In Situ Demonstration of Epstein-Barr Virus Small RNAs (EBER 1) in Acquired Immunodeficiency Syndrome-Related Lymphomas: Correlation With Tumor Morphology and Primary Site

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Some acquired immunodeficiency syndrome (AIDS)-related lymphomas (ARLs) are infected with Epstein-Barr virus (EBV), although the frequency and importance of this association is disputed. Using paraffin section RNA in situ hybridization (ISH) with digoxigenin-labeled riboprobes, we screened 16 central nervous system (CNS) non-Hodgkin’s lymphomas (NHLs), 101 systemic NHLs, and 11 Hodgkin’s disease cases arising in human immunodeficiency virus-seropositive individuals for EBV-encoded small RNA (EBER 1) expression, an EBV gene product transcribed in abundance during latent infection. Tumor cells contained EBV in 85 of 128 ARLs (66%), but infection rates differed with lymphoma type. EBER 1 was expressed in tumor cells in 11 of 11 Hodgkin’s disease cases (100%), whereas other groups have found a higher incidence. A possible explanation for this discrepancy was suggested by a previous EBV-DNA ISH study performed by us. This study showed that the association of ARLs with EBV appeared to vary with morphologic type, with viral genomes being detectable in tumor cells in 65% of immunoblast-rich lymphomas compared with only 20% of Burkitt-type lymphomas. Larger-scale studies to confirm this finding have been hampered by the technical problems involved in detecting EBV in tissues. Paraffin section DNA-ISH using radiolabeled probes is a difficult and time-consuming technique, and may give rise to false-negative results; the demonstration of EBV-DNA by Southern blotting, and of EBV-associated antigens by immunocytochemistry, generally require frozen tumor tissues that are difficult to obtain in large numbers. However, it has recently been shown that an RNA-ISH technique for the detection of EBV-encoded small RNAs (EBERS) can be adapted for use in formalin-fixed, paraffin-embedded tissues. Unlike other EBV

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gene products, EBERs are transcribed in abundance (up to $10^7$ copies per cell) in all forms of EBV latency described to date. Thus, single-figure EBV genome copies can be detected in routine tissue sections using nonisotopic-ISH, making the technique an ideal tool for investigating the disease association of EBV.

In the present study, we examined 128 lymphomas arising in HIV-seropositive individuals for EBER 1-positive cells using a sensitive RNA-ISH technique with digoxigenin-labeled riboprobes. We found a significant correlation between the presence of latent EBV in tumor cells and the morphology and primary site of the lymphomas.

MATERIALS AND METHODS

Tissues. Cases were drawn from a larger unselected series of lymphomas arising in HIV-seropositive patients collected by the French-Danish Study Group on the Pathology of AIDS-Related Lymphomas. The study tumors were chosen at random, with the only criteria for inclusion being the availability of paraffin-embedded tissue for analysis. The 128 ARLs consisted of 117 NHLs and 11 cases of HD. NHLs were classified according to the Kiel classification, modified to include ARL variants as described previously. Equivalent terms from the Working Formulation (WF) have been given where appropriate.

Probes. EBER 1-specific fragments from plasmid pJJ1, kindly provided by Dr. J. Arrand (Manchester, UK), were subcloned into the pBluescript KS vector (Stratagene, La Jolla, CA). In vitro transcription of linearized plasmid template was performed in the presence of digoxigenin-labeled UTP, using either T7 or T3 RNA polymerases, to produce single-stranded digoxigenin-labeled RNA probes complementary (antisense probe) or anticomplementary (sense, negative control probe) to EBER 1 RNA transcripts, as described.

ISH. EBER 1 was shown by RNA/RNA-ISH as described in full elsewhere. Paired paraffin sections from each case were mounted on treated glass slides, enabling test (antisense probe) and control (sense probe) sections to be processed in parallel. After pretreatment with 0.2 N HCl (15 minutes at room temperature) and 20 µg/mL proteinase K (30 minutes at 37°C), Boehringer, Mannheim, Germany), approximately 20 µL of hybridization mixture, consisting of digoxigenin-labeled riboprobe (2 ng/µL), 50% deionized formamide, 2X SSC (1X SSC = 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate, pH 7.6), 10% dextran sulphate, and 200 µg/mL tRNA, was applied per section. After hybridization overnight at 50°C, excess probe was removed by high stringency washing in 50% formamide/0.2X SSC for 60 minutes at 37°C and sections were treated with 20 µg/mL RNase A (Boehringer) for 30 minutes at 37°C. Hybrids were detected by a five-step alkaline phosphatase, antialkaline phosphatase (APAAP) procedure using monoclonal antidigoxin (Sigma, St Louis, MO) and with new fuchsin substrate. Some cases were also evaluated for EBV-DNA using a previously described ISH technique with 32P-labeled EBV BamHI W fragments.

Controls. Paraffin-embedded pellets of EBV-infected lymphoid cell lines (Raji, B95-8, and P3HR1) and EBV-positive infectious mononucleosis and HD cases from HIV-seronegative individuals served as positive controls for EBER-ISH.

RESULTS

The NHLs were all high-grade diffuse lymphomas. Immunophenotyping (data not shown) identified a single T-cell lymphoma of anaplastic large-cell type (previously reported in Pallesen et al); the remainder were of B-cell origin, except for seven cases in which cell lineage could not be established in paraffin section. Sixteen lymphomas showed primary parenchymal CNS involvement. In 14 cases, the lymphoma was confined to the brain; the other 2 cases presented with primary brain lymphoma that subsequently spread to sites outside the CNS. Cases of disseminated lymphoma with secondary brain involvement were not included in this group. Most of the CNS lymphomas were of immunoblastic type, often with pronounced polymorphism and plasmacytic differentiation (WF: large-cell immunoblastic, plasmacytoid); a single CNS lymphoma showed polymorphic B-cell proliferation (WF: not recognized). The systemic ARLs fell into a variety of histologic categories (Table 1). However, most could be assigned to one of two broad morphologic groups, as described previously. One group is immunoblast-rich/large-cell lymphomas. These consisted primarily of lesions with many immunoblasts or their variants, often showing pronounced tumor-cell polymorphism. Included in this group were immunoblastic lymphomas (WF: large-cell immunoblastic, plasmacytoid); polymorphic centroblastic lymphomas, (WF: diffuse large noncleaved cell); anaplastic large-cell lymphomas (WF: not recognized); and polymorphic B-cell proliferation (WF: not recognized). The second group is Burkitt-type lymphomas. These included typical Burkitt's lymphomas and cases with atypical features (Burkitt-like lymphomas) (WF: small noncleaved cell, Burkitt and non-Burkitt types).

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Table 1. EBV-Encoded EBER 1 Expression in Tumor Cells of ARLs (n = 128)

<table>
<thead>
<tr>
<th>Histology*</th>
<th>No. of Cases Tested</th>
<th>EBER 1 Positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS lymphoma†</td>
<td>16</td>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td>Systemic lymphoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB, monomorphic (WF: DLNCC)</td>
<td>6</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>CB, polymorphic (WF: DLNCC)</td>
<td>8</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>PBP (WF: NR)</td>
<td>6</td>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>IBL (WF: RBC)</td>
<td>44</td>
<td>36</td>
<td>82</td>
</tr>
<tr>
<td>ALCL (WF: NR)</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>HD-type (WF: SNCC)</td>
<td>35</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>HD</td>
<td>11</td>
<td>11</td>
<td>100</td>
</tr>
</tbody>
</table>

Total ARLs | 128 | 85 | 66 |

Abbreviations: ALC, anaplastic large cell; BL, Burkitt; CB, centroblastic; DLNCC, diffuse large noncleaved cell; IB, immunoblastic; IBL, large-cell immunoblastic, plasmacytoid; NR, not recognized; PBP, polymorphic B-cell proliferation; SNCC, small noncleaved cell.

* Kiel classification with modifications. Equivalent terms from the WF are given in parentheses.
† Primary CNS lymphoma, confined to the brain (n = 14) or with secondary spread outside the CNS (n = 2).
‡ Including one ALC of T-cell type.
EBER 1 expression in AIDS-related lymphomas

EBER 1 expression was detected in 46 of 60 immunoblast-rich/large-cell lymphomas (77%), but in only 12 of 35 Burkitt-type lymphomas (34%), with this difference being significant ($\chi^2$ test, $P = .0001$). Within each of the groups, no morphologic differences could be discerned between EBV-positive and EBV-negative tumors. However, among immunoblast-rich/large-cell lymphomas, there was a trend for EBV to be associated with extranodal disease. Thus, 29 of 35 extranodal (83%) and 17 of 25 nodal (68%) immunoblast-rich/large-cell lymphomas were EBER 1 positive ($\chi^2$ test, $P = .3$). With only rare exceptions (see below), EBER 1 was detected in essentially all viable, identifiable tumor cells in EBV-positive ARLs, irrespective of morphologic type. However, in many cases, there was clearly variation in the strength of signal (and, by implication, in the level of EBER 1 gene expression) between tumor cells within technically adequate areas of the sections. Differences in patterns of EBER 1 staining were primarily a reflection of the number and type of virus-negative reactive cells associated with the individual lesions. All EBV-positive Burkitt-type ARLs showed

![Image](https://via.placeholder.com/150)

**Fig 1.** Detection of EBER 1 expression by RNA-ISH in paraffin sections of ARLs (A through D). (A) Primary CNS immunoblastic lymphoma. (Left) EBV-positive tumor cells surround blood vessels and infiltrate cerebellum. (Right) At higher power, strongly stained lymphoma cells can be distinguished from smaller EBER-negative cerebellar granular cells. (B) EBV-positive Burkitt-type lymphoma. All tumor cells express EBER 1. No signal is seen over adjacent connective tissue. (C) HD, mixed cellularity. (Left) HRS cells express EBER 1. (Right) At higher power, a Reed-Sternberg cell shows strong nuclear EBER 1 reactivity with relative nucleolar sparing. (D) EBV-negative centroblastic lymphoma. EBER 1 expression was seen in very rare reactive lymphocytes, two of which are illustrated. Note the absence of staining in the surrounding lymphoma cells.
the same, homogeneous pattern, with sheets of EBER 1-positive tumor cells interspersed with low numbers of virus-negative starry-sky macrophages and other reactive cells (Fig 1B). A similar pattern was seen in some immunoblastic lymphomas. Other immunoblast-rich/large-cell lymphomas, particularly the more polymorphic lesions, showed an apparently heterogeneous EBER 1 pattern caused by the presence of scattered groups of EBV-positive tumor cells separated by extensive reactive infiltrates. Again, all recognizable lymphoma cells in these lesions appeared to carry the virus. In two technically adequate cases (one immunoblastic and one polymorphic centroblastic lymphoma), patterns of EBV expression differed from those described above, with EBER 1 positivity being confined to a minority (some 10%) of tumor cells. All 11 AIDS-related HD cases contained EBER 1-positive Hodgkin and Reed-Sternberg (HRS) cells (Fig 1C). Although there was variation in the total number of viral-positive tumor cells, all identifiable HRS cells appeared to be labeled in technically adequate sections.

ARLs with EBER 1-negative lymphoma cells frequently contained small non-neoplastic EBER 1-positive lymphocytes, either within the tumor itself (Fig 1D) or, especially, in adjacent benign nodal tissue when this was included in the tumor block. This finding not only attested to the sensitivity of the EBER-ISH method, but also provided an internal positive control of the technique in many cases. Although these cells were usually rare, larger numbers could be detected in occasional cases. However, they always represented less than 0.1% of all cells present, and this pattern was clearly different from that seen in the two ARLs described above in which a minor (but substantial) population of EBV-positive tumor cells was found. The viral copy number in these reactive cells was below the lower limit of detection using DNA-ISH.

All cases previously found to contain EBV-DNA by ISH were also positive for EBER 1 (data not shown). In addition, EBER 1 expression was detected in five ARLs that had been either technically unsatisfactory or (false) negative when analyzed for EBV-DNA by isotopic ISH. Fourteen of the 17 NHLs had been previously analyzed for EBV-DNA in frozen tissue using Southern blotting (data not shown). These results were in agreement with those of EBER-ISH in 13 of the cases, with the exception being a Burkitt-type ARL negative for both EBV-DNA and EBER 1 by ISH, but apparently positive for EBV-DNA by Southern blotting. Scattered EBER 1-positive reactive cells were clearly identifiable in this lymphoma, suggesting that the Southern blot result did not reflect virus in tumor cells.

**DISCUSSION**

There is good evidence that some ARLs contain EBV, although there has been disagreement as to the frequency of this association. In the present study, we have made use of a highly sensitive ISH technique to screen a large series of ARLs for EBER 1 expression, an EBV gene product transcribed in abundance during latent infection. We identified the virus in tumor cells in 85 of 128 ARLs (66%), but found clear differences in tumor-cell viral carriage rates according to lymphoma type. EBV was present in all 11 cases of HD (100%), in 15 of 16 cases of CNS NHL (94%), and in 46 of 60 systemic immunoblast-rich/large-cell lymphomas (77%). In contrast, tumor-cell EBV was found in only 12 of 35 Burkitt-type (WF; small noncleaved cell) lymphomas (34%) and in 1 of 6 monomorphic centroblastic (WF; diffuse large noncleaved cell) lymphomas (17%). These results are in keeping with our previous findings from a smaller ISH survey of EBV-DNA in ARLs.2

Both we and other groups have confirmed the sensitivity and specificity of EBER-ISH performed with riboprobes.17,20,21,23 The high target copy number allows the use of nonradioactive probe labels, making the technique ideal for rapid and accurate screening of many paraffin-embedded specimens for latent EBV infection. Comparison of the technique used in this study with Southern blot analysis of frozen tissue for EBV-DNA suggests that EBER-ISH is equally, if not more, sensitive at detecting the virus. In addition, ISH provides useful topographic information concerning the nature of the infected cells in an EBV-positive lesion, which is not available with destructive techniques such as standard polymerase chain reaction (PCR). Indeed, many of the EBV-negative ARL tissue blocks could be shown to contain EBER 1-positive small benign lymphocytes (corresponding to the latently infected small lymphoid cells present in the peripheral blood and tissues of normal EBV-seropositive individuals) that might have been a source of false-positive results if PCR had been used to detect tumor-cell EBV.

Previous DNA-ISH studies of ARLs have described a variety of patterns of lymphoma cell EBV positivity, ranging from cases in which all tumor cells carry the virus to cases in which only a fraction of cells in the lesion appear to be infected.2,16,22 Using EBER-ISH, it is apparent that in technically adequate sections essentially all recognizable tumor cells in the great majority of EBV-positive ARLs (of all morphologic types) are infected with the virus, although there is frequently variation in the strength of signal and, by implication, in the level of EBER 1 gene expression. Thus, the variable patterns of EBV infection described previously appear to be caused by low sensitivity of the DNA-ISH technique used combined with differences in the degree of non–tumor-cell infiltration in the various ARL types. That essentially all tumor cells in EBV-positive ARLs show viral gene expression indicates that infection probably preceded clonal expansion, suggesting a pathogenetic role for the virus in these tumors. The absence of EBV from all but a few of the nonmalignant lymphoid cells in each case provides further evidence that viral infection of tumor cells is not a coincidental event. Only in rare ARL cases did we find EBER 1 expression confined to a minor population of tumor cells. This finding implies that in these lesions (1) EBV gene transcription had been downregulated in most cells; (2) EBV genomes had been lost from a proportion of the cells during malignant progression; (3) infection had occurred after clonal expansion; or (4) the cases were in fact polyclonal or oligoclonal lesions in which EBV infection had not occurred in all clones.

Our study is in keeping with previous reports that have
found a close association between EBV and primary AIDS-related CNS lymphoma.\textsuperscript{17,25,26} In particular, our findings confirm those of MacMahon et al,\textsuperscript{17} who used a similar EBER-ISH technique to detect EBV in each of 18 cases of primary CNS-ARL. Taken together, these two series indicate that sensitive techniques are (with rare exceptions) able to detect EBV in the malignant cells of essentially all primary CNS-ARLs, providing strong circumstantial evidence that EBV is of pathogenetic importance in the development of these tumors. CNS involvement has also been reported to be disproportionately common in lymphomas arising in immunosuppressed allograft recipients,\textsuperscript{27} suggesting that parallels may exist between these tumors and CNS-ARLs. In contrast, EBV is only infrequently detected in tumor cells in primary CNS lymphomas arising in apparently immunocompetent individuals.\textsuperscript{28}

MacMahon et al\textsuperscript{17} have suggested that the pathogenesis of primary CNS lymphoma in AIDS may be distinct from that of other ARLs. Our findings indicate a rather more complex picture. Comparing CNS-ARLs with all systemic ARLs, a significant difference in the prevalence of tumor-cell EBV positivity was indeed found ($\chi^2$ test, $P < .02$). However, if CNS-ARLs are compared only with systemic immunoblast-rich/large-cell lymphomas, then the difference in EBV prevalence is reduced ($\chi^2$ test, $P > .2$). This reflects the highly significant differences found in EBV carriage rates comparing Burkitt-type lymphomas (which do not occur as primary brain tumors) with other ARLs. Moreover, in systemic immunoblast-rich/large-cell lymphomas, extranodal disease was particularly associated with EBV, with the frequency of viral infection approaching that of primary brain lymphomas. Thus, although primary CNS ARLs show a close association with EBV, there is evidence that the virus may play an equally important part in the development of a substantial proportion of systemic immunoblast-rich/large-cell lymphomas. In keeping with this finding, we believe that the tumor-cell morphology shown by EBV-positive primary CNS and systemic immunoblast-rich/large-cell ARLs is comparable. Furthermore, we have reported elsewhere that these two groups of tumors show a similar range of both EBV latent gene expression and tumor cell adhesion and activation molecule phenotype, suggesting that most CNS-ARLs and a major subgroup of systemic immunoblast-rich/large-cell lymphomas are primarily EBV-driven lymphoproliferations comparable to lymphomas arising in allograft recipients.\textsuperscript{13}

In contrast, tumor cells in most cases of Burkitt-type ARL do not contain EBV. Such tumors frequently show chromosomal translocation with c-myc gene rearrangement,\textsuperscript{14} but the nature of other factors involved in their development remain unknown. We have previously proposed that an analogy may be drawn between Burkitt-type ARLs and sporadic Burkitt's lymphomas in HIV-seronegative patients, most of which are not EBV associated.

In addition to the differences observed between immunoblast-rich/large-cell and Burkitt-type lymphomas in morphology and in EBV association, further evidence that the two groups of tumors may be pathogenetically distinct has come from clinical and immunologic studies. Thus, HIV-seropositive patients with immunoblast-rich/large-cell lymphomas have significantly lower CD4 cell counts and more often a history of previous AIDS-defining illnesses (both indicating more severe immunosuppression) compared with patients with Burkitt-type ARL.\textsuperscript{6,7}

Cases of HD in HIV-seropositive patients are being reported with increasing frequency, particularly in European patients.\textsuperscript{3} Although it is not clear whether the incidence of HIV-related HD is greater than would be expected compared with the general population, these lesions do appear clinically to be unusually aggressive.\textsuperscript{29} Previous studies have suggested a close association between EBV and HIV-related HD. Such cases are significantly more likely to contain EBV-DNA in their HRS cells than are HD cases in HIV-seronegative individuals.\textsuperscript{3} Similarly, HRS cell expression of EBV-encoded latent membrane protein 1 (LMP 1) was reported in each of 16 cases of HIV-related HD,\textsuperscript{30} compared with only about one-half of HD cases in HIV-seronegative patients.\textsuperscript{3} The present study provides further evidence of a consistent, and we believe pathogenetically important, association between EBV and HIV-related HD.

**Acknowledgment**

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In situ demonstration of Epstein-Barr virus small RNAs (EBER 1) in acquired immunodeficiency syndrome-related lymphomas: correlation with tumor morphology and primary site

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