Multiple Genetic Lesions in Acquired Immunodeficiency Syndrome-Related Non-Hodgkin’s Lymphoma

By Paola Ballerini, Gianluca Gaidano, Jerry Z. Gong, Vittorio Tassi, Giuseppe Saglio, Daniel M. Knowles, and Riccardo Dalla-Favera

Non-Hodgkin’s lymphoma (NHL) develops in about 5% to 10% of acquired immunodeficiency syndrome (AIDS) patients. The vast majority of AIDS-NHL are clinically aggressive B-cell NHL that are histologically classified as small noncleaved cell lymphoma (SNCC), large cell immunoblastic plasmacytoid lymphoma (LC-IBPL), and large noncleaved cell lymphoma (LNCC). In an attempt to understand the molecular pathogenesis of these tumors, we have investigated the involvement of dominantly acting oncogenes (c-myc, N-, K-, H-Ras), tumor suppressor genes (p53, RB1), and Epstein-Barr virus (EBV) infection in 27 AIDS-NHL samples (16 SNCC, 5 LC-IBP, and 6 LNCC). The following lesions were detected in AIDS-NHL: EBV infection (10/24; 41.6%), c-myc rearrangement (19/24; 79.1%), Ras mutation (4/27; 14.8%), and p53 loss/mutation (10/27; 37.0%). These lesions are not uniformly distributed, but, rather, cluster with specific types of AIDS-NHL: EBV infection is preferentially associated with LC-IBPL (4/4; 100%), while it is present in only a fraction of SNCC (5/16; 31.2%) and LNCC (1/4; 25%); c-myc oncogene activation clusters with SNCC (16/16; 100%), whereas it is less frequent in LC-IBPL (1/4; 25%) and LNCC (2/4; 50%); p53 inactivation is restricted to SNCC (10/16; 62.5%) and consistently associated with c-myc activation. These data show that AIDS-NHL are associated with multiple genetic lesions that involve both proto-oncogenes and tumor suppressor genes and may accumulate in the relatively short period of time (4 to 6 years) between human immunodeficiency virus infection and AIDS-NHL development. These genetic lesions differ in the various AIDS-NHL subtypes, suggesting the involvement of distinct molecular pathways.

INDIVIDUALS AFFECTED by acquired immunodeficiency syndrome (AIDS) develop non-Hodgkin’s lymphomas (NHL) at a frequency 60 times higher than the general population. AIDS-related NHL (AIDS-NHL) have been consistently reported in human immunodeficiency virus (HIV)-infected individuals since the outbreak of the AIDS epidemics in 1982, and since 1985 the development of high-grade NHL in high-risk populations has been included among the diagnostic criteria of AIDS. Their incidence has steeply increased in the last few years because of improved treatment and the longer survival of AIDS patients. Indeed, some estimates project that 10% to 20% of all new NHL cases in the United States may eventually be related to AIDS.

AIDS-NHL are almost invariably derived from B cells. They usually present as a systemic NHL, although the gastrointestinal (GI) tract and the central nervous system (CNS) represent the primary site of the tumor in a significant fraction of cases. Among systemic AIDS-NHL, 70% of the cases are classified according to the Working Formulation as high-grade NHL and display a histology consistent with small noncleaved cell lymphoma (SNCC) or large cell immunoblastic-plasmacytoid lymphoma (LC-IBPL), while 30% of cases are of intermediate-grade, histologically defined as diffuse large noncleaved cell lymphoma (LNCC). When compared with histologically similar lymphoid neoplasms arising in immunocompetent patients, systemic AIDS-NHL display distinctive features, including poor prognosis and the frequent involvement of extranodal sites, particularly the GI tract, liver, CNS, and bone marrow (BM).

Many reports have focused on the clinico-pathological features of AIDS-NHL; however, far less is known about the genetic alterations underlying the pathogenesis of these tumors. Previous observations have indicated an association with chromosomal translocations involving the c-myc oncogene. In addition, Epstein-Barr virus (EBV) infection of the tumor cells has also been detected in 40% to 50% of systemic AIDS-NHL, and it has been suggested to play a pathogenetic role based on the observation that viral infection precedes clonal expansion in these tumors. Although EBV infection associated with c-myc oncogene activation is sufficient for the malignant transformation of human B cells in vitro, the identification of AIDS-NHL lacking one or both of these lesions suggests that alternative genetic lesions may be involved in the development of AIDS-NHL in vivo. In this respect, the role of several dominantly acting oncogenes relevant to B-cell lymphomagenesis, such as bcl-2 and bcl-1, as well as the presence of viral genomes other than EBV, has been ruled out by previous studies. On the other hand, the role of tumor suppressor gene inactivation has not yet been investigated, although recent evidence suggests a role for these lesions in the pathogenesis of certain lymphoid malignancies.

Based on these observations, this study has been aimed at a comprehensive analysis of a relatively large panel of cases to identify lesions associated with the pathogenesis of the various subtypes of AIDS-NHL. We found that AIDS-NHL can be characterized by the accumulation of multiple distinct genetic lesions, including EBV infection, c-myc oncogene activation, and loss of tumor suppressor genes. These lesions cluster with specific subtypes of AIDS-NHL, suggesting a role for distinct molecular pathways in the pathogenesis of these tumors.
genetic lesions within the same tumor. These lesions differ in various AIDS-NHL histologic subtypes, suggesting that alternative molecular pathways may be associated with AIDS-lymphomagenesis.

MATERIALS AND METHODS

Pathologic samples. Biologic samples of lymph node, BM, peripheral blood, or other involved organs from 27 patients with AIDS were collected during standard diagnostic procedures. Approval was obtained from the institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki. In all instances, the specimen was collected at diagnosis before specific therapy. Most samples were derived from patients referred to institutions within the United States (Department of Pathology, New York University, New York, NY; and Division of Surgical Pathology, Department of Pathology, Columbia University, New York, NY); three cases were derived from the Università di Torino, Italy. The histology of all cases has been reviewed by D.M.K. to ensure uniformity of diagnostic criteria. Diagnoses were based on analysis of hystopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of Ig gene rearrangement. For most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytofluorimetric analysis. For most samples, a monoclonal cell suspension of greater than 95% viability was prepared by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation.

DNA extraction and Southern blot analysis. DNA was purified by digestion with proteinase K, extraction with phenol/chloroform, and centrifugation.

DNA probe. The organization of the c-myc locus was analyzed by hybridization of EcoRI and HindIII digested DNA to the human c-myc probe MC413RC, representative of the third exon of the c-myc gene. The presence of the EBV genome was investigated with a probe specific for the EBV genomic termini (5.2-kb BamHI fragment). To study the RBI locus, two subclones were derived from the full-length RBI cDNA (kind gift of Dr S. Friend, Harvard Medical School, Charlestown, MA) and used in Southern Blot analysis: probe 5'-RBI (7.4-kb Hpal/EcoRI fragment) spanning exons 1 through 9, and probe 3' RBI (EcoRI/EcoRI 3.9-kb fragment) spanning exons 10 through 27.

Oligonucleotide primers. All the oligonucleotides used for polymerase chain reaction (PCR) amplification in this study were synthesized using an Applied Biosystems (Foster City, CA) synthesizer. Names and sequences of p53 primers used in the present study have been previously reported. To analyze the first exon-first intron boundary region of the c-myc gene the following pairs of primers derived from the c-myc locus sequence were used: F5', 5'-GACTGACAACTCAACACCC-3'; G5', 5'-CTCGACGACACCCCTCTTTC-3'; and F3', 5'-GGTGCTACCTCCTGTGTTTCAA-3'; G3', 5'-ATTACCTCCAATCAGT-C-3'; and 5'-TTTACCCCTGACAGTTCTCTG-3' (nucleotides 3041 through 3060). The oligonucleotides used to amplify sequences of K-Ras and H-Ras exons 1 and 2, as well as N-Ras exon 2, have been previously described; the following primers have been used to amplify sequences of N-Ras exon 1: N1A, 5'-GACTGGTACAACTCAACACCC-3'; and N1B, 5'-GGTGCTACCTCCTGTGTTTCAA-3'; and 5'-TTTACCCCTGACAGTTCTCTG-3'. The following pairs of primers were derived from the sequence of the RBI gene and were used to study RBI exons 10 through 27: exon 10, RBI 10-5, 5'-GTGTGCTCCTGACATGTTATCT-3'; and RBI 10-3, 5'-TACCTATATCATGATTCAACC-3'; exon 11, RBI 11-5, 5'-GATGCATAAGCAACAAATTGG-3'; and RBI 11-3, 5'-GAAACACTATAAGCCTGAT-3'; exon 12, RBI 12-5, 5'-CCITCATGCTTAACACTT-3'; and RBI 12-3, 5'-AGAATCACTATTTAATTCT-3'; exon 13, RBI 13-5, 5'-TCTCTGTTACAGTATCTCTC-3'; and RBI 13-3, 5'-ATACAGGCTGAAAGATGCT-3'; exon 14, RBI 14-5, 5'-CAGTGAGACTCTCATTCAAAA-3'; and RBI 14-3, 5'-TCCCAAGGGTCGGTTGATTG-3'; exons 15 and 16, RBI 15b, 5'-ATGCAGCCACAAAAAATGTT-3'; and RBI 15b, 5'-AAAGAAACACACACACCTTAAAA-3'; exon 17, RBI 17-5, 5'-CTCAAGGGTTAAATCTTTCAT-3'; and RBI 17-3, 5'-CCATATGCATATGAAATAGTATAT-3'; exon 18, RBI 18-5, 5'-ATATGATCCCCTTGGAAATA-3'; and RBI 18-3, 5'-GACCTCATTGCTTAAATCTCA-3'; exon 19, RBI 19-5, 5'-GTATGATATACTCTGATCC-3'; and RBI 19-3, 5'-ACATGTATGGACCGACTA-3'; exon 20, RBI 20-5, 5'-TCTATTGTTAACTTAAAGT-3'; and RBI 20-3, 5'-ATCATGTTAAAGTAAAGTAGAT-3'; exon 21, RBI 21-5, 5'-CCATGTAAATTAATCTTGGC-3'; and RBI 21-3, 5'-GTATGTTATTTGATGTAGAT-3'; exon 22, RBI 22-5, 5'-ATAUTGTCCTTCTACAGTCC-3'; and RBI 22-3, 5'-GGCTGGACCTATTACATTGAGA-3'; exon 23, RBI 23-5, 5'-TCTATTGTTAAGGTGCAGG-3'; and RBI 23-3, 5'-CCTTGGTACCACAAATATCTCC-3'; exon 24, RBI 24-5, 5'-GCTTACCCTCTGTGCAAATTGG-3'; and RBI 24-3, 5'-TGAGGTTTTTGGATATGATC-3'; exon 25, RB 25-5, 5'-GGTTCTACATGTAAGACCC-3'; and RB 25-3, 5'-GACCTGTCGTGACTCTC-3'; exon 26, RB 26-5, 5'-GCACTTACATTGGAATAAC-3'; and RB 26-3, 5'-GACCTCTGTGAACTACCA-3'; RB 27-5, 5'-GCACTTGCCAACTTACCA-3'; and RB 27-3, 5'-CTGTGAGACAACTGATTC-3'.

Single strand conformation polymorphism (SSCP) analysis. SSCP analysis was modified from Orita et al. Briefly, PCRs were performed with 100 ng of genomic DNA, 10 pmol of each primer, 2.5 mmol/L dNTPs, 1 μCi of [α-32P]dCTP (NEN, Boston, MA; specific activity, 3,000 Ci/mmol; 1 Ci = 37 GBq), 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl2, 0.01% gelatin, 0.5 U Taq polymerase (Cetus, Emeryville, CA), in a final volume of 10 μL. Thirty cycles of denaturation (94°C), annealing (annealing temperatures were optimized for each pair of primers), and extension (72°C) were performed as previously reported. Briefly, 10 pmol of one of the two primers used for DNA amplification was labeled with [γ-32P] adenosine triphosphate (ATP) (NEN; specific activity, 3,000 Ci mmol) by means of T4 polynucleotide kinase (Biolabs, Beverly, MA) at 37°C, Neutralized, transferred to Duralose filters (Strategene, La Jolla, CA) and neutralized. Transferred to Duralose filters (Strategene, La Jolla, CA). DNA was digested with restriction endonuclease, electrophoresed in a 0.8% to 1% agarose gel, denatured, neutralized, transferred to Duralose filters (Strategene, La Jolla, CA) and hybridized to probes that had been 5'P-labeled by the random primer extension method. Filters were washed in 0.2 X SSC (NaCl/Na citrate) 0.5% sodium dodecyl sulfate (SDS) (NaDodSO4) for 2 hours at 60°C and then autoradiographed using intensifying screens (Quanta III; Dupont, Boston, MA).

For personal use only.on October 30, 2017. by guest

www.bloodjournal.org
after electrophoresis in low-melting point agarose (BRL, Gaithersburg, MD), were denatured and annealed in the presence of 2 pmol of labeled primer. Sequencing reactions were performed with reagents supplied in a T7 polymerase sequencing kit (USB, Cleveland, OH), following the manufacturer’s specifications. Both strands were sequenced for each DNA segment analyzed.

Analysis of data. Statistical analysis of data was performed using Fisher’s exact test.”

RESULTS

Twenty-seven cases of systemic AIDS-NHL (16 SNCCL, 6 LNCCL, 5 LC-IBPL) were included in the present study; the main clinical features of each patient are reported in Table 1. In all cases tested, a monoclonal pattern of Ig gene rearrangement was observed by Southern Blot analysis using a JH probe on EcoRI, HindIII, or BamHI DNA digests (see Table 1; not shown). The molecular analyses performed included the study of EBV infection, the activation of c-myc and Ras family proto-oncogenes and the inactivation of RB1 and p53 tumor suppressor genes.

EBV infection. On viral infection of the host cell, the termini of the linear intermediate form of EBV DNA are joined intracellularly to form covalently closed episomal DNA. Because EBV genomic termini contain a variable number of tandem repeats, their heterogeneity provides a viral marker that is distinct for each infection event and is a mean to analyze clonality in the EBV-infected tissues. Therefore, when using a probe that recognizes the configuration of episomal viral marker, a monomorphic pattern of episomal EBV genome documents a single infection event preceding clonal expansion of the infected cell.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Risk Group</th>
<th>Histotype</th>
<th>Site</th>
<th>Clinical Stage</th>
<th>Monoclonality</th>
<th>EBV</th>
<th>c-myc</th>
<th>Ras</th>
<th>p53</th>
<th>RB1</th>
<th>No. of Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK808</td>
<td>35</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>Skin</td>
<td>IV</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>DK824</td>
<td>28</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>LN</td>
<td>II B</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>DK573</td>
<td>47</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>UK</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>DK2138</td>
<td>36</td>
<td>M</td>
<td>IVDA</td>
<td>SNCCL</td>
<td>Liver</td>
<td>III B</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>RDF767</td>
<td>29</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>Extrausual</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>DK88D5</td>
<td>UK</td>
<td>42</td>
<td>F</td>
<td>HE</td>
<td>SNCCL</td>
<td>LN</td>
<td>UK</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RDF1168</td>
<td>42</td>
<td>F</td>
<td>HE</td>
<td>SNCCL</td>
<td>PB</td>
<td>IV B</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>RDF784</td>
<td>53</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>Gingiva</td>
<td>I E</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>RDF830</td>
<td>39</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>LN</td>
<td>IV</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>DK1715</td>
<td>49</td>
<td>M</td>
<td>UK</td>
<td>SNCCL</td>
<td>LN</td>
<td>UK</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>RDF955</td>
<td>UK</td>
<td>41</td>
<td>M</td>
<td>UK</td>
<td>LN</td>
<td>Kidney</td>
<td>IV</td>
<td>Y</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>RDF956</td>
<td>UK</td>
<td>41</td>
<td>M</td>
<td>UK</td>
<td>SNCCL</td>
<td>BM</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RDF1294</td>
<td>34</td>
<td>M</td>
<td>IVDA</td>
<td>SNCCL</td>
<td>LN + CNS</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>RDF1295</td>
<td>26</td>
<td>F</td>
<td>HE</td>
<td>SNCCL</td>
<td>Abdomen</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>DK560</td>
<td>29</td>
<td>M</td>
<td>HO</td>
<td>SNCCL</td>
<td>LN</td>
<td>IV</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>RDF1296</td>
<td>23</td>
<td>M</td>
<td>IVDA</td>
<td>SNCCL</td>
<td>BM</td>
<td>IV</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>RDF773</td>
<td>41</td>
<td>M</td>
<td>IVDA</td>
<td>LNCC</td>
<td>LN</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>DK1202</td>
<td>41</td>
<td>M</td>
<td>IVDA</td>
<td>LNCC</td>
<td>Spleen</td>
<td>B</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>DK1452</td>
<td>55</td>
<td>M</td>
<td>HO</td>
<td>LNCC</td>
<td>Parotid</td>
<td>E</td>
<td>Y</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DK1617</td>
<td>36</td>
<td>M</td>
<td>HO</td>
<td>LNCC</td>
<td>Liver</td>
<td>UK</td>
<td>Y</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RDF782</td>
<td>53</td>
<td>M</td>
<td>HO</td>
<td>LNCC</td>
<td>LN</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>RDF834</td>
<td>54</td>
<td>M</td>
<td>HO</td>
<td>LNCC</td>
<td>LN</td>
<td>UK</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>DK63</td>
<td>47</td>
<td>M</td>
<td>HO</td>
<td>LC-IBPL</td>
<td>LN</td>
<td>IV</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>DK64</td>
<td>31</td>
<td>M</td>
<td>HO</td>
<td>LC-IBPL</td>
<td>Plural</td>
<td>IE</td>
<td>Y</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: UK, unknown; M, male; F, female; HD, homosexual; HE, heterososexual with infected partner; IVDA, intravenous drug abuser; SNCCL, small noncleaved cell lymphoma; LNCCL, large noncleaved cell lymphoma; LC-IBPL, large cell immunoblastic plasmacytoid lymphoma; LN, lymphonode; PB, peripheral blood; BM, bone marrow; CNS, central nervous system; Y, yes, indicating the presence of a predominant monoclonal population within the specimen (see text); ND, not done; +, loss or mutation; −, normal.

* Patients were staged according to the Ann Arbor staging system.”

Table 2. Frequency of Molecular Lesions in AIDS-NHL According to Histopathologic Classification

<table>
<thead>
<tr>
<th>Histotype</th>
<th>EBV</th>
<th>c-myc</th>
<th>Ras</th>
<th>p53</th>
<th>RB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCCL</td>
<td>5/16</td>
<td>16/16</td>
<td>3/16</td>
<td>10/16</td>
<td>0/16</td>
</tr>
<tr>
<td>LNCCL</td>
<td>1/4</td>
<td>2/4</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>LC-IBPL</td>
<td>4/4</td>
<td>1/4</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Abbreviations: SNCCL, small noncleaved cell lymphoma; LNCCL, large noncleaved cell lymphoma; LC-IBPL, large cell immunoblastic plasmacytoid lymphoma.
Fig 1. Analysis of EBV termini heterogeneity in AIDS-NHL. DNAs were digested with BamH1 and subjected to Southern blot hybridization using a DNA probe specific for the fused termini of the EBV genome. HL-60, a myeloid leukemia cell line, is used as a negative control. The EBV-positive clonal B-lymphoma cell line Daudi and a lymphoblastoid cell line derived by EBV infection of normal polyclonal B cells (CB33) were used as controls for polymorphic and monomorphic EBV termini, respectively. Representative samples of AIDS-NHL, both positive (DK64, RDF1068, DK63) and negative (RDF1168) for the presence of EBV sequences, are shown.

As shown in Tables 1 and 2, EBV infection was detected in virtually all the LC-IBPL tested (4/4), while only in a minority of SNCCL (5/16) and LNCCL (1/4). A single intense band was present in all the positive cases, indicating monoclonal infection (Fig 1). The size of the hybridizing fragments was consistent with the expected size of fused EBV termini, indicating that EBV is present in the episomal form in infected tumor cells. No small fragments, whose presence would suggest a linearized or integrated form of EBV genome, were detected in any of the AIDS-NHL studied (Fig 1).

The intensity of the hybridization signal is related to the copy number of EBV episomes in the tumor biopsies. After densitometric analysis normalized against a control cell line containing approximately 100 to 200 copies/cell, the estimated number of episomes in the AIDS-NHL samples varies from few to 100 copies/cell (data not shown). Immunofluorescence experiments performed using human α-EBNA-1 antisera showed that EBV was present and expressed within the neoplastic population (data not shown).

Overall, these results define that EBV infection in AIDS-NHL (1) is a frequent event in LC-IBPL, while restricted to only a minority of SNCCL and LNCCL, and (2) where present, precedes clonal expansion, suggesting a pathogenetic role.

c-myc oncoprotein activation. Translocations involving chromosome 8 may lead to c-myc deregulation by different molecular mechanisms in distinct tumor types. In t(8;14) typically associated with sporadic Burkitt’s lymphoma (BL) as well as in a subset of large cell NHL, c-myc is activated by truncations within its first exon, first intron, or flanking sequences, while t(8;14) of endemic Burkitt lymphoma and the variant translocations [t(2;8), t(8;22)] found in sporadic and endemic Burkitt lymphoma are characterized by point mutations or small rearrangements within regulatory regions of the c-myc gene spanning the first exon-first intron border. The pattern of chromosome 14 involvement in t(8;14) is also heterogeneous: in sporadic BL c-myc recombines with the switch region of the IgH locus, whereas the JH region of IgH is preferentially affected in endemic BL.

While c-myc truncations can be detected by Southern blot analyses using restriction enzymes cutting outside c-myc sequences (eg, EcoRI and HindIII), mutational analyses (by PCR-SSCP and PCR-direct sequencing) are required to detect the small mutations in the region surrounding the first exon-first intron border. Therefore, the combination of these two approaches allows the definition of the exact frequency of c-myc involvement as well as the identification of the precise molecular mechanism of c-myc activation in a given tumor type.

Southern blot analyses after digestion with EcoRI and/or HindIII showed a rearranged c-myc allele in a sizable fraction of samples of AIDS-NHL (see Fig 2 for representative examples). Cases displaying a c-myc germline arrangement by Southern blot analyses after digestion with EcoRI and/or HindIII showed a rearranged c-myc allele in a sizable fraction of samples of AIDS-NHL (see Fig 2 for representative examples). Cases displaying a c-myc germline arrangement by...
Southern analysis was studied further to search for small deletions/insertions or point mutations in the region surrounding the first exon-first intron by the PCR-SSCP approach. Two DNA fragments, spanning 165 nucleotides 5' and 179 nucleotides 3' of the first intron-first exon boundary, which are known to harbor mutations in all cases of endemic BL and in those cases with variant translocations (J. Murphy, R. D.-F., unpublished observation, July 1992), were enzymatically amplified. Preliminary experiments using 10 samples harboring independent mutations had demonstrated a 100% concordance between the PCR-SSCP and PCR-direct sequencing approaches (unpublished observation). Mutations were not detected in any of the AIDS-NHLs studied, indicating a normal configuration of the c-myc locus in these cases (not shown).

Altogether, structural lesions indicative of c-myc oncogene activation were detected in 79.1% (19/24) of AIDS-NHL cases tested. According to histology, c-myc lesions were uniformly present in SNCCCL cases (16/16), but limited to a fraction of non-SNCCCL AIDS-NHL (3/8; see Tables 1 and 2). A statistically significant difference in the frequency of c-myc lesions was observed when SNCCCL were compared with LC-IBPL (P = .008), but not when SNCCCL were compared with LNCCL. Based on the location of c-myc breakpoints on chromosome 8 and on the preferential involvement of the IgH switch region on chromosome 14 22,43-50 (and data not shown), AIDS-SNCCCL resemble sporadic BL.

Ras mutations. Activation of the Ras family proto-oncogenes by single nucleotide substitutions at codons 12, 13, and 61 has been associated with a large number of human tumor types. Among lymphoid malignancies, it has been shown that Ras gene mutations are specifically restricted to multiple myeloma and acute lymphoblastic leukemia.42,52 NHL (not including AIDS-NHL) have been consistently found to lack Ras mutations in panels derived from the United States and Europe (and our unpublished results, December 1991), although sporadic NHL cases from Japan may display H-Ras activation.53 The status of the N-, H-, and K-Ras genes in AIDS-NHL was evaluated by a combination of PCR-SSCP and PCR-direct sequencing techniques. For each sample, genomic DNA was amplified with specific pairs of primers to obtain PCR products spanning 110 to 120 bp across codons 12, 13, and 61 of the N-, K-, and H-Ras genes. Analogously to the case of p53,31 in the case of the Ras genes (N-Ras exon 1 PCR fragment was used as an example) dilution curve experiments demonstrated a 1% sensitivity of the SSCP assay in our conditions. Mutations involving one of the Ras genes were detected in four cases of AIDS-NHL (4/27; 14.8%; see Fig 3.

Fig 3. Analysis of Ras mutations in AIDS-NHL by PCR-SSCP (panels A and C) and PCR-direct sequencing (panels B and D). Representative samples are shown for N-Ras exon 1 (codons 12, 13; panels A and B) and N-Ras exon 2 (codon 61; panels C and D). PCR-SSCP (panels A and C): samples were scored positive for mutations when the migration pattern in the SSCP assay was different from the normal control (N). A positive control, harboring a known mutation within the PCR sample tested, was also included for each fragment analyzed (N-Ras exon 1: MOLT-4; N-Ras exon 2: HL-60). PCR-direct sequencing (panels B and D): AIDS-NHL samples scored as positive by the PCR-SSCP assay were further studied by genomic sequence analysis. Each mutation is matched to a normal control DNA. Coding strands are shown for cases DK824/DK64 and noncoding strands are shown for cases RDF955/RDF956. Arrows point to bands corresponding to mutated base pairs. RDF956 and DK64 display a mutation at N-Ras codon 12 and N-Ras codon 61, respectively (see also Table 3).
Table 3. Ras Mutations in AIDS-NHL

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histotype</th>
<th>Ras Gene</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino Acid Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDF956</td>
<td>SNCL</td>
<td>N</td>
<td>12</td>
<td>GGT→GAT</td>
<td>Gly→Asp</td>
</tr>
<tr>
<td>RDF1168</td>
<td>SNCL</td>
<td>N</td>
<td>61</td>
<td>CAA→CAT</td>
<td>Gln→His</td>
</tr>
<tr>
<td>RDF1296</td>
<td>SNCL</td>
<td>K</td>
<td>12</td>
<td>GGT→GAT</td>
<td>Gly→Asp</td>
</tr>
<tr>
<td>DK64</td>
<td>LC-IBPL</td>
<td>N</td>
<td>61</td>
<td>CAA→CAT</td>
<td>Gln→His</td>
</tr>
</tbody>
</table>

Abbreviations: SNCL, small noncleaved cell lymphoma; LC-IBPL, large cell immunoblastic plasmacytoid lymphoma.

for representative examples), including three SNCL and one LC-IBPL (Tables 1 and 2).

The characteristics of Ras mutations in AIDS-NHL are described in Table 3; in three cases the N-Ras gene was involved (two A→T transversions at codon 61 and one G→A transition at codon 12), while in the remaining case, a G→A transition was detected at K-Ras codon 12. The preferential involvement of the N-Ras gene, in contrast to the K- or H-Ras genes, is in agreement with the mutational spectrum usually found in hematologic malignancies.31

In conclusion, Ras mutations, though infrequent and limited to a subset of AIDS-NHL, appear to be a distinctive feature of AIDS-NHL in contrast to NHL of similar histology arising in nonimmunocompromised hosts.

RB1 gene analysis. The human RB1 genomic locus spans approximately 200 kb and is composed of 27 exons.45 Among the several mechanisms of RB1 inactivation described in human tumors, point mutations, introducing stop codons or splicing errors, are most frequently encountered (80% of the lesions), while gross rearrangements or large intra-genic deletions represent a more rare event.54

Southern blot analysis allows the identification of gross rearrangements or partial deletions within the locus, as demonstrated by various studies.22,33; therefore, as a first step, HindIII digest from 24 AIDS-NHL were probed with 0.9- and 3.8-kb RB1 cDNA clones, spanning the 5’ (exons 1 through 9) and 3’ (exons 9 through 27) regions, respectively. A normal genomic pattern was observed in all cases tested (data not shown).

We then undertook a thorough mutational analysis of the RB1 locus by the PCR-SSCP technique (Fig 4). We focused on exons 10 through 27, which include two RB1 domains (between amino acids 393 to 572 and 646 to 772, respectively), which are essential for RB1 function and overlap the location of most naturally occurring mutations of the RB1 gene in human tumors.55,56 Individual exons and corresponding intronic sequences at both ends, in all cases including splicing sites, were PCR-amplified from genomic DNA of the AIDS-NHL cases. Tumor samples harboring known mutations (kind gift of Dr D. Yandell, Harvard Medical School, Boston, MA) were used as positive controls for most exons. Most AIDS-NHL samples tested showed a normal electrophoretic pattern, indicating the absence of mutations within the RB1 domains studied (Fig 4); three cases displayed an altered SSCP migration in exon 26, which, on direct sequence analysis, was found to be caused by a previously reported population polymorphism.57

Overall, our comprehensive analyses by a variety of technical approaches suggests that AIDS-lymphomagenesis is independent of RB1 inactivation.

**p53 gene inactivation.** p53 inactivation in human tumors is most frequently caused by point mutations in the coding sequence of exons 5 through 9 in one allele with or without loss of the other allele.58,59 To define the occurrence of p53 lesions in AIDS-NHL, a two-step strategy was devised, as previously reported.31 p53 exons 5 through 9 were analyzed in genomic DNA of 27 AIDS-NHL cases by the PCR-SSCP technique (Fig 5A). Fragments displaying an altered electrophoretic mobility by SSCP analysis were then reamplified in a separate reaction and analyzed by PCR-direct sequencing.
to confirm and characterize the nature of the mutation (representative examples are shown in Fig 5B).

The overall rate of p53 mutations in AIDS-NHL was 37% (10/27; Table 2). However, p53 mutations were not randomly distributed among the different histologic types of AIDS-NHL; rather, they were found only in SNCCL (10/16; 62.5%) and were consistently absent in all the LNCCCL and LC-IBPL tested (Tables 1 and 2). Analysis of the results by the Fisher's exact test showed that the p53 mutation rate is significantly different in SNCCL compared with both LC-IBPL \( (P = .046) \) and LNCCCL \( (P = .024) \).

The characteristics of p53 mutations in AIDS-NHL are described in Table 4. Overall, p53 inactivation in AIDS-NHL occurs by molecular mechanisms similar to that reported in other types of tumors.\(^ {58, 59} \) In particular, the mutational spectrum identified in AIDS-NHL does not significantly differ from what reported in NHL arising in the immunocompetent host.\(^ {31} \) In most instances, the mutation was represented by a single base-pair substitution (10/13 p53 lesions observed), while three samples displayed gross rearrangements within the amplified product (two deletions and one insertion). The single base-pair substitutions found in AIDS-NHL led to missense (nine events) or nonsense (one event) mutations. Transitions at CpG dinucleotides, the most frequent type of mutation in many human cancers,\(^ {58, 59} \) occurred in 6 of 10 point mutational events. In three AIDS-NHL samples, two p53 lesions were observed in the same tumor, either affecting two different exons of the gene (DK808 and RDF1296; Table 4) or involving two codons of the same exon (DK1715; Table 4). In three other patients, the presence of a p53 mutation was accompanied by the loss of the other allele, as documented by the absence of the wild-type sequence. Our pre-

Fig 5. Analysis by PCR-SSCP and PCR-direct sequencing of the p53 gene in AIDS-NHL. PCR-SSCP (panel A): representative examples are shown for p53 exons 5, 7, and 8. Samples were scored as positive when their migration pattern differed from the normal control (N). PCR-direct sequencing (panel B): samples scored as positive in the PCR-SSCP assay were subjected to genomic sequence analysis to confirm and characterize the mutation. Each mutation is matched to a control sample. The codon involved is indicated. Coding strands are shown for cases RDF956/DK64, RDF830/DK2092, and RDF1168/RDF827. Arrows point to bands corresponding to mutated base pairs. AIDS-NHL RDF956 displays a 26-bp deletion within exon 5; the deleted sequence is indicated in brackets and is normally represented in the control sample DK64.
DISCUSSION

We have investigated the pathogenesis of AIDS-NHL by studying the frequency of EBV viral infection and the involvement of several oncogenes and tumor suppressor genes in these tumors. Overall, our results suggest that AIDS-NHL are associated with multiple genetic lesions underlying distinct molecular pathways in different histologic types, and that some of these pathways represent a peculiar feature of AIDS-NHL as opposed to NHL of similar histology developing in the immunocompetent host.

The association between EBV infection and lymphoid tumors is well established in immunodeficiency settings, including AIDS. A pathogenetic role of EBV in AIDS lymphomagenesis is supported by the well-recognized finding that EBV transforms human B cells in vitro as well as by several lines of indirect evidence. First, it has been shown that one of the immunologic defects present in AIDS selectively impairs the immunosurveillance of EBV infected cells, resulting in their oligoclonal expansion. Second, there is a positive correlation between the appearance of EBV in B-cell clones at the stage of persistent generalized lymphadenopathy (PGL) and the eventual development of EBV-positive NHL. Third, EBV-termini analysis (this report) shows that EBV infection precedes clonal expansion of the malignant cell in all cases. Taken together, these observations suggest that EBV infection, in the context of immunodeficiency, leads to an expansion of a B-cell population at risk for additional genetic accidents leading to NHL. However, it remains to be investigated whether the expression of the putative EBV transforming proteins EBNA-2 and LMP are maintained in AIDS-NHL cells, or is repressed, as it is the case for endemic and sporadic BL.

The presence of EBV infection is not uniformly distributed throughout the different histotypes of AIDS-NHL. Among SNCL the presence of EBV genome is limited to a fraction of samples, similar to that reported for sporadic BL developing in the immunocompetent host. On the contrary, we show that EBV infection represents a distinctive feature of systemic AIDS-LC-IBPL, being present in virtually all the samples tested. Because other NHL characterized by an immunoblastic phenotype, namely AIDS-NHL arising in the CNS and NHL arising in allograft recipients, also display a high rate (virtually 100%) of EBV infection, it is possible that a common genetic mechanism distinctly involving EBV may be associated with the pathogenesis of these malignancies.

This study extends previous observations that c-myc plays an important role in AIDS lymphomagenesis. c-myc is activated in a significant fraction (79.1%) of AIDS-NHL, including 100% of SNCL and a subset of LNCL and LC-IBPL. The molecular mechanisms leading to c-myc activation in AIDS-NHL are similar to sporadic BL, as opposed to endemic BL, in many respects. First, c-myc breakpoints consistently occur within the first exon, first intron, or flanking sequences of the gene in both AIDS-NHL and sporadic Burkitt lymphoma (and this report). Second, the truncated c-myc recombines with the switch region of the IgH locus and the timing of the recombination event is believed to parallel the timing of IgH isotype switching, occurring at a relatively late stage of B-cell development. Finally, the association with EBV infection in AIDS-SNCL (~40%; this report and refs 10 and 22) is very similar to sporadic BL, rather than to endemic BL (100%). Altogether, the example of AIDS-NHL, as well as endemic BL and tumors developing in immunodeficient animals, suggests that c-myc activation is a genetic event frequently associated with lymphomagenesis in certain conditions of immunodeficiency, particularly those associated with chronic B-cell stimulation. A possible explanation for this phenomenon is that c-myc oncogene activation, besides deregulating B-cell growth and differentiation, may also cause other cellular alterations that are positively selected in immunodeficient states. One such alteration may be the downregulation of the integrin receptor LFA-1, which has been shown to be controlled by c-myc in B cells and which is involved in a variety of biologic functions including cell conjugate formation between B cells and cytotoxic T cells and natural killer cells.

p53 inactivation represents a novel finding associated with AIDS-NHL; its pathogenetic relevance is underscored by the specific clustering of p53 lesions with the SNCL histotype. In AIDS-SNCL, p53 inactivation is detected at high frequency (62.5%), almost twice that observed in tumors of the same histology observed in the immunocompetent host.

**Table 4. p53 Mutations in AIDS-NHL**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histotype</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino Acid Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK808</td>
<td>SNCL</td>
<td>213</td>
<td>CGA → TGA</td>
<td>Arg → End</td>
</tr>
<tr>
<td>DK573</td>
<td>SNCL</td>
<td>248</td>
<td>CGG → CAG</td>
<td>Arg → Gin</td>
</tr>
<tr>
<td>DK2138</td>
<td>SNCL</td>
<td>248</td>
<td>CGG → TGG</td>
<td>Arg → Trp</td>
</tr>
<tr>
<td>RDF767</td>
<td>SNCL</td>
<td>245</td>
<td>GGC → AGC</td>
<td>Gly → Ser</td>
</tr>
<tr>
<td>RDF1168</td>
<td>SNCL</td>
<td>248</td>
<td>CGG → CAG</td>
<td>Arg → Gin</td>
</tr>
<tr>
<td>RDF830</td>
<td>SNCL</td>
<td>172</td>
<td>GTT → CTT</td>
<td>Val → Leu</td>
</tr>
<tr>
<td>DK1715</td>
<td>SNCL</td>
<td>281</td>
<td>GAC → GAG</td>
<td>Asp → Glu</td>
</tr>
<tr>
<td>RDF956</td>
<td>SNCL</td>
<td>284</td>
<td>ACA → ATA</td>
<td>Thr → Ile</td>
</tr>
<tr>
<td>RDF1296</td>
<td>SNCL</td>
<td>273</td>
<td>CGT → TGT</td>
<td>Arg → Cys</td>
</tr>
</tbody>
</table>

Abbreviation: SNCL, small noncleaved cell lymphoma.

* The PCR-amplified fragment displayed an abnormal size on agarose gel and the mutation was confirmed by the PCR-SSCP technique; further analysis by PCR-direct sequencing could not be informative because of the superimposition of the other allele.

† The wild type sequence at the mutation site could not be detected, implying loss of one allele, or was faintly visible, consistent with the presence of residual normal cells.
The frequent simultaneous occurrence of p53 inactivation and c-myc deregulation in the same tumor in vivo, observed in AIDS-NHL as well as in BL and L3-type B-cell acute lymphoblastic leukemia arising in the general population, suggests that cells carrying an activated c-myc oncogene may be under pressure to subsequently delete a p53-dependent pathway. This hypothesis is supported by several preliminary observations suggesting that the c-myc protein may be involved in regulation of p53 gene expression.

Finally, mutations of the Ras family proto-oncogenes are present in AIDS-NHL, although in a limited fraction of cases (14.8%). Ras activation appears to be a peculiar feature of AIDS-NHL in contrast with NHL of the same histologic type arising in the immunocompetent host. Indeed, in more than 40 NHL samples of intermediate- and high-grade histology derived from non-AIDS patients, no Ras mutations were detected using a combination of technical approaches (and our unpublished data, December 1991). Several mutagenic carcinogens are capable of inducing Ras mutations; however, since the Ras-positive AIDS-NHL samples were derived from untreated patients, these mutations cannot be attributed to the well-established mutagenic effects of antiviral therapy, although this possibility must also be explored in future studies in treated patients.

Overall, this study documents that the repertoire of genetic lesions differs substantially in different histologic types of AIDS-NHL, and, therefore, AIDS-lymphomagenesis may be associated with distinct molecular pathways. Our analyses of c-myc, p53, RB1, Ras family genes, and EBV infection would suggest that at least two independent patterns of genetic lesions are observed in these tumors: on one side, NHL displaying SNCCCL histology are strictly associated with c-myc deregulation and p53 inactivation, while EBV infection is present only in a subset of these tumors; on the other hand, a monoclonal EBV infection is consistently associated with systemic LC-IBPL AIDS-NHL, where additional unknown lesions may be necessary.

Finally, it is noteworthy that lymphomagenesis in the AIDS patient may be characterized by the accumulation of multiple (up to four) genetic lesions within a relatively short time (4 to 6 years). Other presently uncharacterized lesions may also be present based on the finding that chromosome 6 deletions, involving the site of two putative tumor suppressor genes, are detectable in a sizable fraction of these tumors. The rapid multilesional development of AIDS-NHL is in contrast with the general concept, mainly derived from solid tumors, that a long time period (30 to 40 years) is commonly associated with multistep tumorigenesis. The high proliferative activity of AIDS-NHL may at least in part account for this discrepancy; however, it remains to be explored whether AIDS-associated lymphomagenesis involves the rapid stochastic accumulation of genetic lesions caused by intrinsic genomic instability, or the mutually dependent selection of a number of synergizing lesions (eg, c-myc deregulation and p53 inactivation).

ACKNOWLEDGMENT

We are grateful to Dr D. Yandell for providing us with DNA samples harboring RB1 mutations, to Dr S. Friend for the generous gift of the RB1 cDNA clone, to Dr K. Dunn for help with statistical analysis, and to Drs B. Tycko and J. Krowleski for critically reading the manuscript.

REFERENCES

3. Centers for Disease Control: Revision of the case definition of acquired immunodeficiency syndrome for national reporting—United States. MMWR 34:373, 1985
17. Joachim HL, Dorsett B, Cronin W, Maya M, Wahl S: Acquired immunodeficiency syndrome-associated lymphomas: Clinical, patho-
logic, immunologic, and viral characteristics of 111 cases. Hum Pathol 22:659, 1991


56. Hu Q, Dyson N, Harlow E: The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J 9:1147, 1990


Multiple genetic lesions in acquired immunodeficiency syndrome-related non-Hodgkin’s lymphoma

P Ballerini, G Gaidano, JZ Gong, V Tassi, G Saglio, DM Knowles and R Dalla-Favera