Restricted expression of Epstein–Barr virus (EBV)-encoded, growth transformation-associated antigens in an EBV- and human herpesvirus type 8-carrying body cavity lymphoma line

Laszlo Szekely,1 Fu Chen,1 Norihiro Teramoto,1 Barbro Ehlin-Henriksson,1 Katja Pokrovskaja,1 Anna Szeles,1 Agneta Manneborg-Sandlund,1 Mia Löwbeer,1 Evelyne T. Lennette2 and George Klein1

1 Microbiology and Tumour Biology Centre (MTC), Karolinska Institute, S-17177 Stockholm, Sweden
2 Virolab Inc., Berkely, CA 94710-1509, USA

A body cavity lymphoma-derived cell line (BC1), known to carry both Epstein–Barr virus (EBV) and human herpes virus type 8 (HHV-8; or Kaposi’s sarcoma-associated herpesvirus, KSHV), was analysed for the expression of EBV-encoded, growth transformation-associated antigens and cellular phenotype by immunofluorescence staining, Western blotting, RT–PCR and flow cytometry. A similar phenotypic analysis was also performed on another body cavity lymphoma line, BCBL1, that is singly infected with HHV-8. Phenotypically, the two lines were closely similar. Although both lines are known to carry rearranged immunoglobulin genes, they were mostly negative for B-cell surface markers. Both expressed the HHV-8-encoded nuclear antigen (LNA1). Similarly to Epstein–Barr nuclear antigen type 1 (EBNA1), LNA1 was associated with the chromatin in interphase nuclei and the mitotic chromosomes in metaphase. It accumulated in a few well-circumscribed nuclear bodies that did not co-localize with EBNA1. BC1 cells expressed EBNA1, LMP2A and EBV-encoded small RNAs but not EBNA2–6, LMP1 and LMP2B. They were thus similar to type I Burkitt’s lymphoma cells and latently infected peripheral B-cells. Analysis of the splicing pattern of the EBNA1-encoding message by RT–PCR showed that BC1 cells used the QUK but not the YUK splice, indicating that the mRNA was initiated from Qp and not from Cp or Wp.

Introduction

Human herpes virus type 8 (HHV-8; or Kaposi’s sarcoma-associated herpesvirus, KSHV) is a recently discovered human gammaherpesvirus that is the probable aetiologic agent of both AIDS-associated and sporadic Kaposi’s sarcomas (Chang & Moore, 1996; Kedes et al., 1996; Miles, 1996). The virus has also been found in primary effusion lymphomas (PEL), also called body cavity lymphomas (BC-1) (Cesarman et al., 1995a; Nador et al., 1996), in the multicentric Castleman’s disease (Gessain et al., 1996) and in the dendritic cells of multiple myeloma patients (Rettig et al., 1997). HHV-8 is not ubiquitous like Epstein–Barr virus (EBV), but is present in a minority of people in all human populations (Gao et al., 1996b). Body cavity lymphomas that are often, but not always, associated with AIDS, frequently carry both EBV and HHV-8 (Carbone et al., 1996; Said et al., 1996). Cell lines have been established from both single HHV-8-positive and double HHV-8/EBV-positive body cavity lymphomas (Arvanitakis et al., 1996). The latter provide a unique opportunity to study the relations between two latent and closely related herpesviruses.

EBV can establish at least three different types of virus latency. The full expression of all nine growth transformation-associated viral proteins, EBNA1–6, LMP1, -2A and -2B is only found in lymphoblastoid cell lines (LCLs), immunoblastic lymphomas, and in Burkitt’s lymphoma (BL) lines that have drifted to an immunoblastic phenotype after in vitro culturing. The six EBNA1s are spliced from a single giant message, initiated from one of several alternative promoters in the C or W region (W/C programme) (Bodescot & Perricaudet, 1986; Bodescot et al., 1987). In type I latency, found in BL cells in vivo and in derived cell lines that have maintained a representative cellular phenotype (Rowe et al., 1986), as well as in latently infected normal B-cells in vivo (Chen et al., 1995), only EBNA1 and LMP2 are expressed. The former is initiated from a promoter in the Q region (Q programme) (Chen et al., 1995; Schaefer et al., 1995). Latency type II, found in all non-B-cells that carry the virus, including Hodgkin’s and T-cell lymphomas, nasopharyngeal and other carcinomas, is identical
to type I latency as far as the EBNA s are concerned, but differs by the constitutive expression of LMP1 (Fahraeus et al., 1988; Hitt et al., 1989; Pallesen et al., 1991). The EBV-encoded small RNAs (EBERs) are expressed in all three types of latency.

Latent EBV thus modifies its expression depending on the phenotype of the host cell. Full expression of all six EBNA s and the three LMPs is only known to occur in immunoblasts. The resting B-cell and its closest counterpart, the BL cell that is driven by the immunoglobulin/myc translocation, as well as T-cell lymphomas and carcinoma cells, apparently lack some immunoblast-specific component that is needed to activate the full programme. Non-B-cells can express LMP1 constitutively. This does not occur in lymphocytes of the B-lineage, due to a cellular repressor that can be overridden by EBNA2 (Fahraeus et al., 1993). In BL cells and normal resting B-cells, LMP1 remains suppressed.

The body cavity lymphomas lines BC1 and BCBL1, which were the subjects of the present study, represent a different cell type within the B-lineage, probably a pro- or pre-B-cell. It appeared to be of interest to examine the EBV expression pattern in the double-infected BC1 cell. We found that it corresponds to type I latency, as in the BL cells but no other lymphomas so far studied.

Methods

Cell culture and karyotype analysis. All cell lines were cultured at 37 °C, in Iscove’s medium containing 10% FCS and 50 mg/ml gentamycin. Absence of mycoplasma contamination was monitored by periodic staining with Hoechst 33258. The cell lines used in this study have been described in the references (Cesarman et al., 1995; Ehlin-Henriksson et al., 1987; Renne et al., 1996; Pokrovskaja et al., 1996). Karyotype analysis of G-banded chromosomes was done following the protocol of Seabright (1971).

Immunofluorescence staining, image analysis and flow cytometry. Indirect immuno-fluorescence staining and anti-complement immunofluorescence reaction (ACIF) with monospecific absorbed human sera or with unabsorbed serum with high anti-EBNA titre were performed as previously described (Jiang et al., 1991; Szekely et al., 1995). Briefly, cells were regularly fixed with methanol-acetone (1:1) for at least 10 min and rehydrated in PBS. The first antibodies were diluted in blocking buffer (2% BSA, 0.2% Tween-20, 10% glycerol, 0.05% NaN3 in PBS) in the proportion of 1:1 and 1:10–50, respectively, depending on the individual antibodies. The secondary antibody, FITC-conjugated rabbit anti-mouse or anti-human IgG (Dakopatts), was diluted in blocking buffer (1:20). The slides were mounted with 70% glycerol, 2.5% Dabco (Sigma) pH 8.5 in PBS. For double immunofluorescence staining, serum of a Kapo’s sarcoma patient with high anti-LNA1 activity was used together with mouse monoclonal anti-EBNA1 OT1x (1:200; provided by J. M. Middelop, Organon Teknika, The Netherlands) and followed by FITC-conjugated anti-human IgG (1:100; Dakopatts) and biotinylated goat anti-mouse IgG1 (1:100; Pharmingen). The latter was detected by rhodamine-conjugated streptavidin (1:200; Dakopatts). The anti-EBNA activity of the polyclonal human anti-LNA1 serum was eliminated by absorption with acetone-fixed IB4 and IARC171 cells. No nuclear staining was detected when these LCLs were stained with the absorbed serum.

Images were generated using a Leitz DM RB microscope, equipped with Leica PL Fluorat x 100, x 40 and PL APO Ph x 63 oil immersion objectives. Leica L4, Tx and A composite filter cubes were used for the FITC, Texas Red and Hoechst 33258 fluorescence, respectively. The pictures were captured with a Hamamatsu dual-mode cooled CCD camera (C4880), recorded and analysed on a Pentium PC (133 MHz, 32 Mb RAM) computer equipped with an AFG VISIONplus-AT frame grabber board using the Hipic3.2.0 (Hamamatsu), Image-Pro Plus (Media Cybernetics) and Adobe Photoshop image capturing and processing software.

Flow cytometry measurements were carried out as previously described (Pokrovskaja et al., 1996).

Western blotting, RT–PCR and in situ hybridization. Detection of membrane-blotted proteins was carried out as previously described (Pokrovskaja et al., 1996; Szekely et al., 1993) with the modification that chemiluminescence (ECL kit, Bio-Rad) was used to detect the peroxidase-conjugated goat anti-human IgG. RT–PCR for the analysis of EBV gene expression as well as of promoter usage was carried out as described (Chen et al., 1995).

For EBER in situ hybridization the cells were fixed in 4% paraformaldehyde and were subsequently treated for 10 periods of 10 min each in the following solutions: distilled water, 0.2 M HCl, twice in PBS, PBS containing 5 µg/ml proteinase K, PBS, distilled water, 70% ethanol and finally 100% ethanol. After drying, the cells were hybridized with EBER probes (Dakopatts) following the manufacturer’s instructions. After washing in 50% fluorescent antibody–2× SSC and 1× SSC, the EBER1 signal was detected by the combination of mouse monoclonal anti-FITC (1:100; Dakopatts) and rabbit anti-mouse FITC-conjugated antibodies. EBER2 was detected by the combination of rhodamine-conjugated sheep anti-digoxigenin (1:10; BMH) and rhodamine-conjugated rabbit anti-sheep immunoglobulin (1:50).

Results

Phenotypic characterization and karyotype analysis

The expression of B-cell markers was analysed by immunofluorescence staining and flow cytometry. The LCLs IARC171 and BCBL1 lines

<table>
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<tr>
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<td>%</td>
<td>mFI</td>
<td>%</td>
<td>mFI</td>
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<td>–</td>
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</tr>
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<td>–</td>
<td>0</td>
<td>–</td>
<td>Rael</td>
<td>65</td>
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* mFI, mean fluorescence intensity (measured by flow cytometry).
Table 2. Karyotype analysis

<table>
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<tr>
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<th>Observed/analysed metaphases</th>
<th>BCBL1 (41–48, XY) Observed/analysed metaphases</th>
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<tr>
<td>t(1;3)(q12;q29)</td>
<td>8/8</td>
<td>dup(1q21;44)</td>
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<tr>
<td>9q+</td>
<td>4/8</td>
<td>2p–</td>
</tr>
<tr>
<td>12q–</td>
<td>5/8</td>
<td>6p+</td>
</tr>
<tr>
<td>13p+</td>
<td>8/8</td>
<td>8p+</td>
</tr>
<tr>
<td>15p+</td>
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<td>16q+</td>
<td>1/8</td>
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<td>5/8</td>
<td>14q+†</td>
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<td></td>
<td></td>
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<tr>
<td></td>
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</table>

* and — indicate added and missing pieces to the given chromosome region, respectively.
† 14q+ does not resemble the myc/IgH translocation because the added piece is bigger than in the 14q+ in BLs with t(8:14).

and Nad20 and the BL cell lines BL29, BL41, P3HR1, Daudi and Rael were used as positive and negative controls, respectively. The results are summarized in Table 1. Both body cavity lymphoma lines were negative for most B-cell markers, including immunoglobulins. Only a small fraction of the cells showed some expression of B-cell markers, such as CD10, CD39 and CD77. The mean fluorescence intensities of the few positive cells were much lower than in the positive controls.

Karyotype analysis of Giemsa-stained metaphases revealed multiple chromosomal aberrations in both lines. There was no consistent, common change (Table 2).

Analysis of latency-associated EBV gene expression by immunofluorescence, Western blot and in situ hybridization

Immunofluorescence staining of BC1 cells with monoclonal antibodies against EBNA1, EBNA2 and EBNA5 as well as with monospecific absorbed human sera against EBNA1, EBNA3, EBNA4 and EBNA6 detected only EBNA1. The staining intensity showed some cell to cell variation but essentially all BC1 cells were EBNA1-positive. Only a very small fraction (< 0.1%) of cells showed entry into the EBV lytic cycle. EBV-negative BCBL1 cells and the EBV-carrying LCLs IARC171 and CBMI-RAL-STO served as negative- and positive-staining controls.

Immunoblotting of total cell lysates from IARC171 LCL with a human serum with high anti-EBNA titres detected EBNA1, EBNA2 and the high molecular mass EBNA1s (3, 4 and -6) (Fig. 1A). Only a single specific band was identified in the BC1 lysate, absent from the EBV-negative BCBL1. It was approximately 75 kDa in size, well within the range of the known EBNA1 size variation among the different EBV strains. Testing the same cell lysates with affinity-purified human antibodies that reacted only with the Gly–Ala repeats of EBNA1 identified the single band in the BC1 lysate as EBNA1 (Fig. 1C).

Fluorescent in situ hybridization with EBER1- and EBER2-specific probes showed that both small RNA species were present in the nuclei of BC1 but not in BCBL1 cells (Fig. 2A).

RT–PCR assessment of viral gene expression and splicing patterns

As shown in Figs 3 and 4, RT–PCR detected EBNA1 and LMP2A mRNA expression in BC1 cells but not in EBNA2, LMP1 or LMP2B. Promoter usage for EBNA1 transcription was studied by primers detecting the YUK and QUK splicing.
Fig. 2. For legend see facing page.
EBV in body cavity lymphoma

Fig. 2. (A) Detection of EBER1 (green) and EBER2 (red) in BC1 but not in BCBL1 cells by fluorescence in situ hybridization. DNA is stained with Hoechst 33258 (blue). (B) Double immunofluorescent staining of LNA1 (green) with LCL-absorbed human HHV-8-positive polyclonal serum and of EBNA1 (red) with mouse monoclonal antibody. BC1 cells show no significant overlap (white) between the two proteins. DNA is stained with Hoechst 33258 (blue). (C) Association of LNA1 (green) with chromosomes in prometaphase and metaphase in BCBL1 cells. DNA is stained with Hoechst 33258 (blue).

Fig. 3. RT–PCR detects only LMP2A but not EBNA2, LMP1 or LMP2B mRNA in BC1 cells. B95-8-, IB4-, CBMI-RAL-STO- and LMP2A-transfected A431 cells served as positive and BCBL1 cells as negative controls. Arrowheads point to the specific PCR products. Low molecular mass bands on the LMP1 and LMP2B pictures are PCR primers.

Fig. 4. BC1 cells use the QUK but not the YUK splice pattern, suggesting that the latency-associated Qp, and not the Cp or Wp, promoter is used to express EBNA1 in these cells, in contrast to the LCL control IB4 cell line that uses Wp. B95-8 uses both Qp and Cp promoters.

patterns, characteristic for the Cp/Wp and Qp promoter usage, respectively. BC1 cells were only positive for the QUK, not the YUK, splice pattern. The B95-8 controls were positive for both as previously shown (Chen et al., 1995). The BC1 pattern is the same as in phenotypically representative BL cells, including in vivo tumours and recently established lines with the corresponding phenotype.

Expression and distribution of the HHV-8-encoded nuclear antigen (LNA1)

Western blotting with sera from human immunodeficiency virus-positive Kaposi’s sarcoma patients with high anti-HHV-8 titre showed that the major specific band corresponded to the high molecular mass (p226 and p234) LNA1 (Fig. 1B) (Rainbow et al., 1997). The same sera detected, by immunofluorescence, the coarsely speckled, sharply outlined blocks of nuclear antigen for LNA1 present in all interphase nuclei (Gao et al., 1996b; Simpson et al., 1996). LNA1 remained associated with the chromosomes during mitosis (Fig. 2C). There was no difference in the staining pattern of the EBV-carrying BC1 and EBV-negative BCBL1 lines.

It has been previously shown that EBNA1, alone among the EBV-encoded growth transformation-associated proteins, is chromatin-associated both in interphase and metaphase nuclei. This was also true in the BC1 line, where the EBNA1 staining was mainly restricted to well-defined nuclear dots, detected both by ACIF staining with monospecific human sera.
and by indirect immunofluorescence with rabbit polyclonal or mouse monoclonal antibodies. Double staining of BC1 nuclei showed that LNA1 and EBNA1 occupy different nuclear domains, without any colocalization (Fig. 2B).

**Discussion**

EBV expression in latently infected cells is regulated in a host cell phenotype-dependent fashion. Immunoblasts induced by EBV transformation *in vitro*, established LCLs and the immunoblastic lymphomas of immunocompromised individuals express the full set of EBNA s and all three LMPs. We can refer to this as the full programme. BL cells in vivo and derived cell lines that have maintained the BL-representative (type I) phenotype, as well as normal B-cells that carry latent virus, use a restricted programme and express only EBNA1 and LMP2A. Non-B-cells that harbour the virus genome, such as certain T-cell lymphomas, Hodgkin’s lymphoma and nasopharyngeal carcinoma, express a variant of the restricted programme, characterized by the additional expression of LMP1.

Change from restricted to full expression occurs in B-cells in connection with immunoblastic transformation. Examples include the phenotypic drift of EBV-carrying BL lines from type I to a more immunoblastic (type II–III) phenotype during prolonged *in vitro* culturing (Rowe *et al*., 1987) or by the outgrowth of latently EBV-infected normal B-cells or chronic lymphocytic leukaemia cells into established lymphoblastoid lines (Lewin *et al*., 1995).

Cell fusion experiments between LCLs and different B- and non-B-cells showed that the full expression programme manifested in the C p promoter-initiated polycistronic EBNA1–6 transcript was switched off in hybrids between LCLs and non-B-cells that have acquired the phenotype of the non-B-parent, while the restricted monocistronic EBNA1 transcript was turned on (Altio k *et al*., 1992; Contreras-Brodin *et al*., 1991; Contreras-Salazar *et al*., 1989).

Our phenotypic characterization of the two BC lines, together with earlier evidence (Gao *et al*., 1996a), showed that both lines have a closely similar phenotype. Their B-cell origin is supported by the IgH rearrangement, previously detected with a JH probe (Cesarman *et al*., 1995b). Most B-cell markers were absent, however, including the pan-B marker CD19, CD21 and immunoglobulin heavy and light chains.

A minority of the cells expressed some B-cell markers, such as CD10, CD39 and CD77, in a small fraction of the cells and at a low level. This phenotype does not correspond to any conventional B-cell subtype. It is possible that the cells of the two BC lines correspond to an unusual pro- or pre-B-cell. However, EBV transformed pro- and pre-B-cells have been grown in culture previously, but their phenotype was similar to immunoblastic LCLs and they expressed the full EBV programme (Altio k *et al*., 1989).

Recently, in parallel experiments, Horenstein *et al.* (1997) analysed five EBV/KSHV double-infected primary effusion lymphomas in order to determine the EBV gene expression pattern and had the same results as those presented above.

Two conclusions can be drawn from these findings. First, the use of the restricted, rather than the full, programme by the virus in the BC1 cell is consistent with the widely accepted notion that the full programme can only be expressed in immunoblasts. This is the first confirmation of that conclusion in a non-immunoblastic B-cell-derived lymphoma line that is not of BL origin. Second, the finding further reinforces the impression that B-cell clones that maintain a restricted programme during proliferation can only be derived from established malignancies *in vivo*. *In vitro* transformation selects for proliferating immunoblasts that express the full programme. Immunoblastic proliferation can occur *in vivo*, but only in immunodeficient systems. This is consistent with the high immunogenicity of EBNA2–6 for T-cells.

Cells infected *in vivo* that have a restricted programme can only proliferate *in vitro* if they are driven by some EBV-unrelated stimulus. BL cells are driven by c-myc that has been constitutively activated by immunoglobulin/mucr translocation. It may be surmised that the BC lines are driven by HHV-8. This is consistent with the existence of the EBV-negative, HHV-8-positive BCBL1 line. Alternatively, their immortality may stem from genetic changes, as suggested by the numerous, but essentially non-overlapping, chromosomal anomalies found in the two lines.

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