The Epstein-Barr Virus (EBV) is associated with a variety of lymphoid and epithelial neoplasms, including Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), posttransplant lymphoproliferative disorders (PTLD), and undifferentiated nasopharyngeal carcinomas (NPC). In these tumors, monoclonal viral genomes have been identified in virtually all tumor cells indicating that EBV infection takes place early in the neoplastic process and suggesting an important role for the virus in tumor development. The nature of the contribution of EBV, however, remains uncertain. Studies of EBV gene expression in virus-carrying cell lines and in human tumor biopsy specimens have identified three distinct forms of virus latency. Lymphoblastoid cell lines (LCLs), generated by in vitro immortalization of B lymphocytes with EBV, express an array of virus-encoded proteins including six nuclear antigens (EBNA1, 2, 3A, 3B, 3C, -leader protein [LP]) and three latent membrane proteins (LMP1, 2A, 2B). The small EBV-encoded nonpolyadenylated nuclear RNAs (EBERs) are expressed. This form of latency, termed latency I, is also encountered in some posttransplant lymphoproliferative disorders. In EBV-positive cases of HD, the EBERs, EBNA1, and the LMPs are expressed (latency II), whereas in Burkitt’s lymphoma (BL) only the EBERs and EBNA1 have been detected (latency I). We have studied the expression of EBV proteins in 17 cases of EBV-positive endemic BL by immunohistology. Expression of LMP1 was seen in variable proportions of tumor cells in two cases and EBNA2 was detected in some tumor cells in three other cases. Also, the BZLF1 trans-activator protein was expressed in a few tumor cells in 6 cases, indicating entry into the lytic cycle. A phenotypic drift from latency I to latency III has been observed previously in some BL cell lines. Our results suggest that a similar phenomenon may occur in BL in vivo and indicate that the operational definition of EBV latencies is not easily applied to human tumors.

The clinicopathologic data are summarized in Table 1. All cases were from areas in Africa where Burkitt’s lymphoma is endemic (8 from Uganda, 9 from Malawi) and collected between 1972 and 1994. Biopsy specimens were from children, aged between 2 and 16 years. The clinicopathologic data are summarized in Table 1. All cases showed histologic features characteristic of Burkitt’s lymphoma. The clinicopathologic data are summarized in Table 1. All cases showed histologic features characteristic of Burkitt’s lymphoma.
munostaining with the CD20 monoclonal antibody (MoAb), L26, confirmed a B-cell phenotype of the tumor cells in all cases. Staining with a CD3-specific rabbit antiserum (Dako, High Wycombe, UK) showed the presence of small numbers of scattered reactive T lymphocytes admixed with the tumor cells in all samples.

**Immunohistology.** The MoAb, Ber-H2, directed against the CD30 lymphocyte activation antigen and the bcl2-specific MoAb, 124, were obtained from Dako. For the detection of LMP1, the MoAbs CS1, CS2, CS3, and CS4 were used either alone or in a commercially available combination (Dako). In addition, a newly produced murine IgG1 subclass LMP1-specific MoAb, LMPO24, was used. This antibody was generated by immunizing BALB/c mice with purified plasma membranes from LMP1-expressing insect cells that were infected with a recombinant LMP1 baculovirus. The recombinant baculovirus was a kind gift of Dr F. Gräser (Universitätsklinikum des Saarlandes, Homburg, Germany) and has been described previously.34 Spleen cells from an immunized mouse were fused with the nonsecreting myeloma fusion partner, Sp2/0-Ag14; several hybridomas were found to produce antibodies reactive with LMP1 in fixed cell smears and in Western blotting assays. The LMPO24 clone was selected for use in this study because of its strong and specific reactivity in immunohistochimical staining of paraffin-embedded tissues. The MoAb, PE2, was used for the detection of the EBNA2 protein, and the BZ1 MoAb was employed for the detection of the BZLF1 trans-activator protein.35 All MoAbs were applied to paraffin-sections following antigen retrieval as described.31 In brief, dewaxed and rehydrated paraffin sections were subjected to microwave irradiation in 1 dm³ of 0.01 mol/L citrate buffer for 40 minutes at 750 W. Sections were then stained using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method as described.31

**In situ hybridization.** The plasmids pBSJJ11 and pBSJJ22 harboring inserts specific for EBER1 and EBER2, respectively, and the plasmid pBSW containing the BamHI W fragment of the EBV genome were employed in this study.32 Following digestion with suitable restriction enzymes, single stranded RNA probes were generated from these plasmids in sense and antisense direction using T3 and T7 RNA polymerases (Gibco-BRL, Paisley, UK) and 35S-dUTP (> 1000 Ci/mmol, Amersham, Little Chalfont, UK) as described.

The antisense probes derived from the plasmids pBSJJ11 and pBSJJ22 were mixed to increase sensitivity, and the corresponding sense probes were also mixed. In situ hybridization was performed overnight as described, using a hybridization mixture containing 50% deosinated formamide, 2 × SSC (0.3 mol/L NaCl, 0.03 mol/L sodium citrate, pH 7.6), 10% dextran sulphate, 10 mmol/L dithiothreitol (DTT), 0.2 mg/mL yeast tRNA, and 10³ cpm of labeled probe per slide. After hybridization, slides were washed for at least 4 hours in 5% deosinated formamide, 1 × SSC, 10 mmol/L DTT at 52°C, rinsed in 0.1 × SSC, and dipped in a 1:2 dilution of Ilford G5 emulsion. Slides were exposed at 4°C for 3 to 10 days, developed, fixed, and counterstained.

**Controls.** Paraffin sections of a tonsil from a patient with acute infectious mononucleosis (IM) were used as positive control in all experiments. EBER in situ hybridization showed numerous EBV-infected lymphoid blasts in extranodal areas as described previously.35 A large number of cells expressing mRNA derived from the BamHI W fragment of the EBV genome was also seen in the same areas. Immunohistology with the CS1-4 mixture of MoAbs and with the MoAb, LMP024, showed many LMP1-positive large lymphoid blasts. Similarly, many cells showing an EBNA2-specific staining were seen with the MoAb, PE2. Staining with the BZ1 MoAb showed scattered smaller lymphoid cells expressing the BZLF1 protein of EBV.

Preliminary experiments with the CS1, CS2, CS3, and CS4 MoAbs on sections from an IM tonsil showed large numbers of LMP1 expressing lymphoid blasts with each individual MoAb as seen with the CS1-4 mixture. However, the CS3 and particularly the CS4 MoAbs produced a strong staining of many nuclei and a cytoplasmic labeling of endothelial cells in addition to the specific staining. This has been occasionally observed previously with the CS1-4 mixture.35 Serial dilution of the CS3 and CS4 MoAbs showed that at tissue culture supernatant dilutions of about 1:500, the nuclear staining had disappeared almost completely, while the specific staining of EBV-infected lymphoid blasts was still clearly visible. A weak staining of endothelial cells remained, but this did not interfere with the interpretation of results. The CS1, CS2, and LMP024 MoAbs did not show any background staining at dilutions of 1:50.

**RESULTS**

**EBV status of tumors.** In situ hybridization with the EBER1- and EBER2-specific probes showed the presence of EBV in virtually all tumor cells of all 17 BL cases (Fig 1 and Table 1).

**LMP1 expression.** Immunohistology with the CS1-4 mixture of MoAbs showed labeling of a substantial number of tumor cells in one case (Fig 2A and case no. 7 in Table 1). Positive cells tended to show focal aggregation. Because of the focal distribution of these cells the proportion of labeled tumor cells was difficult to assess. While in some areas up to 40 CS1-4-positive cells were found per high power field (Fig 2A), the vast majority of tumor cells was unlabeled. The proportion of CS1-4-positive cells was estimated to be less than 1% of the total tumor cell population. Another case (case 17 in Table 1), displayed very few cells labeled with the CS1-4 MoAbs, again representing well under 1% of all tumor cells. To assess the specificity of this staining result, the CS1, CS2, CS3, and CS4, and LMP024 MoAbs were applied separately to these cases. All five antibodies showed the same staining pattern in both cases confirming the specificity of the staining result (Fig 2B). While the majority of LMP1-expressing cells shared the morphologic features of BL tu-

<table>
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Abbreviations: M, male; F, female; +, virtually all tumor cells positive; +/-, small proportion of tumor cells positive; +/sc, isolated scattered tumor cells positive; -, negative.
Fig 1. In situ hybridization with radiolabeled EBER-specific probes shows accumulation of silver grains over virtually all tumor cell nuclei of this Burkitt's lymphoma, indicating the presence of Epstein-Barr virus.

In tumor cells, some cells had enlarged nuclei with prominent nucleoli.

**EBNA2 expression.** Immunohistology with the EBNA2-specific MoAb, PE2, resulted in nuclear staining of some tumor cells in three cases (Fig 3A and B and Table 1). The largest number of EBNA2-positive cells was observed in case no. 3. In this case, labeled cells showed a focal distribution pattern and staining was often weak, making assessment of the number of EBNA2-expressing cells difficult. However, the vast majority of tumor cells did not show EBNA2-specific staining and thus the overall proportion of stained cells was small (<1%). Only very few scattered cells labeled with the PE2 mAb were seen in the other 2 cases.

Notably, these 3 cases did not show any LMP1-expressing cells (Table 1). Most PE2-positive cells shared the morphology of tumor cells, but a few isolated cells with a more immunoblast-like appearance were noticed in 1 case. To ascertain the specificity of this staining, in situ hybridization with RNA probes derived from the plasmid pBSW was performed on these 3 cases. This probe detects mRNA transcripts derived from the BamHI W fragment of the viral genome, which are transcribed in type III latency, and are part of the primary transcript encoding for EBNA2, as well as the other EBNAs. Scattered cells expressing this RNA were detected in one of the three cases with PE2-positive cells (not shown).

**BZLF1 expression.** Using the BZLF1-specific MoAb, BZ1, nuclear staining of isolated tumor cells was observed in 6 BL cases (Fig 4 and Table 1). Invariably, these cells showed the morphologic features of BL tumor cells (Fig 4).

**CD30 and bcl2 expression.** Staining with the MoAb Ber-H2 of 16 cases showed a few CD30-positive lymphoid cells in 1 case (case no. 5 in Table 1), and this case also displayed some EBNA2-positive tumor cells. However, the CD30-expressing cells had the morphologic features of lymphoid blasts and were located at the edge of the tumor and thus probably represented reactive lymphoid cells rather than transformed tumor cells. The 5 cases containing LMP1 or EBNA2 expressing cells were stained with a bcl2-specific MoAb, 124. In all 5 cases, bcl2 expression was restricted to small lymphoid cells. No bcl2 expression in tumor cells was observed.
Fig 3. Immunohistology with the MoAb PE2 shows expression of EBNA2 in isolated tumor cells (arrows) of Burkitt's lymphoma case no. 5 (A) and case no. 3 (B) using the APAAP technique.

Fig 4. A single tumor cell is seen expressing the BZLF1 protein of EBV as detected by APAAP immunohistochemistry with the MoAb BZ1 (case no. 16).
DISCUSSION

In this study of 17 EBV-positive endemic BL cases, variable but small numbers of LMP1-expressing cells were observed in 2 cases and isolated EBNA2-positive tumor cells were detectable in 3 other cases. In the remaining 12 cases, EBV latent gene expression was restricted to the EBERs, consistent with a type I latency. A few isolated BL cells expressing the BZLF1 trans-activator protein of EBV were observed in 6 cases. This protein induces the switch from latent to replicative EBV infection. Thus, expression of the BZLF1 protein suggests that a small minority of BL cells in some cases may enter into the lytic cycle. This is in keeping with the occurrence of occasional virus-producing cells in some BL cell lines and with the recent demonstration using Southern blot hybridization of replicating EBV DNA in some BL biopsy specimens.34

The detection of some EBNA2- and LMP1-positive cells in a proportion of endemic BL cases was unexpected in the light of previous studies consistently demonstrating a type I latency in BL.17,18 However, these studies have employed methods based on the extraction of proteins and nucleic acids, such as Western or Northern blotting. While these techniques allow the analysis of EBV gene expression in the majority cell population, they may not be sufficiently sensitive to detect different expression patterns in small fractions of the total cell population. Also, these studies have examined mainly early passage BL cell lines and thus the number of BL biopsy specimens analyzed for EBV gene expression reported in the literature is very small.

Several control experiments were performed to assess the specificity of our immunostaining results. The 2 cases with LMP1-positive cells as detected using the CSI-4 mixture of MoAbs were examined using these four MoAbs separately and also with a newly generated LMP1-specific MoAb, LMP024. Identical staining patterns were obtained in both cases with all five MoAbs. In 1 of the 3 cases displaying scattered cells labeled with the EBNA2-specific MoAb, small numbers expressing RNAs derived from the BamHI W fragment of the EBV genome were identified. These transcripts are part of the EBNA2 mRNA expressed in Lat III.9 Thus, these control experiments provide additional evidence for the specificity of our immunostaining results.

Expression of LMP1 may occur during lytic infection in B cells.35 However, in our cases, there was no correlation between LMP1 and BZLF1 expression (Table 1). A few BZLF1-positive BL cells were seen in the case showing the most LMP1-positive cells (case no. 7, Table 1), but these were infrequent in comparison to the LMP1-positive cells and they also showed a different pattern of distribution. Thus, the presence of LMP1-positive cells in these two biopsy specimens clearly reflects the expression of LMP1 in the context of latent infection.

The expression of LMP1 has been demonstrated previously in 3 of 11 EBV-positive acquired immunodeficiency syndrome (AIDS) related BL cases.36 Thus, the detection of LMP1-positive cells in our cases no. 7 and 17 (Table 1) raises the question as to the possibility of human immunodeficiency virus (HIV) infection in these patients. However, the biopsy specimen from case no. 7 was obtained in 1982 before the outbreak of the AIDS epidemic. This tumor had developed in an ovary of a young girl. In case no. 17, the tumor had developed in the mandible of a 12-year-old girl in 1994. Therefore, both cases would seem to represent typical examples of endemic BL. A recent autopsy study from Cote d'Ivoire has also concluded that BL is not a common AIDS-associated complication in Africa, particularly in children.37

Thus, while we cannot definitely rule out this possibility, it seems very unlikely that these cases represent AIDS-related lymphomas.

In 12 of our endemic BL cases, the immunohistologic results were in keeping with a tight Lat I form of EBV infection as described in previous studies.17,19 The detection of EBNA2 and LMP1 in some tumor cells of 5 cases demonstrates that forms of virus latency other than Lat I may be realized in endemic BL. It is interesting to note in this context that LMP1-positive cells were not detected in those cases showing EBNA2-expressing cells. Thus, it appears that in some BL cases, a proportion of tumor cells may display a Lat II that is characterized by the expression of EBNA1 and the LMPs only. A similar phenotype has been demonstrated by Hamilton-Dutoit et al36 in 3 of 11 EBV-positive AIDS-related Burkitt-type lymphomas. The expression of EBNA2 in the absence of LMP1 is more unusual, as EBNA2 has been shown to induce LMP1 expression in B cells.38 However, this phenotype has been observed previously in some posttransplant lymphomas.12 Moreover, this form of latency is clearly realized in a proportion of cells in PTLDs and AIDS-related lymphomas with Lat III where the number of EBNA2-positive cells may be much greater than that of LMP1-positive cells (unpublished observation). It has to be emphasized, however, that even in those 5 cases with LMP1- or EBNA2-positive cells, the vast majority of tumor cells exhibited a phenotype consistent with Lat I.

In vitro, some BL cell lines show a spontaneous phenotypic drift from Lat I to Lat III.17 Thus, the detection of LMP1 or EBNA2 in BL cells suggests that such a drift may also occur in vivo. Alternatively, it is possible that the BL cells expressing LMP1 or EBNA2 represent earlier stages in tumor development before the establishment of a tight Lat I. While the first possibility appears more likely, it is, at present, not possible to clarify this issue with certainty. A step in this direction would be a more detailed phenotypic characterization of BL cells expressing EBNA2 or LMP1 to see if the phenotypic changes associated with the drift from Lat I to Lat III in vitro are reflected in these tumor cells in vivo. Our preliminary analysis suggests that CD30, which is expressed in type III but not in type I BL cell lines,17 is not upregulated in EBNA2 or LMP1 expressing BL cells in vivo. Similarly, expression of the bcL2 oncogene was not observed in these BL cases. This is surprising in view of the recently demonstrated ability of LMP1 to upregulate bcL2 expression in BL cell lines.39 However, a few EBNA2- or LMP1-expressing cells were noticed that displayed an immunoblast-like morphology, and this problem will have to be addressed in more detail using frozen biopsies that will allow a broader range of antigens to be analyzed.

The phenotypic analysis of LMP1 or EBNA2 expressing
expression of EBNAZ in some tumor cells suggests a drift pattern prevailing in the majority cell population. Thus, the operational definition of different forms of EBV latency is possibilities will have to be addressed at the transcriptional level in future studies. Our observations demonstrate that in those cases showing EBNA2-positive cells, LMP1 expression was not observed. During the switch from Lat I to Lat III, EBNA2 expression precedes LMP1 expression. Thus, it seems possible that EBNA2-expressing BL cells are eliminated by CTLs before the EBNA2-induced upregulation of LMP1 expression can occur. If the relatively small number of T cells detected in BL biopsies is sufficient to mediate such an effect remains to be established. In summary, this is the first demonstration of EBV latent proteins other than EBNA1 in tumor cells of endemic BL. The expression of EBNA2 in some tumor cells suggests a drift towards Lat III, as seen in some BL cell lines in vitro. In other cases, expression of LMP1 has been observed without detectable EBNA2 expression, raising the possibility that BL cells may, under certain conditions, sustain Lat II. These possibilities will have to be addressed at the transcriptional level in future studies. Our observations demonstrate that in virus-associated human tumors, individual tumor cells may sustain a form of EBV latency that is different from the pattern prevailing in the majority cell population. Thus, the operational definition of different forms of EBV latency is not easily applicable to human tumors in vivo.

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REFERENCES


Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma

G Niedobitek, A Agathanggelou, M Rowe, EL Jones, DB Jones, P Turyaguma, J Oryema, DH Wright and LS Young