Variable Morphology of Human Immunodeficiency Virus-Associated Lymphomas With C-MYC Rearrangements

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Burkitt lymphoma (BL) and immunoblastic lymphoma (IL) are the most frequent lymphoid tumors encountered in human immunodeficiency virus (HIV)-infected patients. Tumors with a morphology intermediate between BL and IL, and the existence of Burkitt's type translocations in some IL cases makes the classification of these tumors sometimes unclear. We have studied 14 cases of BL and IL in c-myc rearrangement. Of seven tumors with morphology of IL cases makes the classification of these tumors some-

ment and four were associated with EBV. Five tumors with morphology of IL were associated with EBV and devoid of c-myc rearrangement. Three were polyclonal representing EBV-driven lymphoproliferations similar to those observed in transplant recipients. Two tumors, one with a morphology of IL and the other intermediate between IL and BL were monoclonal, associated with EBV, and harbored a c-myc rearrangement. We propose that these last two tumors represent cases of BL that have adopted an immunoblastic morphotype in the context of acquired immunodeficiency syndrome (AIDS), reflecting the morphologic evolution of Burkitt lymphoma cells observed in culture.

MALIGNANT B-cell proliferations including non-Hodgkin’s lymphomas (NHL) are well known complications of human immunodeficiency virus (HIV) infection.1-6 These are intermediate- and high-grade malignant NHL, according to the international working formulation.7 Large-cell lymphomas, of the immunoblastic or of the large noncleaved type, peripheral or involving the central nervous system,2,8 and small noncleaved cell lymphoma of the Burkitt type constitute the majority of these tumors.9 Peripheral polyclonal lymphomas not associated with Epstein-Barr virus (EBV),10 and atypical undifferentiated large-cell lymphomas have also been reported.11 Occurrence of NHL in HIV-infected patients has been initially attributed to the state of immunodeficiency, as observed in patients with primary immunodeficiency or after organ transplantation.12,13 Posttransplant lymphomas have been proposed to be the consequence of the uncontrolled proliferation of EBV-infected cells giving rise to proliferations of extremely variable morphology.14-17 Large-cell lymphomas of the immunoblastic type are particularly frequent in cardiac transplant recipients.18 Lymphoproliferations in transplant recipients usually do not possess chromosomal t(8;22) translocations affecting the c-myc locus.10,19,20 They may be either polyclonal or monoclonal, as suggested by immunophenotypic or by Ig gene rearrangement analysis.17,21-24 However, in addition to immunoblastic lymphomas, HIV carriers have also a high risk of small noncleaved cell lymphomas principally of the Burkitt type (BL), a complication only exceptionally identified to date in congenital or acquired immunodeficiencies other than acquired immunodeficiency syndrome (AIDS).1,3 BL is characterized by its monoclonality and one of the typical t(8;14), t(2;8), or t(8;22) translocations affecting the c-myc locus. However, association with EBV is not a constant feature.25,26 Several studies have pointed out the different characteristics of these two types of lymphomas in the context of HIV infection. Immunoblastic lymphoma (IL) and large-cell lymphomas occur usually in patients with severe immunodeficiency with an extremely low count of CD4+ lymphocytes. These tumors do not respond to chemotherapy and have a poor prognosis.2 In contrast, BL generally occurs in patients with less severe immunodeficiency, is often preceded by a generalized reactive lymphadenopathy, and responds better, at least initially, to chemotherapy.1,3 Previous studies on the presence of EBV in BL among HIV carriers have described a viral association in about one-half of the cases.1,3,27-32 These tumors display the characteristic chromosomal translocations affecting the long arm of chromosome 8 (8q24).29,31-34 However, analysis of tumors from HIV-infected patients has shown the existence of Ig/c-myc translocations in cases of IL.31,35 The pathologic diagnosis of these tumors in HIV-positive individuals is sometimes difficult, with presence of numerous atypical morphologic features and existence of tumors with morphologies intermediate between IL and BL.36,37 For these reasons we have undertaken a combined molecular and morphologic analysis of 14 of these tumors to precisely distinguish between BL and IL in the context of HIV infection. Our results suggest the possibility that in the course of the HIV infection, BL cells may adopt morphologic characteristics of IL cells in vivo, a phenomenon also observed when BL cells are explanted in vitro in culture.
LYMPHOMAS IN HIV-INFECTED PATIENTS

MATERIALS AND METHODS

Pathologic Samples

Tumoral biopsies obtained from patients infected by HIV were used for simultaneous morphologic and molecular analyses. Cases with partial tumoral infiltration were systematically excluded from our study. Tumors from 14 patients satisfied the conditions cited above.

Part of material was formalin-fixed and embedded after standard procedures. DNA was extracted from the rest of the primary material, with the exception of case 1 and 2 where in vitro cultured cell lines established from the initial tumors were used (IARC BL 115 and IARC BL 134).

These tumors were diagnosed and classified, according to the international working formulation (Table 1). All pathologic findings, except cases 1 and 2, were reviewed by the members of the “French Study Group of Pathology for HIV-Associated Tumors.” Cases 1 and 2 were diagnosed and typed in Lyon (Centre Léon Bérard).

DNA Isolation and Southern Blotting

Total cellular and viral DNA was prepared by sodium dodecyl sulfate (SDS) cell lysis, proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation.

Ten micrograms of DNA were digested with 50 IU of the appropriate endonucleases (Bethesda Research Laboratories, Bethesda, MD).

Digested DNAs were electrophoresed on a 0.7% to 0.9% agarose gel in TBE (0.045 mol/L Tris-Borate 0.0025 mol/L EDTA) buffer, hydrolyzed for 15 minutes in 0.25 N HCl, denatured for 30 minutes, and renatured before being transferred onto Hybond N filter (Amersham, Amersham, UK) according to Southern analysis. Probes were labeled with 32P by multiple random priming (Amersham) and hybridized with the filter at 65°C in 0.5 mol/L NaH2PO4, pH 7.2, 1% bovine serum albumin (BSA), 7% SDS.

Filters were washed at 60°C for 30 minutes in 2X SSC, 0.1% SDS, and autoradiographed using intensifying screens (Quanta III; Dupont, Wilmington, DE).

DNA Probes

Rearrangements of the c-myc gene were studied by hybridization of BamHI-, EcoRI-, HindIII- and Xba I-digested DNA to probes specific of the joining region (JH) and of the Cμ gene (Fig 1B). The χ light chain was studied using a 2-kb J χ and a 2.5-kb C χ fragment.

Search for EBV DNA was done using a probe specific for the BamHI-W internal repeats of the virus,44 and the 1.9-kb Xho I fragment located near the right end of the linear viral genome.44

RESULTS

Patients and Pathologic Characteristics

Table 1 summarizes the main clinical and pathologic characteristics of our cases. Fourteen tumor samples were collected in four French hospitals (Hôpital Pitié-Salpêtrière, Paris; Hôpital Beaujon, Paris; Hôpital d’Argentueil; Centre Léon Bérard, Lyon). Seven patients were homosexual, four were heterosexual with multiple partners, one came from an endemic country, and two were intravenous (IV) drug users. Ages ranged from 22 to 50 (mean, 38) years.

Pathologic examination was performed by members of the “French Study Group of Pathology for HIV-Associated Tumors.” Tumors typed as IL or BL according to the international working formulation were selected for molecular analysis. All tumors were B-cell lymphomas, according to immunologic studies performed on frozen sections using monoclonal antibodies to detect B-cell antigens and Ig heavy and light chains (data not shown). Seven tumors (cases 1 through 7) were diagnosed as small noncleaved cell lymphoma of Burkitt type. Six tumors were IL, of which 5 (cases 9 through 13) showed marked plasmacytic differentiation. One sample (case 14) was typed as lymphoproliferative with plasmacytic differentiation (LP) according to the initial description of Nalesnik et al22 in posttransplant lymphoproliferative diseases. Case 8 was diagnosed as intermediate between IL and BL. This tumor possessed features of small noncleaved cell lymphoma of the Burkitt type. The architecture was composed mainly of large cells with interspersed macrophages, giving rise to a “starry sky” pattern. A high level of apoptosis and a strong cohesiveness of the cells were observed. However, there was a polymorphism in the size of the tumor cells with presence of an important percentage of small cells (Fig 2). Most of the large cells possessed a large unique nucleolus, as seen in IL in contrast to the multiple and paracentral nucleoli of the small cells.

The majority of the cases had a nodal presentation. Extralodal presentation was observed in 5 patients: 1 had a subcutaneous mass, 3 had buccal involvement, and 1 had splenomegaly. Four patients had bone marrow (BM) involvement at diagnosis (Table 1). Two patients (cases 5 and 8) have developed a generalized reactive lymphadenopathy before onset of the lymphoma.

Patients received polychemotherapy, except for two: one representative of the first or third exon of the c-myc gene38 (Fig 1A). Rearrangements 3' of c-myc in pvt-1 or bvr-1 regions were analyzed using the probes 455 and 550.39 These genomic probes represent parts of the bvr-1 and pvt-1 loci, respectively situated 140 and 260 kb 3' of the c-myc locus, which are frequently involved in the t(2;8) and t(8;22) variant translocations3940 (Fig 1A). The search for rearrangements 5' of c-myc was performed using the probe p3809j.31 SS (kindly provided by F. Haluska) situated at 175 kb 5' of c-myc.42

The organization of the IgH locus was analyzed by hybridization of BamHI-, EcoRI-, HindIII- and Xba I-digested DNA to probes specific of the joining region (JH) and of the Cμ gene (Fig 1B). The χ light chain was studied using a 2-kb J χ and a 2.5-kb C χ fragment.

Table 1. Pathologic Diagnosis and Clinical Characteristics of the HIV+ Patients with NHL

<table>
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<tr>
<th>Case</th>
<th>Sex/Age</th>
<th>Risk Factor</th>
<th>Major Location</th>
<th>Diagnosis</th>
<th>Treatment</th>
<th>Survival (mo)</th>
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<td>BL</td>
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<td>BL</td>
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<td>M/31</td>
<td>Ho</td>
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<td>BL</td>
<td>PC</td>
<td>Dead (7)</td>
</tr>
<tr>
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<td>BL</td>
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</tr>
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<td>BL</td>
<td>PC</td>
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<tr>
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<td>BL</td>
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<td>BL</td>
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<tr>
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<td>IL</td>
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<tr>
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<td>IL</td>
<td>PC</td>
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<td>M/30</td>
<td>Het/splenic</td>
<td>Spleen</td>
<td>LP</td>
<td>PC</td>
<td>Dead (1)</td>
</tr>
</tbody>
</table>

Abbreviations: Het/xPart: heterosexual with multiple partners; Ho, homosexual; IVD, intravenous drug user; LN, lymph node; SCM, subcutaneous masses; IB, intra-buccal; BL, Burkitt’s lymphoma; IL, immunoblastic lymphoma; LP, lymphoproliferative with plasmacytic differentiation; PC, polychemotherapy.
died shortly after diagnosis and the other could not be observed.

Detection of myc Rearrangements

In eight tumors, we identified rearrangements within the c-myc gene (Fig 3A) Because genetic polymorphisms in the c-myc gene are extremely rare, we interpret these as a result of chromosomal rearrangements. In case 9, with a diagnosis of IL, a faint rearranged c-myc fragment was observed. Therefore, the Southern analysis was repeated using EcoRI and HindIII, confirming the initial result (Fig 3B). In one tumor classified as BL, a rearrangement on chromosome 8 could neither be detected by Southern blotting using a c-myc, pvt-1 or bvr-1 probe, nor using a probe detecting tumor classified as BL, a rearrangement on chromosome 8 Therefore, the Southern analysis was repeated using EcoRI and HindIII, confirming the initial result (Fig 3B). In one tumor classified as BL, a rearrangement on chromosome 8 could neither be detected by Southern blotting using a c-myc, pvt-1 or bvr-1 probe, nor using a probe detecting tumor classified as BL, a rearrangement on chromosome 8 Therefore, the Southern analysis was repeated using EcoRI and HindIII, confirming the initial result (Fig 3B). In one tumor classified as BL, a rearrangement on chromosome 8 could neither be detected by Southern blotting using a c-myc, pvt-1 or bvr-1 probe, nor using a probe detecting tumor classified as BL, a rearrangement on chromosome 8 Therefore, the Southern analysis was repeated using EcoRI and HindIII, confirming the initial result (Fig 3B)

Chromosome 14 Breakpoints

3p. Breakpoints were assumed to be located within the switch μ region if two criteria were fulfilled: (1) hybridization of the JH and Cμ probes to different EcoRI and BamHI fragments indicating a noncontiguous configuration of JH and Cμ, and (2) comigration of fragments visualized by a Cμ and a c-myc exon 3 probe after digestion with BamHI, EcoRI, and HindIII. According to these criteria, 3 of the 8 cases with c-myc gene rearrangement (cases 2, 5, and 9) had a breakpoint within the μ switch region (Table 2).

JH. In two cases (cases 4 and 8) Cμ and JH probes hybridized to identical BamHI and EcoRI fragments, indicating a contiguous configuration of JH, 3μ, and Cμ on both alleles. However, hybridization with JH showed a third fragment different in size from the nonarranged JH gene provided by the stromal cells. This third rearranged JH fragment did not hybridize to a Cμ probe. Visualization of a third fragment was taken as an indication that the joining region is transected on one of both Ig heavy-chain alleles49 (Fig 4). The most likely explanation is that one heavy chain is functionally rearranged and that the other is fused to chromosome 8 sequences within the region covered by the JH probe.

Breakpoints in the IgH gene locus of the three other tumors could not be localized by this method (Table 2), probably because the rearrangements affected the genes of other Ig heavy-chain isotypes that have also been shown to participate in c-myc translocations.

Study of Clonality

Southern blot analysis of Ig genes permits to discriminate between monoclonal or polyclonal proliferations. In the case of a monoclonal proliferation, neoplastic cells present one or two identical rearrangements in all cells corresponding to a rearrangement on one or both Ig alleles. Thus, monoclonality is shown by the presence of one or two bands in a position different from that of the unrearranged germ-line configuration as exemplified in Fig 4.

In contrast, polyclonal proliferations are composed of neoplastic cells carrying different Ig rearrangements. Each is expected to give a discrete band but the signal is too weak to be identified, and in fact nothing but the germline band can be identified by Southern blot analysis. Using the Cμ and the JH probes and at least two enzymes, all BL were monoclonal, whereas IL or LP were composed of monoclonal or polyclonal proliferations (Table 3). The Southern blot hybridization of two polyclonal proliferations using three different enzymes and the Cμ and JH and Jκ probes is presented in Fig 5.

Presence of EBV Sequences

The presence of EBV was detected in all cases of IL and LP and in 4 of 7 BL using the BamHI-W and the 1.9-kb XhoI probe (Table 3). The intensity of the signals obtained after hybridization was higher in monoclonal, than in polyclonal cases (Fig 6) with the exception of case 4 (not shown). For the three polyclonal cases, comparison with a cell line containing two copies of EBV DNA (Namalwa) indicated the presence of about one to two copies per cell.

Fusion of the terminal repeats gives rise to the episomal conformation of the EBV genome in lymphoid tumors. The number of terminal repeats, and thus the size of the fused terminal fragment, is variable in different infected cells. Southern blot hybridization using the 1.9-kb XhoI probe has been used to study the clonality of EBV-carrying tumors.45 A single fused terminal fragment is interpreted as evidence for monoclonality, the presence of two or more terminal fragments is taken as evidence for oligoclonality or polyclonality,45,49,51

Seven of the 8 monoclonal proliferations were monoclonal according to the criteria described above (Fig 6A). However, one case (case 1, Fig 6B) showed one or two additional bands after digestion with BamHI, HindIII, and XhoI. The monoclonality of this case is beyond any doubt. The molecular analysis of Ig and c-myc rearrangements of EBV-carrying tumors indicated by Southern blot analysis of Ig gene rearrangements, oligoclonality or polyclonality was confirmed using the EBV terminal repeat probe and the restriction enzymes BamHI and EcoRI (case 14, Fig 6C). The restriction enzyme HindIII generates very large fragments spanning the termini that cannot be resolved by conventional electrophoresis in case of polyclonality. All other cases showed one major band on the background of a smear, as exemplified for case 12 in Fig 6C. This type of pattern is not conclusive for either monoclonality or polyclonality, as detailed in the Discussion. Therefore, diagnosis of monoclonality or polyclonality is based on the unequivocal results obtained by the analysis of Ig gene rearrangements.

DISCUSSION

t(8;14), t(8;22), and t(2;8) translocations leading to rearrangements between the c-myc and the Ig loci are a constant
characteristic of BL, a tumor composed of small non-cleaved cells with high mitotic activity. The so-called "small noncleaved nonBurkitt lymphoma", made of small cells with a pleomorphism in size and shape and with unique central nucleoli, has also been found to harbor one of the typical BL translocations, and is considered as a variant of BL. The small to medium size of these tumors, as well as the high nuclear cytoplasmic ratio, clearly differentiate these tumors from immunoblastic lymphomas that represent a monotonous proliferation of large cells with a large nucleus with a central unique nucleolus and a large amount of cytoplasm.
Among the 14 tumors studied here, 7 had the characteristic morphologic features of BL. All 7 tumors were of monoclonal origin; 6 showed a c-myc rearrangement and 4 harbored EBV DNA sequences. Case 1 was clearly monoclonal with regard to Ig and c-myc rearrangements. However, it showed more than one band after hybridization with the 1.9-kb Xho I probe. This shows that additional bands detected by this probe have to be interpreted with caution. They are not indicative for oligoclonality or polyclonality per se. There is a number of reasons why monoclonal tumors may harbor more than one EBV-terminal fragment. One obvious reason is the coexistence of episomal and integrated copies as recently reported for Burkitt’s lymphoma cells.57 Another possibility that is difficult or impossible to exclude is homologous recombination among individual episomal genomes. Finally, reinfection of cells carrying already EBV genomes has been reported to occur in vitro.58

Our data confirm previous studies showing the presence of EBV in about one-half of the BL cases in HIV+ individuals1,26,31 (Table 3). Remarkably, neither of the patients with
BL presented with opportunistic infections or Kaposi sarcoma at the time of diagnosis (Table 3). This is in line with the observation of Kalter et al13 that BL is often an early or even the first manifestation of AIDS.

Four of 6 lymphomas with morphologic features of IL as well as the case diagnosed as lymphoproliferation were associated with EBV and were devoid of c-myc rearrangement. Three of them were polyclonal and 2 were monoclonal according to the analyses of the Ig gene rearrangements. The polyclonal cases exhibited a comparatively small number of viral genome copies per cell and it remains an open question as to whether in fact all cells carried the viral DNA within the pathologic lesions. However, even if the EBV load is low in these lymphoproliferations compared with the monoclonal cases including EBV+ Burkitt lymphomas, the detection of viral sequences within a clinical specimen by conventional Southern blotting is indicative of an EBV-associated lymphoproliferation. In 50 HIV+ patients with persistent generalized lymphadenopathy, it has not been possible to detect EBV by conventional Southern blotting.59 EBV has only been detected in persistent generalized lymphadenopathy from HIV-infected patients by polymerase chain reaction (PCR).60

Clonality was studied not only by Ig gene rearrangement analyses, but also using an EBV terminal repeats specific probe. Analyses of two of the polyclonal cases with the terminal repeat probe of EBV showed a smear with one band predominating (Fig 6C). At first sight, this suggested the presence of a minor occult clonal population within the polyclonal lymphoproliferations that remained undetected by Ig gene rearrangement analysis. However, this conclusion is unlikely to be correct for the following reasons. Herpes viruses carry quite variable numbers of terminal repeats at the ends of their linear viral genome. However, the variation in terminal repeat copy number is much smaller if the number of terminal repeats from the left and right end of individual linear DNA molecules is compiled.61,62 The reason is probably that only DNA molecules within a given range of length can be packaged. Circular genomes contain the terminal repeats from both ends. Therefore, the number of repeats is less variable than the number of repeats from individual ends. Because the number of repeats follows a Poisson distribution, molecules with an average number of repeats will predominate. This is exactly what has been observed by us and others, ie, a minor band in the middle of a smear (Fig 6C).60 Therefore, the existence of a band on the
Fig 4. Southern blot analysis of case 8 using the JH gene as a probe. Fibroblasts are used as control. DNAs were digested with EcoRI (lanes 1 and 4), HindIII (lanes 2 and 5), and XbaI enzymes (lanes 3 and 6), and hybridized with a 3.1-kb EcoRI-HindIII JH probe. Lanes 1, 2, 3 represent the germline configuration (fibroblasts). Lanes 4, 5, 6 represent DNA digestions of case 8. Fragment sizes are given in kilobases (kb).

Table 3. Immune Status of Patients at the Time of Diagnosis and Characteristics of the Tumors

<table>
<thead>
<tr>
<th>Case No.</th>
<th>OI</th>
<th>KS</th>
<th>CD4</th>
<th>Diagnosis</th>
<th>Clonality*</th>
<th>c-myc</th>
<th>EBV†</th>
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<td>LP</td>
<td>P</td>
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</table>

CD4⁺ lymphocytes: (N) 729 ± 253/mm³.
Abbreviations: OI, opportunistic infections; KS, Kaposi sarcoma; BL, Burkitt’s lymphoma; IL, immunoblastic lymphoma; LP, lymphoproliferation with plasmacytic differentiation; R, rearranged; G, germline; M, monoclonal; P, polyclonal; ND, not done.
* Results on clonality are based on Southern blots hybridized with JH, Ck, and Cκ probes, which in all cases gave concordant results.
† Analyzed with an BamHI-W as well as the 1.9-kb XhoI probe.
sis of these tumors. They may represent a new disease entity of HIV-infected patients, as proposed for the EBV− peripheral polyclonal lymphomas, or they may represent a modification of tumors also encountered in patients without AIDS. We favor the latter hypothesis for the following reasons. In vitro, studies have clearly shown that BL cells have the potential to progress towards different morphotypes. Tumor cells, suspended from a biopsy and seeded into culture, present as a uniform population of small cells. However, after only a few passages in culture, they may give rise to cells with quite different morphology. Some tumors retain the typical morphology of BL in vitro, others evolve towards a morphology indistinguishable from IL. EBV− cell lines were more stable in culture in maintaining small size (16 of 19 EBV− cell lines), whereas EBV+ lines displayed a much higher tendency to develop in large cells (19 of 33 cell lines). Of the cases with large irregular morphology, 7 of 8 were EBV+, and progression to the immunoblastic morphotype has been observed exclusively within EBV+ cases. This finding suggests an active role of the virus in vitro, cultured cells for morphologic progression towards increased cell size and irregular or immunoblastic morphotype. The
Fig 6. Southern blot analysis using the EBV 1.9-kb Xho I probe flanking the terminal repeats. After digestion with BamHI (B) and/or EcoRI (E), HindIII (H), and Xba I (X). Cases shown in (A) and (B) are monoclonal; those shown in (C) are polyclonal according to Ig gene rearrangements.
change in morphology most likely reflects the change in the phenotype of Burkitt lymphoma cells after explantation of the tumor cells into culture described by Rickinson et al. 66-69

Thus, we hypothesize that the two EBV+ tumors with a c-myc rearrangement have adopted morphologic features of IL caused by the severe perturbation of the immune system in these patients. Immunohistologic analyses are in progress to correlate the morphologic features of these tumors with the pattern of cell-surface antigen as well as viral-gene expression. Another interesting correlation has recently been observed between morphology and interleukin-6 (IL-6) expression in lymphomas that had developed in HIV seropositive and negative patients. 70 A high number of IL-6 producing stromal cells has been observed in IL but not in BL. Remarkably, the case with a morphology intermediate between IL and BL reported here has been included in this study and was found to harbor a high number of IL-6-expressing cells. This raises the question as to whether IL-6 is responsible for morphologic transformation towards immunoblasts. IL-6 receptor has been reported to be expressed in immunoblasts. It will also be interesting to see whether EBV can modulate expression of this receptor.

ACKNOWLEDGMENT

We are most grateful to K. Lenner (Kiel) for expert morphologic advises on difficult cases and to H. Stein (Berlin) for helpful discussion. We would like to acknowledge F. Haluska (Boston) for supplying some probes and M. Pulik, A. Gauthier (Argenteuil), J.P. Flejoux (Beaujon), J. Gabbarre (Paris), and T. Philip (Lyon) for making available the samples.

APPENDIX


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Variable morphology of human immunodeficiency virus-associated lymphomas with c-myc rearrangements. The French Study Group of Pathology for Human Immunodeficiency Virus-Associated Tumors, I

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