Detection and Localization of Epstein-Barr Viral Genomes in Angioimmunoblastic Lymphadenopathy and Angioimmunoblastic Lymphadenopathy-like Lymphoma

By Lawrence M. Weiss, Elaine S. Jaffe, Xian-Fang Liu, Yuan-Yuan Chen, Darryl Shibata, and L. Jeffrey Medeiros

We studied 23 cases of angioimmunoblastic lymphadenopathy (AILD) and AILD-like lymphoma for evidence of Epstein-Barr virus (EBV) using the polymerase chain reaction (PCR) and in situ hybridization studies. EBV nucleic acid sequences were found by either PCR or in situ hybridization in 96% of the cases. There was a wide range in the number of EBV-positive cells among the different cases as detected by in situ hybridization. The EBV-positive cells most often possessed nuclei of intermediate to large size. Double-labeling immunohistochemistry/in situ hybridization studies demonstrated that most of the EBV-positive cells expressed the B-lineage antigen CD20 (as detected by L26), with a minority of the EBV-positive cells stained for the T-lineage associated antigen, CD43 (as detected by Leu 22). The abnormally high amounts of EBV found in AILD and AILD-like lymphoma may be a reflection of decreased immunocompetence in these patients. The presence of EBV-positive B cells may explain the presence of B-cell clones found by others as well as the paradoxical occurrence of B-cell lymphoma in a primary T-cell lymphoproliferative disorder.

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Materials and Methods

Patient samples. All of the cases were seen in the Laboratory of Pathology of the National Cancer Institute (Bethesda, MD) from the period 1982 through 1991. Cases were accepted into the study when a histologic diagnosis of AILD or AILD-like lymphoma was rendered and when paraffin blocks were obtainable for study. The histologic diagnosis of AILD was made according to the original criteria of Frizzera et al.1 When numerous medium- to large-sized lymphoid cells formed clusters or sheets in a lymph node biopsy that otherwise showed features of AILD, a diagnosis of AILD-like lymphoma was made.4 Case 6 has been previously reported (case 1 from the previous study).10 In addition, four normal lymph nodes, three normal tonsils, and three normal spleens were studied for comparative purposes.

PCR studies. Formalin and B5-fixed, paraffin-embedded tissues were cut into 10-µm thick sections and extracted as previously described by us elsewhere.14 The PCR was performed as previously described with primers SL1 and SL3 and a probe, SL2, specific for an 80-bp region of the EBV EBNA 1 gene.14 All positive results were confirmed by Southern blot analysis and hybridization with SL2. As a positive amplification control, primers for the β-actin gene were used for any samples negative for EBV.14 Positive controls consisting of a known EBV-positive undifferentiated nasopharyngeal carcinoma and negative controls consisting of at least five “water” ( assay with all reagents except sample DNA) controls and at least one sample in which the in situ hybridization studies were completely negative for EBV (see below) were performed with each experiment. In addition, to minimize the possibility of carryover at the time of cutting the specimens as a cause for positive results, samples from EBV-negative liver tissues, cut between each AILD sample, were analyzed when consecutive AILD samples were positive for EBV. The specificity and sensitivity of this PCR assay has been previously reported by us.14
**In situ hybridization studies.** The EBV RNA in situ hybridization studies were performed using a 30-base oligonucleotide complementary to the a portion of the EBER 1 gene, a region of the EBV genome which is actively transcribed (up to \(10^7\) copies per cell) in latently infected cells.\(^{11,12}\) The oligonucleotide was biotinylated using methods previously described.\(^{13}\) The procedure used for the in situ hybridization studies has been fully described by us elsewhere.\(^{14}\) Briefly, 10-μm sections cut from paraffin blocks of formalin and B5-fixed tissues were deparaffinized, dehydrated, predigested with pronase, prehybridized, and hybridized overnight at a concentration of 0.25 ng/μL of probe. The B5-fixed tissue sections were pretreated with a 1% iodine xylene solution before boiling ribonuclease A (Boehringer Mannheim, Indianapolis, IN) for 15 minutes to remove heavy metals. After washing, detection was accomplished using avidin-alkaline phosphatase conjugate followed by development of the signal with McGadey’s substrate. A brown or blue-brown color within the nucleus over background levels was considered a positive reaction. The number of positive cells was visually estimated at \(<1\), 1 to 10, 10 to 100, and \(>100\) per medium power field (mpf) using a 15× ocular lens and a 20× objective. This methodology detected EBV RNA from the EBV-infected Raji cell line, but not from the non–EBV-infected T-cell line Molt 3. In addition, cells from tissues infected with herpesvirus I, papilloma virus 16, and adenovirus showed no cross-reactivity. Although the sense strand oligonucleotide could not be used as a negative control (due to partial identity with adjacent antisense sequences), substitution of the probe with 10 other oligonucleotides of identical length and similar G-C content showed no similar staining. Preincubation with 9 KgipL of other oligonucleotides with sufficiently atypical features to warrant suspicion or an outright diagnosis of AILD-like lymphoma;

### Table 1. Summary of Results

<table>
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<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
<th>Diagnosis</th>
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<th>EBV*</th>
<th>Poly d(T)</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>6b</td>
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<td>AILD-L</td>
<td>+</td>
<td>1-10</td>
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</tr>
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<td>&lt;1</td>
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<tr>
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</tbody>
</table>

*Results expressed as number of positive cells per medium power field (see Materials and Methods).
in one of these patients, biopsies at two sites were available for study.

Evidence of EBV was found by either PCR or in situ hybridization in 22 of the 23 cases (96%) (Table 1). Amplification of EBV-specific DNA sequences was found by PCR in 16 of the patients (Fig 1). In four of the negative cases, PCR for actin was also negative, indicating insufficient amplifiable DNA as a possible explanation for the negative results for EBV genomes. Excluding these four cases, EBV was identified by PCR in any of the four lymph nodes with reactive follicular hyperplasia, three normal tonsils, or three normal spleens.

EBV RNA was identified by in situ hybridization in 22 of the 23 patients (96%) (Table 1); the tissue from the negative case contained sufficient RNA for detection, as demonstrated by hybridization for mRNA using a poly d(T) probe. There was a very wide range in the degree of positivity in the different cases, ranging from rare positive cells less than 1 per mpf to greater than 100 positive cells per mpf (Fig 2). There was also variation from slide to slide when two blocks of the same specimen were studied, and even wide variation between areas of the same piece of tissue in some cases. In the four cases in which two biopsies were available for study, the results were similar at the different sites in three cases (cases 6, 7, and 9), while in the fourth (case 12), 1 to 10 positive cells per mpf were seen in an inguinal lymph node biopsy while greater than 100 positive cells per mpf were seen in a subsequent cervical lymph node biopsy obtained shortly thereafter. The positive cells most often possessed nuclei of intermediate to large size, particularly in specimens with greater numbers of positive cells. In specimens with few positive cells, the majority of the positive cells had nuclei of small size. There was no correlation between number of positive cells and the fixative used, histologic diagnosis, site of biopsy, or the age or sex of the patient.

Three of the four lymph nodes with reactive follicular hyperplasia, two of the three normal tonsils, and two of the three normal spleens had detectable EBV RNA by in situ hybridization. However, in all cases only rare positive cells were observed, much less than 1 per mpf.

Double-labeling immunohistochemical/in situ hybridization studies were attempted in 10 cases of AILD and AILD-like lymphoma, and were successful in three instances (Fig 3). The majority of the cells positive for EBV RNA in all three cases, mostly medium-sized and large cells, labeled with the B-lineage marker L26 (CD20) and were unlabeled with the T-cell-associated marker Leu 22 (CD43). These cells were present in areas of diffuse effacement of architecture or interfollicular areas, and generally not in residual germinal centers. A minority of cells positive for EBV RNA, mostly small- and medium-sized cells, were labeled with Leu 22. In all three cases, the majority of both L26 and Leu 22-labeled cells were negative for EBV RNA, although focally, most of the L26-labeled cells were also positive for EBV RNA.

**DISCUSSION**

The results of this study confirm the previous observations of Staal et al. and Knecht et al., and suggest that EBV may be identified in abnormally high amounts in AILD and AILD-like lymphoma. Using our PCR procedure, EBV was not found in any of the four lymph nodes with reactive follicular hyperplasia, three normal tonsils, or three normal spleens, and has not been detected in a total of 39 lymph nodes showing no pathologic abnormality, follicular hyperplasia, or dermatopathic lymphadenitis in previous studies by us using an identical PCR procedure. Yet, EBV was found by PCR in 84% of the cases of AILD and AILD-L in which adequate amplifiable DNA was present. These results were supported by the in situ hybridization findings which identified EBV RNA in 96% of the AILD and AILD-like lymphoma cases. Although this

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**Fig 1.** Results of a Southern blot hybridization of the products of EBV PCR. The numbers at the top refer to the case numbers. Lane N is the negative control, while lane P is a known EBV-positive control specimen. Positive signals are seen in lanes 2, 10, 11, 15, 12B, 16, and 14. Lane 5 shows a possible faint band at 80 bp; a longer exposure autoradiogram showed a definite band at 80 bp in this lane. Lanes 9B, 23, and 9A are negative, and remained negative with a longer exposure autoradiogram.
Fig 2. In situ hybridization for EBV. (A) Case 21 shows rare positive cells, despite negative PCR study. (B and C) Results obtained in case 11, with numerous positive small to intermediate sized cells. (A and B, original magnification × 200; inset and C, original magnification × 600; no counterstain.)
Fig 3. Case 1, double-labeling immunohistochemical/in situ hybridization study. (A and C) Results of double-labeling for CD20 (L26) and EBV. Most EBV-positive cells also show membrane labeling for CD20. (B and D) Results of double-labeling for CD43 (Leu 22) and EBV. Most of the EBV-positive cells show no membrane staining, although occasional cells show definite copositivity for EBV and CD43 (D). (A and B original magnification × 200, C and D original magnification × 600; no counterstain.)
sensitive technique could detect rare positive cells in the majority of normal lymphoid tissues studied, the number of EBV-positive cells in the majority of cases of both AILD and AILD-like lymphoma far exceeded the normal range.

Knecht et al\(^1\) have previously speculated that EBV may be implicated in the pathogenesis of many AILD cases, considering a role both as an etiologic factor and as a modulating element. Their hypothesis would seem to be supported by our data showing the vast majority of cases to contain abnormally high levels of EBV. However, other data we obtained are not fully consistent with these hypotheses. In the three cases in which double-labeling was successful, the majority of the positive cells were identified as B-lineage cells, as evidenced by expression of CD20. Only a minority of cells, mostly small cells, showed colabeling for EBV RNA and the T-lineage antigen CD43. In addition, there was no apparent correlation between the number of positive cells and histologic progression to AILD-L, even within an individual case, although the follow-up may be incomplete in some cases. Furthermore, in case 12, one biopsy contained numerous EBV-positive cells while a second biopsy at another site, with a similar histologic appearance, showed at least 10-fold fewer positive cells.

We believe that it is more likely that the abnormally high levels of EBV are more likely a reflection of the disease process rather than a cause of it. It is well known that patients with AILD have a profound immunologic deficiency, and intercurrent infections are frequent and a common cause of death. It is possible that abnormal immunoregulation leads to the polyclonal, and oligoclonal processes rather than a cause of it. It is well known that abnormal immunoregulation leads to the polyclonal, and oligoclonal proliferation of EBV-positive cells, which are mostly B cells. This could account for the presence of B immunoblasts in a predominantly T-lineage lymphoproliferative process, which may become so prominent that Lukes et al\(^2\) originally considered AILD a primary B-lineage process. Abnormal immunoregulation may also explain the occurrence of clonal rearrangements of the Ig genes observed by some in a subset of AILD patients,\(^3\) and may similarly explain the presence of Ig gene rearrangements that appeared and disappeared over time reported in a previous study.\(^4\) For instance, heavy and light chain Ig gene rearrangements had been found in the tissues from both lymph node specimens of patient 6 (reported in a prior study).\(^5\) Finally, this hypothesis may explain the reported paradoxical occurrence of B-lineage or "Burkitt-like" lymphomas in AILD, a primary T-lymphoproliferative disorder.\(^6\) In fact, the patient from case 8 in the current study died of a B-lineage lymphoma that showed the presence of EBV in a monoclonal pattern reminiscent of lymphomas arising in the setting of transplantation (L. Abruzzo, in preparation). On the other hand, we studied two cases of AILD-like lymphoma with abundant EBV DNA on PCR analysis, and found the Ig heavy chain gene to be in the germline configuration. However, these results may reflect a sampling problem because analysis of the same tissues for the status of the EBV terminus by Southern blotting showed an absence of bands, indicating a low level of EBV genomes in these tissues.

The presence of EBV RNA in occasional CD43-positive cells as demonstrated by the double-labeling studies is intriguing and worthy of further study. CD43 is expressed by normal T lymphocytes, macrophages, and plasma cells.\(^7\) The morphologic features of the EBV-positive/CD43 cells were not consistent with macrophages or plasma cells, but rather with small to medium-sized lymphoid cells. CD43 may also be occasionally expressed on neoplastic B cells,\(^8\) however, the number of EBV/CD43-positive cells did not match the number of EBV/CD20-positive (B-lineage) cells. EBV genomes have now been identified in both reactive and neoplastic T cells.\(^9\) In the current cases, the EBV was not present in a large population of T cells as is generally seen in EBV-positive T-cell lymphoma (L.M.W., unpublished observations, March 1990), but was confined to a minority of cells expressing CD43. It is possible that small T-cell polyclonal and oligoclonal proliferations may occur analogous to the EBV-driven polyclonal and oligoclonal B-cell proliferations.

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