EBV Is Necessary for Proliferation of Dually Infected Primary Effusion Lymphoma Cells

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Abstract

Epstein Barr virus (EBV) and Kaposi's sarcoma–associated herpesvirus (KSHV) are found together in ~80% of primary effusion lymphomas (PEL), but their contribution to these cancers is unclear. We found that dominant-negative derivatives of EBNA1 inhibited EBV-positive PEL cells from forming colonies. Those rare PEL cells that proliferated after expression of the dominant-negative derivatives usually expressed these derivatives at low or undetectable levels and continued to maintain their EBV genomes. Those proliferating cells expressing higher levels of the derivatives expressed mutant derivatives that could not bind DNA. These findings indicate that EBV is required to sustain proliferation, as measured by colony formation of dually infected PEL cells. The dominant-negative derivatives of EBNA1 had no effect on the colony-forming ability of five EBV-negative, KSHV-negative hematopoietic cell lines. Surprisingly, they did inhibit the colony-forming ability of EBV-negative, KSHV-positive PEL cells. The small fraction of cells that continued to proliferate expressed only mutants of the EBNA1 derivatives that could no longer bind DNA. These findings indicate that the site-specific DNA-binding activity of EBNA1 or its derivatives when expressed efficiently in EBV-negative, KSHV-positive PEL cells inhibits their colony formation possibly through their binding to the KSHV genome. [Cancer Res 2008;68(17):6963–8]

Introduction

Primary effusion lymphomas (PEL) are rare and represent a distinct class of B-cell, non-Hodgkin's Lymphomas for which there is no effective therapy and survival is predicted to be less than 1 year postdiagnosis (1). All PELs are infected with Kaposi's sarcoma–associated herpesvirus (KSHV) and most with EBV, but they consistently lack the *c-myc* gene rearrangements typically found in EBV-positive Burkitt's lymphoma (2). PELs frequently occur in AIDs patients, supporting a correlation between immunodeficiency, susceptibility to viral infection, and subsequent tumor development (3–5). It is not clear, however, how each of the viruses found in dually infected PELs (EBV⁺, KSHV⁺) contributes either to the initiation or maintenance of these tumors.

We have found that EBV is necessary for the proliferation of dually infected PEL cells under limiting dilution, as measured by colony formation assays. We forced the loss of EBV from these cells by inhibiting EBNA1, the viral protein on which the plasmid

replication of EBV depends, with dominant-negative derivatives of EBNA1. The majority of dually infected cells in which EBNA1 was inhibited failed to grow to form colonies under limiting dilution. Those that did grow were found not to have their EBNA1 inhibited and to retain EBV. The dominant-negative derivatives of EBNA1 used to inhibit it were either expressed inefficiently or mutated and nonfunctional in the minority of cells that did grow.

We also tested the derivatives of EBNA1 in five EBV-negative, KSHV-negative hematopoietic cell lines and three EBV-negative KSHV-positive PEL lines. Whereas these EBNA1 derivatives had no effect on colony formation by the virus-negative hematopoietic lines, they did inhibit that of the EBV-negative, KSHV-positive PEL cells. We have found that EBNA1 binds multiple sites in the KSHV genome[1] and hypothesize that high levels of DNA-binding derivatives of EBNA1 affect the functions of the KSHV genome. We again found that the EBV-negative, KSHV-positive PELs that did grow to form colonies either expressed the derivatives of EBNA1 inefficiently or expressed nonfunctional mutants of these derivatives.

Materials and Methods

Cell lines. The EBV⁻, KSHV⁺ PELs (BC-3, BCP-1, and BCBL-1) and the EBV⁺, KSHV⁺ PELs (JSC-1 and BC-2) were cultured in RPMI 1640, 10% fetal bovine serum (FBS), 200 units/mL of penicillin, and 200 μg/mL of streptomycin. The EBV⁻, KSHV⁻ hematopoietic cell lines K562, Jurkat, MOLT-4, and BJAB, as well as the EBV-transformed 721 lymphoid clones, were cultured in the same medium as the PELs. 293T cells used for retroviral production express the SV40 T-antigen and were cultured in DMEM and 10% calf serum. Human fibroblasts used in colony formation assays were maintained in DMEM and 10% FBS.

Retroviral production. Retroviruses were produced, concentrated, and titered as described (6).

Retroviral infections. A multiplicity of infection equal to 10 to 15 infectious units per cell as titered on BJAB cells was used wherever PEL or other hematopoietic cells were infected. Infections were done at a concentration of 5×10^5 cells/mL in R10F/50 mmol/L HEPES (pK_a, 7.55), which were placed in 5-mL snap-cap tubes and rocked at 4°C for 1 to 2 h. Cells were then transferred to 15-mL Falcon tubes, brought to 4 mL with R10F, and spun at 1,000 rpm, 4°C for 10 min. After infection, cells were washed with 4 mL of 1× PBS and then resuspended in 10-mL R10F, transferred to a 10-cm plate, and incubated for 48 to 72 h.

Western blotting. Cell lysates were run on 10% polyacrylamide gels and EBNA1 was detected with an alkaline phosphatase–conjugated rat monoclonal antibody.

Detection of EBV and KSHV by fluorescence *in situ* hybridization. Cells were prepared and hybridized with probes that were generated as described (7). Briefly, the DNA probes for the detection of KSHV plasmids were generated by nick translation using alkali-labile digoxigenin-11-dUTP. The DNA template was derived from cosmid Z8 (8). The DNA probe for the detection of EBV plasmids was derived from the *Bam*H W repeated region and generated by nick translation using biotinylated-11-dUTP.

[1] L. Dresang and B. Sugden, unpublished data.

Colony formation assays. Infected cells were sorted based on the levels of fluorescent protein expression by fluorescence-activated cell sorting (FACS) analysis and distributed into the wells of a 96-well plate at dilutions ranging from 1 to 100 cells per well. The efficiencies of colony formation at all dilutions were then averaged. Each well of the plate also contained $\sim 2 \times 10^4$ human fibroblast cells in 200 μL of R10F and were plated no more than 3 d before sorting. Wells containing colonies with >1,000 cells were scored positive whereas those containing few to no cells were scored negative, 14 to 18 d after sorting. The efficiency of colony formation was then calculated using a Poisson distribution.

Results

Inhibiting EBNA1 in EBV⁺, KSHV⁺ PEL cells inhibits their growth under limiting dilution as measured by assays of colony formation. We used retroviral vectors to introduce two derivatives of EBNA1 that function dominant-negatively, DBDmut and deltaUR1, into two EBV-positive, KSHV-positive PEL cell lines to inhibit EBNA1. DBDmut dimerizes, binds DNA site-specifically, and inhibits all known functions of EBNA1 (Fig. 1A). DeltaUR1 lacks a 25-amino-acid region required for the transcriptional activity of EBNA1 and inhibits only the EBNA1 support of transcription (Fig. 1A). Both of these derivatives inhibit colony formation by EBV-positive normal and Burkitt's lymphoma cells and both inhibit their survival by inducing apoptosis (9).

The colony-forming ability of BC-2 and JSC-1 cells was significantly inhibited by the expression of either DBDmut or deltaUR1 (Fig. 1B). DBDmut-infected BC-2 cells formed colonies at 45% the level of control-infected cells, and deltaUR1-infected BC-2 cells at 25% that of control-infected cells (P = 0.02). The colony-forming ability of DBDmut-infected JSC-1 cells was reduced to 10%,
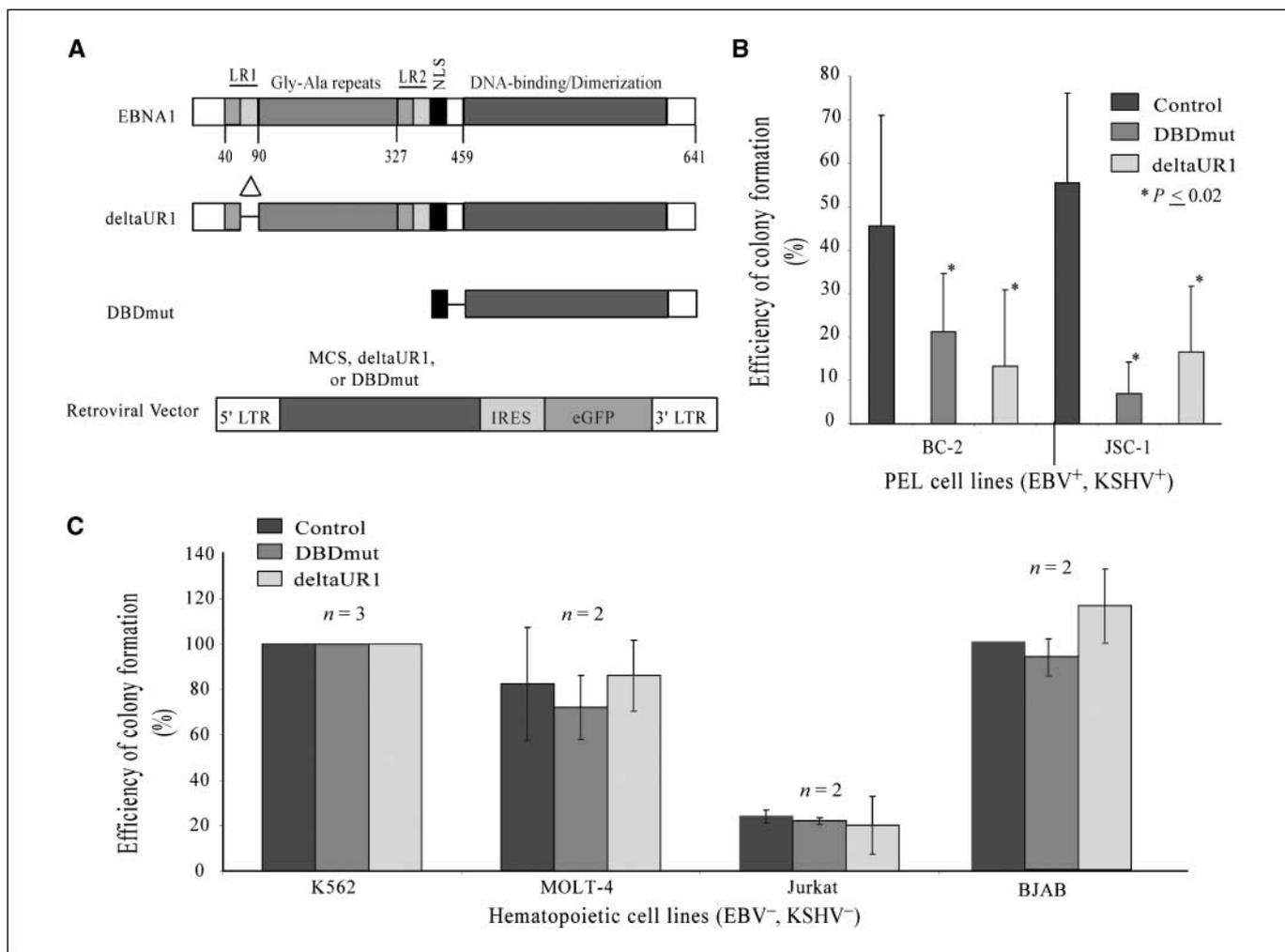

Figure 1. Two derivatives of EBNA1 inhibit EBV⁺, KSHV⁺ PEL cells from forming colonies and do not affect EBV⁻, KSHV⁻ hematopoietic cell lines. *A,* full-length EBNA1 is 641 amino acids in length and contains two regions (*LR1* and *LR2*) responsible for linking DNA, a nuclear localization sequence (*NLS*), and a DNA-binding and dimerization domain (15). This carboxyl-terminal DNA-binding and dimerization domain is not sufficient to support the contributions of EBNA1 to DNA replication and acts as a dominant-negative derivative inhibiting all of these functions (9, 15). Numbers indicate the position of amino acids. The deletion within deltaUR1 spans amino acids 65 to 89 (Delta), rendering EBNA1 transcriptionally defective. DBDmut contains only a nuclear localization sequence and the DNA-binding and dimerization domain and is defective in all of the known functions of EBNA1. DBDmut, deltaUR1, or a multiple cloning sequence as the control was inserted into a retroviral backbone as previously described (9), which coexpresses eGFP. *B,* BC-2 and JSC-1 cells were infected with retroviruses expressing DBDmut, deltaUR1, or the control retrovirus containing only the multiple cloning sequence. Infected cells were sorted by FACS 48 h postinfection, and their efficiency of forming colonies was determined 2 wk after plating. *C,* DBDmut and deltaUR1 do not inhibit EBV⁻, KSHV⁻ hematopoietic cell lines from forming colonies. Two EBV-negative Burkitt's lymphoma cell lines (BJAB and DG75; analyzed in ref. 9), an erythroleukemia cell line (K562), and two T-cell lymphoblastic leukemia lines (MOLT-4 and Jurkat) were tested in assays for colony formation. These cells were infected with control, DBDmut, or deltaUR1-expressing retroviruses, sorted into 96-well plates, and measured for their ability to form colonies 2 wk postplating. *n,* number of independently done experiments.
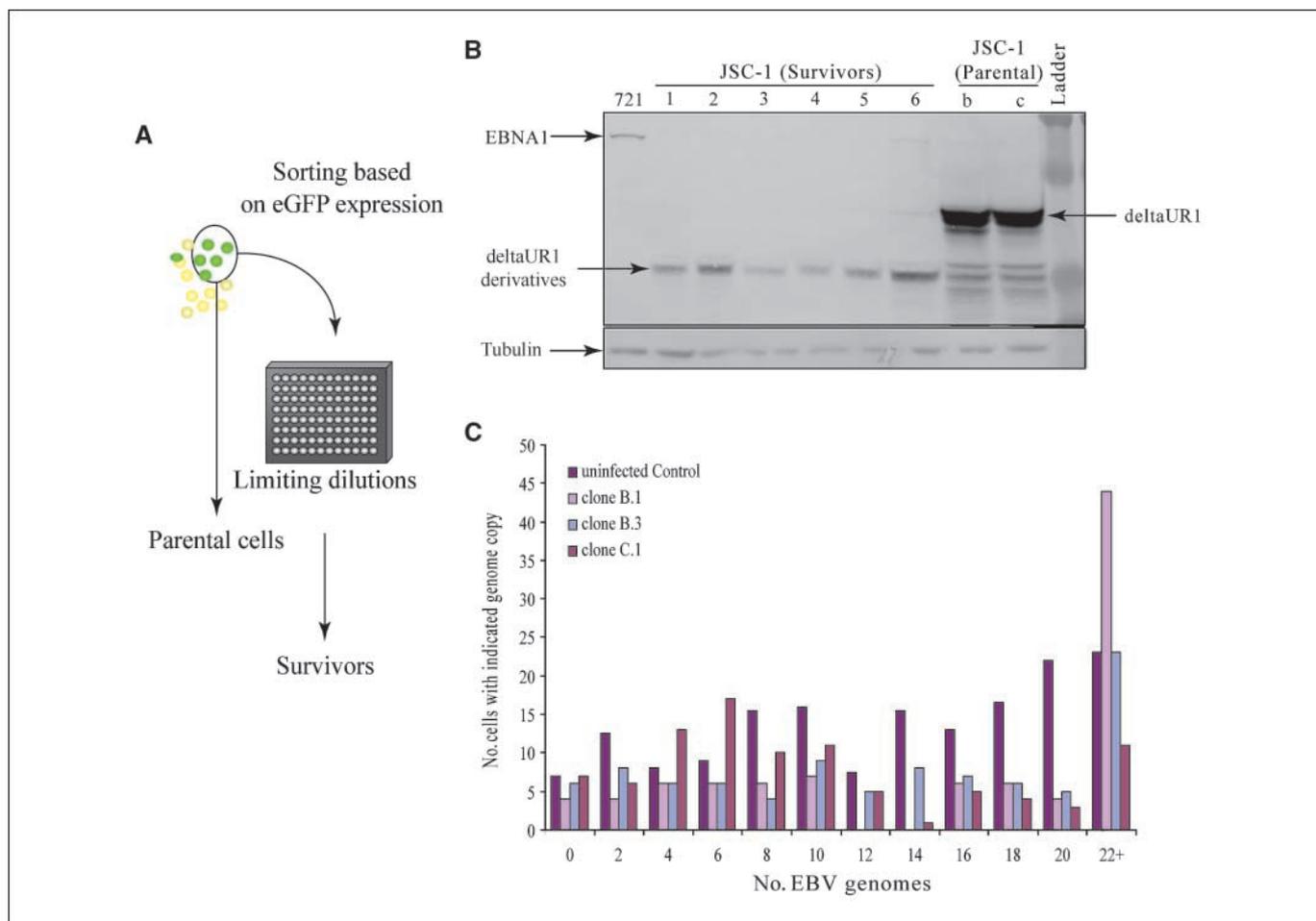
Figure 2. Only JSC-1 cells expressing mutant forms of deltaUR1 and retaining EBV survive to form colonies. *A,* an illustration depicting the two different cell populations analyzed by Western blotting. The Parental population is the initial set of cells transduced with the highest levels of eGFP expression, whereas the Survivors are the transduced cells that successfully formed the clones recovered from colony formation assays. *B,* six lysates chosen from a total of 14 deltaUR1-JSC-1 clones that were the Survivors were analyzed by Western blotting and compared with the corresponding Parental populations (*b* and *c*). In each lane, 3×10^4 cells were loaded. 721 cells were loaded as an EBNA1-positive control and tubulin served as a loading control. *C,* clones of Survivors have distributions of the number of EBV genomes per cell similar to that of uninfected JSC-1 cells. The number of viral DNAs per cell was measured by FISH. The distributions for three representative clones (*B.1, B.3,* and *C.1*) of Survivors are shown along with that of uninfected JSC-1 cells.

and that of deltaUR1-infected JSC-1 cells to 30%, that of the level of control-infected cells ($P < 0.01$).

The EBNA1 derivatives DBDmut and deltaUR1 do not inhibit EBV⁻, KSHV⁻ hematopoietic cell lines from forming colonies. We tested whether these derivatives of EBNA1 could affect EBV-negative, KSHV-negative hematopoietic cells. Two B-cell lines derived from Burkitt's lymphomas (BJAB and DG75; ref. 9), two T-cell lymphoblastic leukemia lines (MOLT-4 and Jurkat), and one erythroleukemia cell line (K562) were infected with retroviruses expressing DBDmut, deltaUR1, or a control retrovirus. Neither derivative of EBNA1 had detectable effects on the colony-forming ability of the five hematopoietic cell lines (Fig. 1*C*; ref. 9).

Those EBV⁺, KSHV⁺ JSC-1 cells that survive to form colonies contain nonfunctional mutants of deltaUR1. Surviving clones from control and deltaUR1-infected JSC-1 cells were expanded from colony formation assays to investigate their escape from the inhibition by EBNA1. Nine control-infected JSC-1 clones and 14 deltaUR1-infected clones from two independent infections were selected and expanded from the colony formation assays (Fig. 2*A*). The signal for intact deltaUR1 (*lanes b* and *c*) was undetectable in most of the 14 clones, which instead expressed a truncated form of

the protein (Fig. 2*B*). The DNA sequences of three of the truncated forms contained a single adenine insertion located at amino acid position 460 of full-length EBNA1. This mutation creates a frameshift resulting in a premature stop codon ∼16 amino acids downstream of the insertion site to yield a protein incapable of binding DNA. Thus, those EBV⁺, KSHV⁺ PEL cells that continue to proliferate in the presence of a dominant-negative derivative of EBNA1 do so only when EBNA1 failed to be inhibited.

All deltaUR1-infected JSC-1 cells that survive to proliferate maintain EBV. We expanded clones of JSC-1 cells infected with deltaUR1 or with the control virus that survived the colony formation assays and tested them for the presence of EBV. Although there was an increase in the number of parental cells lacking EBV when deltaUR1 was present, all 14 deltaUR1-JSC-1 clones that survived limiting dilution maintained EBV as measured by fluorescence *in situ* hybridization (FISH), with their positive proportions being similar to the percent of EBV-positive cells found in JSC-1 cells infected with control virus (Supplementary Table S1). The distribution in the number of EBV genomes per cell paralleled that of JSC-1 clones infected with the control virus as well as that of untransduced JSC-1 cells (Fig. 2*C*). This distribution results from
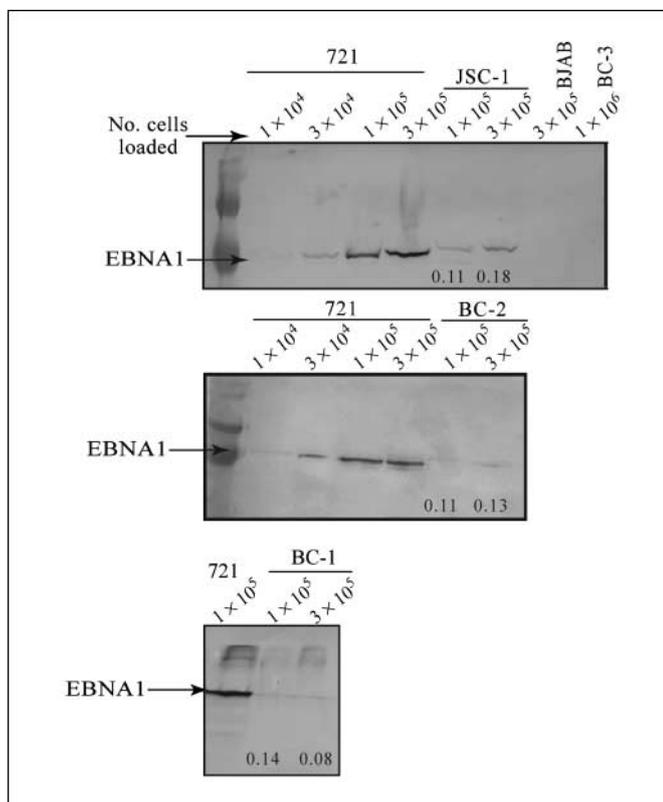
Figure 3. Levels of EBNA1 are atypically low in three EBV-positive PEL cell lines. Lysates from three EBV-infected PEL cell lines, JSC-1, BC-2, and BC-1, were examined by Western blotting to determine the levels of EBNA1. The intensities of the signals for EBNA1 were determined using ImageQuant software version 5.2. The numbers at the bottom of each lane in the Western blots represent the amount of EBNA1 relative to the levels found in EBV-positive 721 cells. Antibody specificity was determined using the EBV-negative cell lines BJAB and BC-3 as well as the EBV-positive cell line 721. The size of EBNA1 detected in each of these cell lines fell within the estimated 72-kDa range as indicated by the size marker located in the first lane of each blot.

defects in the synthesis and partitioning of EBV plasmids (7). These data and the fact that the probe used to detect EBV in these clones covers approximately one third of the viral genome indicate that the EBV DNA in these clones is extrachromosomal as it is in the parental JSC-1 cell. Our finding that all surviving clones of JSC-1 cells maintain EBV indicates that EBV contributes a function to these dually infected PEL cells that is required for their growth under the initial conditions of limiting dilution.

Analyzing the inhibition of colony formation of EBV⁺, KSHV⁺ PEL cells. The reduced colony-forming ability resulting from inhibition of EBNA1 in EBV-infected PEL cells parallels that observed in EBV-infected Burkitt's lymphoma cell lines (9). Inhibiting EBNA1 to force the loss of EBV in Burkitt's lymphoma cells induces apoptosis.[2] We tested whether inhibiting EBNA1 in EBV⁺, KSHV⁺ PEL cells would induce apoptosis in two independent assays and found that it did not (Supplementary Fig. S1). In addition, we tested whether the inhibition of colony formation might result from defects in autocrine signaling that could be rescued by cocultivation with parental PELs cells. These cocultivation

experiments did not rescue the growth of dually infected PELs in which EBNA1 was inhibited (Supplementary Fig. S2).

Levels of EBNA1 are atypically low in three EBV-positive PEL cell lines. EBV-positive normal B cells and Burkitt's lymphoma cells usually express similar, average levels of EBNA1 per cell, although they vary widely in their average number of viral genomes per cell (10). Dually infected PEL cells have average numbers of EBV genomes per cell well within the range of other EBV-positive cells but express one fifth to one tenth the levels of EBNA1 found in
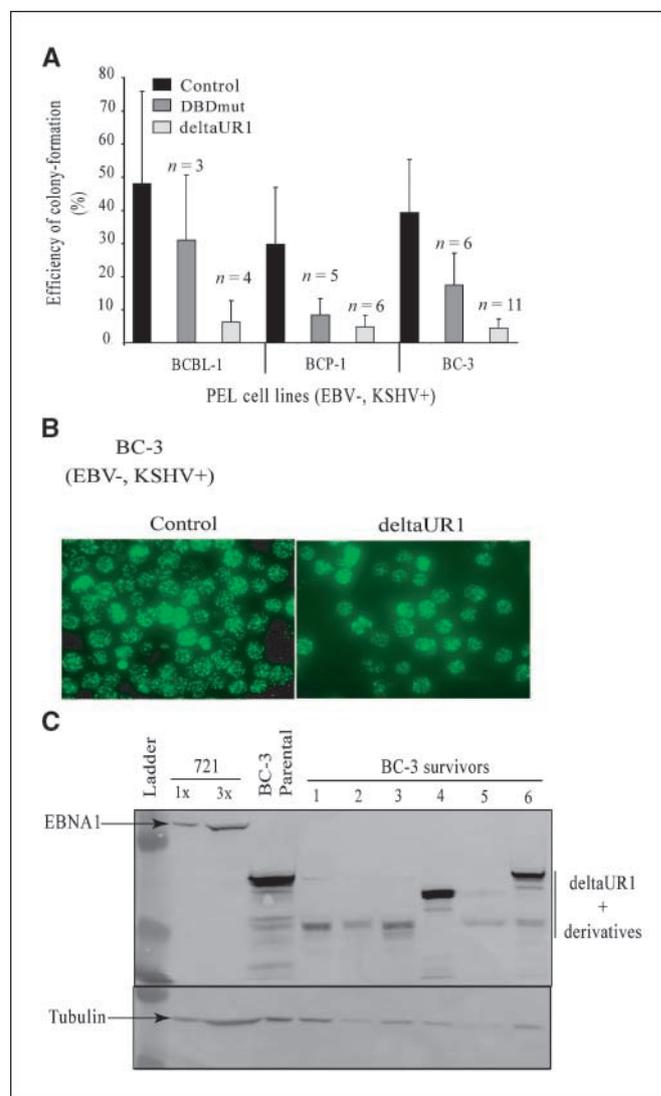

Figure 4. EBV⁻, KSHV⁺ PEL cell lines are inhibited in their ability to form colonies by derivatives of EBNA1, and only BC-3 cells with mutant forms of these derivatives survive to form colonies. *A,* BCBL-1, BCP-1, and BC-3 cells were infected with retroviruses expressing DBDmut, deltaUR1, or the control retrovirus. Infected cells were sorted by FACS for the highest levels of eGFP (5–10% of the total population) 48 h postinfection into 96-well plates. Their colony-forming efficiency was determined using a Poisson distribution. *B,* KSHV DNA was detected in EBV⁻, KSHV⁺ BC-3 cells by FISH and no difference in the number of KSHV genomes in the presence or absence of deltaUR1 expression 10 d postinfection was observed. *C,* six lysates of Survivors from deltaUR1-BC-3 clones were analyzed by Western blotting and compared with a corresponding Parental population. Lysates from 3×10^4 cells were loaded for each lane representing Survivors and 1×10^4 cells for the Parental population. 721 cells were loaded [1×10^4 (*1×*) and 3×10^4 (*3×*)] as an EBNA1-positive control and tubulin was included as a loading control.

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[2] L. Dresang, B. Sugden, and D. Vereide, unpublished data.

other EBV-positive B cells studied (Fig. 3). These findings raise the possibility that efficient expression of EBNA1 is deleterious to KSHV-infected cells and that its expression is likely regulated by the KSHV-encoded protein LANA1, for example, as has been reported (11).

DBDmut and deltaUR1 also inhibit three EBV⁻, KSHV⁺ PEL cell lines from forming colonies. We tested three EBV⁻, KSHV⁺ PEL cell lines (BCBL-1, BCP-1, and BC-3) to determine if DBDmut or deltaUR1 inhibited their ability to form colonies. Surprisingly, these derivatives did inhibit the colony formation of all three cell lines ($P < 0.05$, Wilcoxon rank sum test; Fig. 4A). The inhibition of colony formation of one of these lines, BC-3, did not correlate with an increase in apoptosis or a defect in autocrine signaling (Supplementary Figs. S1 and S2). There was no obvious, detectable decrease in the number of KSHV genomes per cell in DBDmut-infected BC-3 cells 10 days after infection as measured by FISH (Fig. 4B).

DBDmut and deltaUR1 are themselves mutated in BC-3 cells that form colonies in their presence. Six surviving BC-3 clones expressing deltaUR1 were expanded from colony formation assays and the deltaUR1 expressed in them was characterized. Five of these clones expressed little to undetectable levels of deltaUR1, which likely explained their ability to form colonies. All of these clones expressed mutant forms of deltaUR1 that resembled the truncated deltaUR1 mutants detected in the clones of JSC-1 cells that survived the colony formation assays (Fig. 4C). We also examined the DNA sequences of the derivatives of EBNA1 found in the two BC-3 clones expressing DBDmut that survived in colony-forming experiments. Sequencing revealed the same single adenine insertion in the EBNA1 sequence resulting in a frameshift identified in the surviving deltaUR1-infected JSC-1 clones. These findings show that the tested EBV⁻, KSHV⁺ cells that survive limiting dilution express the derivatives of EBNA1 inefficiently and/or express ones defective in binding DNA.

Discussion

These experiments reveal a necessary role for EBV in supporting the proliferation of dually infected PEL cells. They show that inhibiting EBNA1 to force the loss of EBV from these cells inhibits their proliferation under limiting dilution (Fig. 1). When the EBNA1 derivatives, DBDmut or deltaUR1, were introduced into EBV⁺, KSHV⁺ PEL cells, most of the cells failed to grow under limiting dilution as measured by colony-forming assays. The infrequent surviving clones expressed nonfunctional levels of deltaUR1 or nonfunctional mutant forms of either DBDmut or deltaUR1 (Fig. 2). These nonfunctional derivatives could not bind DNA. All 14 clones expanded from the JSC-1 survivors of colony formation assays maintained EBV, showing that EBV is a requirement for dually infected PELs to proliferate under limiting dilution (Fig. 2C; Supplementary Table S1).

We also tested the effects of efficient expression of the two derivatives of EBNA1 in five EBV⁻, KSHV-hematopoietic cell lines and three EBV⁻, KSHV⁺ PEL cell lines on their growth under limiting dilution. The derivatives of EBNA1 had no effect on the growth of the virus-negative cells (Fig. 1C; ref. 9) but did inhibit colony formation of the KSHV⁺ PELs (Fig. 4). Those KSHV⁺ PEL cells that survived these assays expressed either negligible levels of the EBNA1 derivatives or mutants of these derivatives defective in binding DNA. We have found that EBNA1 binds KSHV DNA specifically at multiple sites[1] and hypothesize that the efficient expression of DNA-binding derivatives of EBNA1 on binding KSHV

DNA inhibits one or more functions important for the proliferation of KSHV⁺ PELs. It is possible that the binding of these derivatives of EBNA1 to cellular DNA could affect the growth of KSHV⁺ PEL cells, but we find this a less likely hypothesis because these derivatives have no effects on five EBV⁻, KSHV⁻ hematopoietic cell lines. The hypothesis that the efficient binding of EBNA1 to the KSHV genome inhibits proliferation of PEL cells is also consistent with the atypically low levels of EBNA1 found in dually infected PEL cells (Fig. 3; ref. 11). These dually infected cells likely require low levels of EBNA1 to avoid its inhibiting functions encoded by KSHV but sufficient EBNA1 to maintain their EBV genomes on which they also depend for their growth. We hypothesize that efficient expression of the mutant derivatives of EBNA1 and their ability to bind DNA site-specifically are responsible for the loss of the colony-forming ability of EBV⁺ and EBV⁻ PEL cells. By extension, it is likely that the efficient expression of wild-type EBNA1, which binds DNA as do its derivatives that we used, would similarly result in a reduced ability of PEL cells to form colonies. This likelihood is difficult to test, however, because efficient expression of intact EBNA1 is often toxic to cells whereas its derivatives that we used are not (9).

At first glance, our observation that the expression of two derivatives of EBNA1 inhibits EBV⁻, KSHV⁺ PELs from forming colonies might be inconsistent with our conclusion that EBV is necessary for EBV⁺, KSHV⁺ PELs to form colonies. For example, these derivatives may act in the latter cells by inhibiting *KSHV* gene expression as we speculate it does in EBV⁻, KSHV⁺ PELs. In fact, we think that this notion is likely correct because the deltaUR1 derivative of EBNA1 blocks the transcription, but not replication, of EBV and may force the loss of EBV indirectly. However, our finding that all clones of EBV⁺, KSHV⁺ PELs initially selected to express these derivatives can only grow under limiting dilution if the derivatives are nonfunctional and EBV is retained in the cells indicates that EBV is necessary for the proliferation of this class of PELs.

Multiple studies indicate that KSHV is also necessary to sustain dually infected PELs. For example, inhibition of LANA1 with shRNAs in JSC-1 cells inhibits their proliferation whereas inhibition of v-cyclin and v-flip induces apoptosis in these cells (12). The inhibition of the *vIRF-3* gene of KSHV by shRNA also induces apoptosis in JSC-1 cells (13). The introduction of intact EBV into an EBV⁻, KSHV⁺ PEL, BC-3, renders this experimentally dually infected PEL more tumorigenic in severe combined immunodeficient mice than is the parental, singly infected PEL (14). All of these observations, when coupled with our finding that the forced loss of EBV from naturally dually infected PELs inhibits their proliferation following limiting dilution, indicate that both KSHV and EBV contribute to the maintenance of these dually infected lymphomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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