (number of wells)].

TABLE 2. EBV genomes in newly transformed umbilical cord lymphocytes[a]

Transformed lymphocytes from cell line:	No. of EBV genome equivalents in:	
	Control medium	ACV medium
1	9	3
2	6	9

[a] Duplicate cell lines 1 and 2 from mock- and ACV-treated cultures were established. Total cellular DNA was extracted from 4×10^7 ACV- and mock-treated cells and affixed to nitrocellulose filters. EBV DNA sequences were estimated from levels of hybridization with a ^{3}H-labeled cRNA probe which had been calibrated by hybridization with various known amounts of purified viral DNA. Total DNA per filter was quantitated by a diphenylamine assay (11).

The presence of multiple genome copy numbers in cells transformed in the presence of high concentrations of a viral polymerase inhibitor confirms the findings of Rickinson and Epstein with PAA (16). The almost identical EBV genome number in cells transformed in ACV containing medium and in control medium makes it unlikely that viral DNA polymerase is involved in any amplification of the incoming viral genome. The multiple genome number may reflect replication of the EBV genome by the host polymerase, which is at least 100-fold less sensitive to ACV triphosphate than is EBV DNA polymerase (5). Replication of EBV genomes in the episomal form is unaffected by ACV and other potent inhibitors of herpesvirus DNA polymerases (9). Although transformation is believed to occur according to single-hit kinetics, another possibility is that the transformed cells represent progeny of lymphocytes infected at a multiplicity of infection greater than 1, an event made unlikely by the virus concentrations present in the throat washings used.

These results suggest that functional viral DNA polymerase may not be necessary for induction of cell transformation, but the relationship of early viral replication to the transformation process remains unknown. The replication and maintenance of a limited copy number of EBV episomes may be crucial to transformation, but the apparent role of host rather than viral DNA polymerase in this process makes the use of viral polymerase-active drugs for treatment of EBV-transformed cells of questionable value.

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