RAPID COMMUNICATION

Patterns of Epstein-Barr Virus Infection in Non-Neoplastic Lymphoid Tissue

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Taking advantage of the abundant expression of the small Epstein-Barr virus (EBV)-encoded RNAs (EBERs) in latently infected cells, we have analyzed 72 normal and hyperplastic lymph nodes and three tonsils of acute infectious mononucleosis (IM) for the presence and distribution of EBV+ cells using EBER-specific in situ hybridization, in some cases combined with immunohistologic demonstration of cell type-characteristic antigens. In IM, large numbers of EBV+ lymphoid B blasts were detectable in extracellular areas, whereas germinal centers were generally free of EBV+ cells. In reactive lymph nodes, the frequency of EBV+ cells varied with the degree of lymphoid hyperplasia and underlying immune status. The lowest numbers of EBV+ cells were detected in nonactivated lymph nodes and highest in human immunodeficiency virus-associated lymphadenopathy. If present in these lymph nodes, EBV+ cells were almost exclusively localized to extracellular areas, as also observed in IM. However, in contrast to IM, these cells were mainly small lymphocytes. Furthermore, in some instances, occasional scattered EBV+ cells were seen within germinal centers, and in two cases diffuse expansions of EBV+ cells occurred within a single germinal center each, indicating that under certain circumstances EBV+ B lymphocytes may participate in physiologic germinal center reactions. These findings reflect the interference of EBV with physiologic lymphoid differentiation pathways and provide a link to EBV-associated malignant lymphomas with a postulated origin from germinal center cells.

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THE EPSTEIN-BARR virus (EBV) is well known for its association with certain malignancies, such as undifferentiated nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), and B-cell lymphomas in immunocompromised individuals.1-14 EBV infects more than 90% of the human population worldwide and persists for life in the infected host.5 Upon primary infection, EBV may cause infectious mononucleosis (IM), a self-limiting lymphoproliferative disorder.6 In tonsils from IM patients, EBV-infected lymphoid cells are found in the expanded extracellular areas.7,8 Analysis of serial sections with DNA in situ hybridization and immunohistology has suggested that at this stage EBV-infected cells are largely activated lymphoid B blasts.7,8 Whereas the EBV-infected cells in IM and virus-associated malignancies are well characterized, little is known about latent infection in healthy individuals. However, knowledge about the state of the virus in this environment would be important in understanding the potential role for the virus in the pathogenesis of EBV-associated malignant lymphomas and to elucidate the mode of virus persistence. The application of DNA in situ hybridization techniques to the analysis of latent EBV infection has been hampered by the limited number of target sequences, ie, viral genomes, in latently infected B lymphocytes. The introduction of RNA in situ hybridization for the detection of the EBV-encoded small nuclear RNAs (EBER-1 and EBER-2) has provided a unique tool for the detection of latently infected cells in tissue sections. The EBERs are small nuclear, nonpolyadenylated RNAs that are transcribed at high copy numbers in every known condition of EBV latency, thus providing an abundant target for in situ hybridization.10-12 Moreover, the EBERs are easily detected even in routinely processed tissues, thus facilitating analysis of archival material.10,11 Using this technique, we have analyzed hyperplastic lymph nodes from immunocompetent and immunocompromised individuals for the presence and distribution of EBV+ cells.

MATERIALS AND METHODS

Tissues. Formalin-fixed and paraffin-embedded tissues from 72 lymph nodes and from three tonsils from the files of the Institute of Pathology, Klinikum Steglitz (Berlin, Germany) and of the Department of Pathology, East Birmingham Hospital (Birmingham, UK) were analyzed on serial sections. In all cases, routine histologic and immunohistologic analysis showed reactive alterations (see Results and Table 1), but no involvement by malignant lymphomas or other malignancies. Diagnosis of lymph nodes from human immunodeficiency virus (HIV)-infected individuals followed the classification of Ost et al.13

In situ hybridization. Probes specific for the EBER-1 and EBER-2 RNAs were prepared using the plasmids pBSJJ1 and pBSJJ2, respectively.15 After linearization, [35S]-labeled runoff antisense and sense (control) transcripts were generated using T7 or T3 RNA polymerases (Promega-Biotec, Madison, WI). EBER-1- and EBER-2-specific probes were combined to increase sensitivity. In situ hybridization for the detection of EBV transcripts was performed as described previously.11 In brief, deparaffinized and rehydrated paraffin sections were exposed to 0.2 N HCl and 0.125 mg/mL pronase (Boehringer, Mannheim, Germany), followed by acetylation with 0.1 mol/L triethanolamine pH 8.0/0.25% (vol/vol) acetic anhydride and dehydration through graded ethanols. Slides were hybridized to 2 to 4 × 10^6 cpm of labeled probes overnight at 50°C. Washing and autoradiography was performed as described.

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Table 1. Frequency of EBV+ Extrafollicular Cells

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>EBV+ Cases per Total Cases (%)</th>
<th>Extraculicular EBV+ Cells/0.5 cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectious mononucleosis</td>
<td>3/3 (100)</td>
<td>3</td>
</tr>
<tr>
<td>Normal lymphoid tissue</td>
<td>4/12 (33)</td>
<td>8 4</td>
</tr>
<tr>
<td>LA dermatopathic</td>
<td>2/7 (28)</td>
<td>5 2</td>
</tr>
<tr>
<td>LA Rosai-Dorfman</td>
<td>0/2</td>
<td>2</td>
</tr>
<tr>
<td>Pironier lymphadenitis</td>
<td>9/13 (69)</td>
<td>4 5 3 1</td>
</tr>
<tr>
<td>LA unspecific F</td>
<td>2/4 (50)</td>
<td>2 1</td>
</tr>
<tr>
<td>LA unspecific F/EF</td>
<td>7/10 (70)</td>
<td>3 3 2 2</td>
</tr>
<tr>
<td>LA unspecific EF</td>
<td>9/15 (60)</td>
<td>6 6 1 1</td>
</tr>
<tr>
<td>LA HIV-associated</td>
<td>9/9 (100)</td>
<td>2 3 3 1</td>
</tr>
</tbody>
</table>

Values are numbers of cases.
Abbreviations: LA, lymphadenopathy; F, follicular activation; EF, extrafollicular activation; F/EF, mixed follicular and extrafollicular activation.

Combined immunohistology and in situ hybridization. Immunohistology with monoclonal antibodies (MoAbs) L26 (CD20; Dakopatts, Glostrup, Denmark), A6 (CD45RO; kindly provided by Dr G.G. Aversa, Stanford, CA), and βF1 (anti-T-cell receptor β-chain; T-Cell Sciences, Cambridge, MA) was performed using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique under RNase-blocking conditions. All MoAbs and secondary reagents were diluted in Tris-buffered saline supplemented with 10 mg/mL bovine serum albumin (Sigma, Deisenhofen, Germany), 5,000 U/mL heparin (Sigma), and 1 mg/mL tRNA (Bethesda Research Laboratories, Gaithersburg, MD). After the development of the alkaline phosphatase, slides were subjected to in situ hybridization as described above.

RESULTS

Infectious mononucleosis. Three tonsils from patients with clinically overt infectious mononucleosis were analyzed. In all cases, the examination of routinely stained sections showed an expansion of extrafollicular areas by numerous lymphoid blasts. Using in situ hybridization, large numbers of EBER+ cells were detected in all three IMM tissues. The most prominent feature were sheets of EBER+ lymphoid blasts in the expanded extrafollicular areas (Fig 1a). The number of EBER+ extrafollicular cells exceeded 1,000/0.5 cm² in all three cases. Occasionally, virus-infected cells were also detectable in the follicle mantle. As a rule, the clusters of EBER+ cells spared the germinal centers. However, in one case, occasional EBER+ cells were seen within one germinal center.

Normal lymph nodes. Twelve lymph nodes with or without minimal hyperplasia and/or sinus histiocytosis obtained during mastectomy were considered to represent normal lymphoid tissue. None of these nodes showed any evidence of metastatic tumor. EBV-specific in situ hybridization showed EBV+ cells in the extrafollicular areas of four of these lymph nodes (Table 1). In all four cases, EBER+ cells were rare (Table 1) and showed the morphology of small lymphocytes.

Dermatopathic lymphadenopathy. Seven lymph nodes displayed an activation of extrafollicular areas with CD30+ T-cell blasts, dense sheets of CD1a+ interdigitating cells, as well as melanin-laden macrophages and were thus diagnosed as dermatopathic lymphadenopathy. Small EBV+ extrafollicular cells were detected in two cases (Table 1). Again, the number of EBV+ cells was low.

Rosai-Dorfman lymphadenopathy. Two lymph nodes with massive sinus histiocytosis and hemophagocytosis did not contain any EBER+ cells (Table 1).

Pironier's lymphadenitis. Dilated sinuses filled with mononuclear cells, prominent germinal centers, small clusters of epithelioid cells, and inflammation of the lymph node capsule were seen in 13 cases. Seven of these lymph nodes displayed EBER+ cells (Table 1). These cells were mostly confined to the extrafollicular areas, with involvement of the follicle mantle in three cases (Fig 1b). Rare EBER+ cells were observed within occasional germinal centers in two cases (Fig 1c). The morphology of these cells was mostly consistent with small lymphocytes, but varying proportions of EBV+ lymphoid blasts were also present. The number of EBER+ extrafollicular cells was usually small, exceeding 50 cells/0.5 cm² in only one case (Table 1).

Unspecific follicular and/or extrafollicular hyperplasia. Twenty-nine lymph nodes showing noncharacteristic activation of lymphoid tissue were obtained from patients without clinically known dysfunction of the immune system. The etiology of lymphoid hyperplasia was undetermined in all cases. Four of these cases showed a predominant follicular activation, in 15 cases expansion of the extrafollicular areas with minimal follicular activation was seen, and nine cases displayed follicular and extrafollicular activation to similar extent. EBER+-expressing cells were identified in 50% to 70% of the cases in all three subgroups and their relative number was usually low. However, in some cases, up to 200 EBV+ cells/0.5 cm² were found (Table 1). In the majority of cases, infected cells were localized exclusively in the extrafollicular areas, in some instances with involvement of the follicle mantles. The nuclear morphology of these cells was often masked by dense clusters of grains. However, upon short exposure time with reduced number of grains, it was evident that EBER-specific signals were mainly associated with small lymphocytes. The proportion of EBER+ lymphoid blasts rarely exceeded 20%. In three cases, occasional EBER+ cells were seen within germinal centers that resembled lymphoid blasts. Additionally, one of the cases with unspecific mixed follicular and extrafollicular hyperplasia showed diffuse expansion of EBER+ cells in a single germinal center (Fig 1e and f). In this particular germinal center, EBER+ cells accounted for approximately 40% of
Fig 1. Detection of EBER transcripts in non-neoplastic lymphoid tissues. (a) Large numbers of EBER-expressing lymphoid blasts in the extrafollicular area (tonsil, acute infectious mononucleosis). No EBV-infected cells are detectable in a germinal center. (b) EBER transcripts in occasional small lymphocytes in the extrafollicular area and a follicle mantle (Piringer's lymphadenitis); (c) a single EBER expressing cell within a germinal center (Piringer's lymphadenitis); (d) EBER transcripts in the majority of germinal center cells (HIV lymphadenopathy, follicular involution); and (e and f) EBER-expressing cells in a germinal center showing centroblast-like morphology (follicular and extrafollicular hyperplasia). Paraffin sections and in situ hybridization with [35S]-labeled antisense probes, 3 days of autoradiographic exposure. Original magnification $\times 400$ (a and e), $\times 200$ (b, c, and d), $\times 600$ (f).

the total cell number and most EBER+ cells displayed centroblast-like or centrocyte-like morphology (Fig 1 e and f).

**HIV lymphadenopathy.** Nine lymph nodes from HIV-infected individuals were studied. Follicular hyperplasia without follicular fragmentation, with follicular fragmentation, and follicular involution was seen in three cases each. All lymph nodes contained EBER+ cells (Table 1). The frequency of EBER+ cells was usually higher than in the lymph nodes from immunocompetent individuals. In
one case, the number of extracellular EBER+ cells resembled the situation in IM cases (Table 1). However, the distribution patterns of EBER+ cells were identical in HIV-infected and immunocompetent individuals. In most cases, EBER+ cells were confined to extracellular areas; only rarely scattered EBV+ cells were observed within germinal centers. In one case at the stage of follicular involution, one single germinal center displayed a diffuse expansion of EBER+ cells comprising the vast majority of germinal center cells (Fig 1d).

Combined in situ hybridization and immunohistology. Double labeling was performed on 14 specimens with sufficient numbers of EBER+ cells from patients with infectious mononucleosis, Pirkinger's lymphadenitis, and unspecific and HIV-associated lymphadenopathy (Fig 2). Most, but not all, EBER+ lymphoid blasts in the IM tissues expressed the CD20 antigen. In all other cases, the majority of EBV+ cells were not detected by either B- or T-cell-specific antibodies, whereas neighboring EBV- cells usually showed a strong reactivity. Only occasionally did EBV+ cells express detectable levels of CD20 antigen. No virus-infected T cells were identified in any of the tissues investigated.

DISCUSSION

In contrast to the understanding of EBV-associated malignancies, in which details relating to cell lineage, clonality, and viral gene expression have accumulated during recent years, the Information on EBV infection in non-neoplastic lymphoid tissue of immunocompetent individuals remained fragmentary. An increased frequency of detection by polymerase chain reaction was reported for EBV DNA in hyperplastic lymph nodes,15,16 but the cellular localization of the viral genomes remained unclear. Only IM tissues were investigated in more detail by morphologic techniques.7,8 Taking advantage of the abundance of EBER transcripts in latently infected cells, we have analyzed 72 lymph nodes with varying forms and degrees of hyperplasia for the presence and distribution of EBV-carrying cells using EBER-specific in situ hybridization. Three tonsils from patients with acute IM were included for comparison with previous DNA-DNA in situ hybridization studies. In agreement with these previous investigations, EBER-specific in situ hybridization combined with immunohistology showed large numbers of EBV-infected lymphoid blasts of B-cell type in the extracellular areas of lymphoid tissues in IM.7,8 However, the number of EBV+ cells detected in DNA-DNA in situ hybridization experiments was significantly lower than the number of EBER+ cells reported here. This finding is explained by the comparatively low and variable number of viral episomes per infected cell. As described previously, germinal centers, although present in high numbers, were generally devoid of EBV+ cells, with infected cells thus being restricted to the extracellular areas.7,8

While the majority of hyperplastic lymph nodes from persistently infected individuals also displayed a predominantly extracellular location of EBER+ cells, EBER expression was observed within germinal centers in a small proportion of cases.

EBER expression in extracellular areas. In lymph nodes with no or minimal hyperplasia, EBER+ cells were only rarely detected and these cells were confined to the extracellular areas. A similar pattern was observed in dermatopathic lymphadenopathy, showing activation of the T zone attributable to an underlying disease of the skin. In all these cases, the number of EBER+ extracellular cells was low (Table 1) and the cells were mostly small. The frequency of EBER+ cells in the extracellular zone was markedly increased in lymph nodes with significant follicular and/or extracellular activation of unknown etiology, but even in these groups, between 30% and 50% of the cases did not contain any EBER+ cells (Table 1). The increased frequency of EBER+ cells in these cases in comparison to the nonactivated lymph nodes might indicate an involvement of the virus in the pathogenesis of lymphadenopathy. However, in Pirkinger's lymphadenitis commonly attributable to toxoplasmosis, EBER+ extracellular cells were found at a similar frequency as in hyperplasia of unknown etiology (Table 1). Thus, we assume that every latently EBV-infected individual permanently carries small numbers of circulating EBV+ lymphocytes that, in consequence, are rather infrequent in single sections of normal lymphoid tissue.5 In activated lymphoid tissue provided with a mitogenic stimulus, their number increases along with the general number of lymphocytes, and thus EBER+ cells become more frequently detectable. Differences in the frequency of EBER+ cells between lymph nodes with similar degrees of hyperplasia obtained from different patients may then reflect individual variations in the levels of virus-infected cells, possibly due to the varying efficiency of immunosurveillance. This notion is underlined by the observation of highest numbers of EBER+ cells in lymph nodes obtained from HIV-infected patients. Among all entities, EBER+ extracellular cells were small lymphocytes in most instances, and EBER+ large blast-like cells accounted for up to 30% of the virus harboring cells in rare cases.

EBER expression in germinal centers. In most instances, EBER-expressing cells within germinal centers were scattered and rare, comprising far less than 5% of the total germinal center cell number. The nature of these cells is unclear; however, due to their cytology and sparsity it seems unlikely that they represent genuine germinal center cells, ie, centroblasts and centrocytes. In contrast to these scattered EBV+ cells in germinal centers, two lymph nodes showed a diffuse expansion of EBV+ cells in rare germinal centers. In both cases, the morphology of EBV+ cells was consistent with centroblasts and centrocytes.

In summary, the majority of hyperplastic lymph nodes showed a predominantly extracellular location of EBV+ cells, as also observed in IM. The major difference between primary infection (IM) and persistent infection lies in the number and cytology of EBER+ cells, which in IM are mainly blasts, whereas in persistent infection mainly small EBER+ lymphocytes are found. This observation, in conjunction with the infrequent finding of EBER+ cells
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Fig 3. Model of B-cell differentiation in normal lymphoid tissue after antigen-driven selection and stimulation of a competent B cell with (right) or without (left) interference by EBV (see Discussion).

Within germinal centers, raises questions as to nature and origin of small EBER+ cells, the reasons for the extrafollicular predominance of these cells, and the infrequency of EBER+ cells in germinal centers. Some of these problems may be partially resolved by comparison with the physiologic B-cell differentiation in normal lymphoid tissues (Fig 3).17 B cells with complete antigen receptor gene rearrangement, ie, virgin B cells, are available for natural selection by antigen. This occurs only rarely and therefore the majority of virgin B cells is eliminated by programmed cell death. On the other hand, contact with an antigen prevents cell death and induces extrafollicular blast formation. Immunoblasts in extrafollicular areas may then give rise to short-lived plasma cells or recirculating small B cells. Upon renewed contact with the antigen, complexed with Ig and complement and presented by follicular dendritic reticulum cells, transformation into germinal center cells initially centroblasts and then centrocytes) occurs, followed by clonal expansion of germinal center cells and ultimately resulting in the formation of memory B cells and long-lived plasma cells.17

By infection with EBV, mechanisms normally leading to the elimination of nonselected B cells may be overruled, resulting in rescue from programmed cell death.18,19 Such latently infected cells derived from an extrafollicular blast reaction may then represent the majority of recirculating EBV+ B cells located to the extrafollicular areas of lymphoid tissues. Because reactivity with a specific antigen is not required for the survival of these cells, it is likely that the majority of EBV+ extrafollicular B cells do not have a functional antigen receptor or may even have no antigen receptor at all. In the rare event of antigen-driven selection of EBV+ B cells, these may enter a follicle and participate in the germinal center reaction (Fig 3). This concept would be consistent with the observed low frequency of EBV+ germinal centers. Furthermore, the finding that EBER+ cells may constitute the majority of genuine germinal center cells within individual germinal centers is consistent with the concept of an oligoclonal or monoclonal origin of germinal center cells.17,20 It remains to be elucidated whether EBV+ germinal center cells may further differentiate towards either memory cells or plasma cells. It was shown that most of the EBV nuclear antigen (EBNA)-positive circulating cells arising during early phases of acute IM differentiate towards plasma cells, whereas after 2 to 3 weeks the proportion of Ig+ cells among EBNA+ circulating cells decreased.21 This finding led Robinson et al to suggest either that differentiation of EBV+ cells was altered during the disease or that nondifferentiated EBNA+ cells had a survival advantage over EBNA+ Ig-producing cells.21 It is also noteworthy that immunoblastic lymphomas in acquired immunodeficiency syndrome (AIDS) patients often display plasmacytic differentiation,4 and that EBER+ plasmacytomas may occur (unpublished observation). The absence of EBER+ mature plasma cells in our cases, however, would suggest that differentiation of EBV+ cells in this direction is somehow blocked. The demonstration of EBV within germinal center cells provides a link to virus-associated lymphomas of postulated germinal center cell
origin as BL, lymphocyte predominant HD of nodular subtype, and occasional AIDS-associated malignant lymphomas.\textsuperscript{4,22,23} These findings and the proposed model do not, however, explain the mode and circumstances of T-cell infection by EBV, which obviously must be considered because of the existence of EBV-associated T-cell lymphomas.\textsuperscript{24} Only a minority of the EBER\textsuperscript{+} cells could be unequivocally immunophenotyped as B cells. Neighboring lymphocytes, on the other hand, showed strong reactivity with the T- and B-cell-characteristic antibodies applied, suggesting that EBV-infected cells may downregulate certain differentiation antigens in vivo. This conclusion was also drawn in a recent study of HD tissues that indicated that EBER\textsuperscript{+} nonmalignant small lymphocytes do not express latent membrane antigen (LMP) and EBNA2.\textsuperscript{3,25} The questions arising as to the quality and quantity of the differentiation antigen expression by EBER\textsuperscript{+} cells in reactive lymphoid lesions may possibly be answered using cryostat sections and a broader panel of MoAbs. Further studies on frozen material are also required to correlate viral antigen expression with cellular morphology, particularly with respect to LMP and EBNA2 expression in blasts, which, in contrast to small lymphoid cells, may be LMP\textsuperscript{+}/EBNA2\textsuperscript{+} in tonsils with infectious mononucleosis.\textsuperscript{2} This problem could not be addressed in this series, because most available antibodies directed against EBV-encoded proteins show only limited reactivity or do not react at all in paraffin sections.

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**REFERENCES**

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