Expression of Epstein-Barr Virus Nuclear Antigen 1 Is Associated with Enhanced Expression of CD25 in the Hodgkin Cell Line L428

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Epstein-Barr virus is associated with several human malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease (HD). To examine the effect of Epstein-Barr virus nuclear antigen 1 (EBNA-1) in the pathogenesis of HD, we transfected the gene into the HD cell line L428. EBNA-1 expression was associated with significantly enhanced CD25 expression (interleukin 2 [IL-2]-receptor α chain) in transient and stably transfected L428 cells but did not affect the expression of IL-2 receptor β and γ chains. There was no up-regulation of the B-cell activation molecules CD23, CD30, CD39, CD40, CD44, CD71, and CD54 (intercellular adhesion molecule 1) or enhanced production of IL-6, IL-10, lymphotoxin alpha, and the soluble form of CD25. Stable EBNA-1-expressing L428 cells were nontumorigenic in SCID mice but showed enhanced lymphoma development in nonobese diabetic-SCID mice compared to mock-transfected cells. Hodgkin's disease (HD) is characterized by a disruption of the lymph node architecture with low numbers of Hodgkin and Reed-Sternberg (H-RS) cells surrounded by an abundance of reactive nonmalignant cells (2, 9, 13, 26). This histological pattern of HD is probably due to the production and release of cytokines by H-RS cells which are involved in the biology of HD (19).

In HD, Epstein-Barr virus (EBV) is present in more than 50% of cases and has been suggested to be a critical element in the pathogenesis of the disease (1, 7, 12, 23, 36). Epidemiological studies first indicated a link between EBV and HD and showed an increased risk of developing HD in patients with infectious mononucleosis and elevated EBV-VCA titers. More direct evidence came from molecular studies, which demonstrated the presence of EBV sequences and antigens in H-RS cells (2, 12, 33). The latent membrane protein 1 (LMP-1) is detectable in H-RS cells in the absence of the EBV nuclear antigen 2 (EBNA-2), which can transactivate LMP-1 and a subset of cellular genes in B cells (3, 35). Both EBNA-1 and LMP-2A were also found to be expressed in H-RS cells by immunostaining (7, 11, 24).

EBV is involved in the pathogenesis of a number of different human tumors, including immunoblastic B-cell lymphomas of immunocompromised patients, endemic Burkitt’s lymphoma, certain T-cell lymphomas, and nasopharyngeal carcinoma (NPC) (2, 6, 26). All EBV-associated tumor cells carry episonal copies of the EBV genome, and the EBER genes (small untranslated nonpolyadenylated nuclear viral RNAs) are abundantly expressed. The precise role of EBV in tumor development may be different in each disease, and a different expression pattern of viral genes can be observed in the tumor cells. The least complex case involves immunoblastic B-cell lymphomas, where tumor cell growth appears to be directly EBV driven in a manner analogous to that of the lymphoblastoid cell lines (LCLs) that arise when EBV infects normal resting B cells in vitro or in the absence of a functional immune system. Other EBV-associated tumors show a more restricted pattern of viral gene expression limited to EBNA-1 in Burkitt’s lymphoma only (known as latency type I) or to EBNA-1, LMP-1, and LMP-2A expression in HD and NPC (defined as latency type II) (26).

EBNA-1 is the only latent protein consistently expressed in all EBV-bearing tumor cells. Previously, it has been shown that a subpopulation of B lymphocytes express EBNA-1 mRNA in the absence of EBNA-2 or LMP-1, suggesting that the expression pattern of EBV genes in lymphoma cells of latency type I is the same as that in normal B cells. This expression pattern reflects a strategy of EBV of persisting in a latent state while being unrecognized by immunosurveillance (5). It has been shown that EBNA-1 does not alter the phenotype of stably transfected BJAB lymphoma cells (35), but transgenic mice expressing EBNA-1 specifically in B cells develop B-cell lymphomas (39). The role of EBNA-1 in HD is not clear yet.

We analyzed the effects of EBNA-1 on cell morphology, cell surface antigen, cytokine expression, and tumorigenicity in the HD cell line L428. L428 represents a cell line of B-cell origin and is characterized by the expression of many different surface antigens such as CD30, CD39, CD40, CD44, CD54 (intercellular adhesion molecule 1 [ICAM-1]), CD58 (lymphocyte function-associated antigen 3 [LFA3]), CD70, CD71, CD80 (B7.BB1), and major histocompatibility complex class II molecules. L428 cells are not tumorigenic in immunodeficient nude or SCID mice (9, 34). The cells constitutively express a number of cytokines including tumor necrosis factor alpha, lymphotixin alpha (LT-α), granulocyte-macrophage colony-stimulating factor, and interleukin 6 (IL-6) (19, 30). Furthermore, L428 cells carry rearranged immunoglobulin (Ig) heavy-chain and Ig light-chain genes. Sequence analysis revealed that the rearranged VH gene (VH5) is characterized by a high
number of somatic mutations. Clonally related, highly mutated rearranged Ig genes have also been detected by single-cell analyses in most cases of primary HD (16). This indicates that the L428 H-RS cell, like primary H-RS cells, has probably undergone a complex maturation and differentiation process within the germinal center, where the transformation process may have occurred. This cell line provides a useful model system for analyzing the effect of EBNA-1 gene expression in HD. Therefore, we transfected the EBNA-1 gene in the HD cell line L428 and selected a number of EBNA-1-positive cell clones.

Transfection of EBNA-1 in L428 cells. To obtain stable EBNA-1-expressing cells, L428 cells were cotransfected with pSG5 EBNA-1 expression plasmid or pSG5 vector control and a plasmid coding for puromycin-N-acetyltransferase with an electroporator (Easy-Ject electroporator; Eurogentec, Seraing, Belgium). After transfection, cells were grown under puromycin selection and 60 resistant clones were analyzed for EBNA-1 expression. A total of 15 independent EBNA-1-positive clones and 12 control clones (pSG5 vector) were included in this analysis from three independent transfection experiments. EBNA-1 expression was detected by immunoblotting with an EBNA-1-specific antibody (11) and the enhanced chemiluminescence (ECL) detection kit (Amersham Life Science, Braunschweig, Germany). As depicted in Fig. 1A, the stably transfected L428 clones are characterized by different levels of EBNA-1 expression (indicated by “+” to “+++”), with up to 15-fold-higher levels of EBNA-1 compared to the LCL IARC 304 (Fig. 1B). EBNA-1 was detected over a period of more than 12 months.

Expression of cellular surface antigens. Analysis of cell surface antigens was performed by flow cytometry with the FACScan (Becton Dickinson, Heidelberg, Germany). Values are presented as specific mean linear fluorescence intensities after subtraction of background staining with isotype-matched control. Dead cells were excluded by propidium-iodide staining. The following monoclonal antibodies were used in this study: IgG1 fluorescein isothiocyanate (FITC)-phycoerythrin (PE), IgG2A FITC, anti-CD23-PE–low-affinity Fcε receptor, anti-CD25-PE–IL-2Rα chain, anti-CD54-PE–ICAM-1, and goat anti-mouse Ig-FITC (Becton Dickinson, BDIS, San Jose, Calif.); anti-CD39, anti-CD40-FITC, anti-CD122–IL-2Rβ chain, and anti-IL-2Rγ chain–PE (Pharmingen, San Diego, Calif.); anti-CD44 (R&D Systems, Abingdon, United Kingdom); anti-CD30-FITC (Dako, Glostrup, Denmark); anti-CD4-PE and anti-CD71-FITC–transferrin receptor (Immunotech, Marseille, France). Table 1 summarizes the results of the surface immunofluorescence analyses of five representative EBNA-1-expressing and vector control-fluorescein isothiocyanate (FITC)-phycoerythrin (PE), transfected clones. No changes were found in the expression of the B-cell activation markers CD23, CD39, and CD44, and CD71 and the cell adhesion molecule CD54. All analyzed clones showed low levels of CD23 and CD39 expression, while CD44, CD54, and CD71 were constitutively expressed at high levels without further significant alterations. CD30 and CD40, characteristic surface antigens in most cases of HD and HD-derived cell lines and constitutively expressed in L428 cells, were not affected by EBNA-1 expression.
Induction of CD25 by EBNA-1. As shown in Table 2, the expression of EBNA-1 is associated with a consistent enhanced CD25 expression, compared with CD25 levels in vector control cells. The CD25 expression level on vector-transfected cells ranged from 0.2 to 2.0 with an average mean fluorescence level of 1. In the case of EBNA-1-expressing L428 cells, the expression level of CD25 ranged from 0.2 to 33.8. These cell clones could be divided into two subgroups. Five of fifteen EBNA-1-expressing cell clones were characterized by CD25 expression levels which were comparable to that of control vector-transfected cells (mean fluorescence, 0.2, 0.9, 1.2, 1.6, and 2.0). However, 10 of 15 EBNA-1-expressing L428 clones showed CD25 expression levels which were higher than those in mock-transfected cells. There was a statistically significant difference between CD25 expression levels in EBNA-1-expressing cells and those in control clones as shown by the Mann-Whitney test ($P = 0.001$). One EBNA-1-positive clone (L428E1P MG5) showed a high level of EBNA-1 expression without significant CD25 expression. This could have been due to secondary effects during cell culture. However, the enhanced CD25 surface level correlated in the majority of clones with the level of EBNA-1 expression.

To determine whether L428 cells expressed the high-affinity IL-2R complex consisting of the $\alpha$ chain (CD25), the $\beta$ chain (CD122), and the $\gamma$ chain, we performed surface immunofluorescence stainings for these antigens. EBNA-1-positive and vector control clones showed low staining for the $\beta$ chain, while the $\delta5 \gamma$ chain was constitutively expressed in all L428 clones (Fig. 2A). Since the expression of the $\beta$ chain and the $\delta5 \gamma$ chain of the IL-2R in L428 cells was not altered by EBNA-1, this further underlined the specific effect of EBNA-1 on CD25 expression. To determine whether the increase on the CD25 surface level was associated with changes in IL-2R $\alpha$-chain mRNA levels, total cellular RNA from different transfected clones was analyzed by semiquantitative reverse transcription-PCR (RT-PCR) as previously described (37). L428 cells expressing EBNA-1 contained increased IL-2R $\alpha$-chain mRNA levels compared with vector control cells (Fig. 2B).

CD25 activation by EBNA-1 in L428 cells was also analyzed in a transient assay to confirm that the up-regulation of CD25 was EBNA-1 specific. After cotransfection of pMACS 4.1 (which encodes the extracellular domain of CD4 as a selection antigen) and pSG5 or pSG5-EBNA-1, we enriched transfected cells through immunomagnetic separation of CD4-positive cells with a magnetic cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany). Our results demonstrate that EBNA-1-positive cells express CD25 at up to twofold-higher levels than do mock-transfected L428 cells (Fig. 3). This upregulation of CD25 by EBNA-1 in L428 cells was maximal at 120 h after magnetic sorting.

The high levels of CD25 antigen on stable EBNA-1-express-
ing cells were lost during prolonged cultivation of transfected cells even when the EBNA-1 expression was unchanged in these clones and the cells were grown under permanent selection (Table 3). Therefore, increased CD25 levels in short-time-cultivated stably EBNA-1-expressing cells might reflect a high activation status of these cells. The loss of enhanced CD25 expression suggests a selection process in cell culture which may be directed against the expression of EBNA-1-induced genes. Another possibility is an enhanced shedding of CD25. It has been reported elsewhere that CD25 can be shed from the cell surface and can be detected in serum of HD patients and NPC patients (10, 15). Therefore, we analyzed soluble CD25 in

![FIG. 2. Expression of IL-2R molecules in L428 cells expressing EBNA-1. (A) Surface phenotype analysis of L428 clones for CD25–IL-2R α chain (a), CD122–IL-2R β chain (b), and IL-2R γ chain (c). EBNA-1-expressing L428 cells or vector control cells were stained with isotype controls and corresponding PE-conjugated antibodies directed against CD25, CD122, and IL-2-γ chain. Vector control cells were stained with isotype control (dashed outline and no shading). EBNA-1-expressing clones were stained with isotype control (solid outline with shading) or with specific antibody (solid outline and no shading). In this analysis, the clones L428KoP N6 and L428E1P F1 were used. (B) Semiquantitative RT-PCR of total RNA isolated from L428 cells expressing EBNA-1 or vector-transfected control cells. RNA was reverse transcribed, and defined increasing amounts of cDNA were amplified with both glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific oligonucleotides to normalize the amount of cDNA and IL-2R α-chain-specific oligonucleotides. In both reactions, a control vector at fixed amounts carrying synthetic binding sites for glyceraldehyde-3-phosphate dehydrogenase–IL-2R α-chain oligonucleotides was included (37). For this analysis, clones L428KoP N1, L428KoP N6, L428E1P F1, L428E1P E1, and L428E1P F4 were used (see also Table 2).]

![FIG. 3. Expression of CD25 on the cell surface of transient-transfected L428 cells with EBNA-1 (shaded bars) or vector control (unshaded bars) and pMACS4.1 after MACS separation. The fraction of CD25-positive cells was evaluated 24, 96, and 120 h after MACS separation. A representative experiment of three independent transfection experiments and MACS enrichments is shown.]

<table>
<thead>
<tr>
<th>Transfected clone</th>
<th>CD25 expression at passage</th>
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<tbody>
<tr>
<td></td>
<td>Early</td>
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<tr>
<td>L428E1P E1</td>
<td>21.6</td>
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<tr>
<td>L428E1P E2</td>
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<td>L428E1P F1</td>
<td>33.8</td>
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<tr>
<td>L428E1P F2</td>
<td>9.9</td>
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<tr>
<td>L428E1P F4</td>
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*Expression of CD25 antigens on EBNA-1-expressing L428 clones was analyzed by flow cytometry. The data are presented as mean channel numbers for fluorescence intensity of one representative experiment of five. The mean fluorescence was calculated by using the Becton Dickinson FACScan software. Early passage, 8 to 10 weeks after transfection; intermediate passage, 10 to 20 weeks after transfection; late passage, later than 6 months.*
cell culture supernatants by enzyme-linked immunosorbent assay (Amersham Life Science). No soluble CD25 was detected in the supernatants of EBNA-1-positive cells, revealing that the activation of CD25 by EBNA-1 and following subsequent decrease of surface CD25 was not associated with increased levels of soluble CD25 in the supernatants (data not shown).

This EBNA-1-associated up-regulation of CD25 implies the possibility of IL-2 binding to L428 cells expressing this EBV gene and activating them. To analyze whether IL-2 can preferentially stimulate EBNA-1-positive L428 cells, the cells were incubated with and without recombinant human IL-2 (100 U/ml) (Gibco Life Technologies) for up to 96 h. \(^{3}H\)thymidine incorporation revealed no effect of IL-2 on the proliferation of EBNA-1-positive or -negative L428 cells, while freshly isolated T cells proliferated after addition of IL-2 (data not shown). Since the IL-2R \(\beta\) chain was expressed at low levels in all L428 cell clones analyzed, the effect of IL-2 on the EBNA-1-expressing L428 cells is probably mediated only as a low-affinity signal (14, 19), so that signals of IL-2 are possibly nonmitogenic.

**FIG. 4.** Lymphoma in NOD-SCID mice developed within 5 weeks after injection of EBNA-1-expressing L428 cells (L428E1P F1). (a) Lymphoma developed in the pancreas of a NOD-SCID mouse. The infiltrate has a sharp border towards the pancreas (magnification, \(\times 100\)). (b) Tissue sections were stained for expression of the HD-associated antigen CD30, showing a strong expression on the cell surface (magnification, \(\times 600\)).

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**Notes:**

- Cell culture supernatants by enzyme-linked immunosorbent assay (Amersham Life Science).
- No soluble CD25 detected in EBNA-1-positive cells.
- Activation of CD25 by EBNA-1 not associated with increased soluble CD25 in supernatants.
- EBNA-1-associated up-regulation of CD25 implies IL-2 binding and activation.
- IL-2 does not stimulate EBNA-1-positive or -negative L428 cells.
- IL-2R \(\beta\) chain expressed at low levels.
- Signaling of IL-2 likely as a low-affinity signal.
Cytokine activation by EBNA-1. It has been shown that HD cell lines express a large number of cytokines (14, 19, 29, 30). To investigate whether EBNA-1 can influence cytokine expression, we analyzed the expression of IL-6, IL-10, and LT-α by semiquantitative RT-PCR and enzyme-linked immunosorbent assay. In some but not all EBNA-1-positive clones (5 of 15), enhanced production of IL-6 was detected (800 pg/ml within 48 h compared to 150 pg/ml produced by the control cells), which reveals no direct involvement of EBNA-1. No significant changes were observed for IL-10 (negative) and LT-α (unchanged high-level expression), which are induced by EBV in Burkitt’s lymphoma (14, 20).

Enhanced lymphoma growth by L428 cells transfected with EBNA-1 in mice. The parental cell line L428 is nontumorigenic in SCID mice (34). To test the influence of EBNA-1 on the tumorigenicity of L428 cells, 10^7 cells of EBNA-1 and control transfected L428 cells were injected intraperitoneally into five SCID mice. Mice were sacrificed after 10 weeks. No tumor growth was detected at this time and even when the mice were kept for longer times. However, with NOD-SCID mice 5 of 15 animals developed lymphomas within 5 weeks when EBNA-1-positive cells were inoculated, whereas with vector control-transfected L428 cells no lymphoma development was observed (0 of 15 animals analyzed). In addition, L428 cells expressing LMP-1 (0 of 5 animals analyzed) or EBNA-2 (0 of 5 animals analyzed) did not cause tumor growth in NOD-SCID mice.

The lymphomas that developed from EBNA-1-expressing L428 cells were characterized by a diffuse infiltration in various organs (pancreas, liver, bowel, kidney, skin, and muscle) (Fig. 4a) with characteristics of H-RS cells, and many mitotic and apoptotic cells were present; however, the bystander cell population typical for HD was missing due to the absence of T and B cells in the mice. Immunohistochemical examination revealed a high CD30 expression on the lymphoma cells (Fig. 4b). Our result indicates that EBNA-1 can promote lymphoma growth in this animal system. The enhanced tumorigenicity of EBNA-1-positive L428 cells is compatible with results obtained in a transgenic mouse model where it was shown that the expression of EBNA-1 in B cells induced malignant B-cell lymphomas, frequently of follicular-center cell origin (39) demonstrating a tumorigenic potential for EBNA-1. However, the expression level of EBNA-1 detected in L428 cells in vitro was up to 15-fold higher than that in the LCL, suggesting that a high level of EBNA-1 is needed to induce phenotypic changes or tumor growth. In addition, primary H-RS cells and HD cell lines represent in most cases a population of germinal-center B cells with a high degree of preactivation of surface antigens and cytokine expression and thus represent a different status of activation compared to Burkitt’s lymphoma cells. When expressed in Burkitt’s lymphoma, EBNA-1 has no effects on the surface phenotype, while the expression of the recombinase-activating genes RAG1 and RAG2 seems to be associated with EBNA-1 expression in some but not all analyzed Burkitt’s lymphoma lines. In the Burkitt’s-like lymphoma line BJAB or in different LCLs, RAG1 and RAG2 expression was not detected (21, 28). Thus, the effects of EBNA-1 are probably different in several cell types, depending on the differentiation stage and the preactivation of the cells (38).

The description of these specific effects of EBNA-1 on HD cells now provides an opportunity to dissect the mechanism by which EBV alters gene expression in HD and thus may contribute to the transformation and immune escape mechanisms of the transformed H-RS cells.

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