Rapid Communication

Rearrangements of the BCL-6 Gene in Acquired Immunodeficiency Syndrome-Associated Non-Hodgkin's Lymphoma: Association With Diffuse Large-Cell Subtype

By Gianluca Gaidano, Francesco Lo Coco, Bihui H. Ye, Darryl Shibata, Alexandra M. Levine, Daniel M. Knowles, and Riccardo Dalla-Favera

Acquired immunodeficiency syndrome (AIDS)-associated non-Hodgkin's lymphomas (AIDS-NHL), a major source of morbidity and mortality among AIDS patients, are derived from B cells and can be classified into two main histologic categories, small noncleaved cell lymphoma (SNCLL) and diffuse large-cell lymphoma (DLCL). DLCL includes two histologic subsets, ie, large noncleaved cell lymphoma (LNCLL) and large cell-immunoblastic plasmacytoid lymphoma (LC-IBPL). Several studies have shown that AIDS-SNCCL is associated with the clonal accumulation of multiple genetic lesions, including Epstein-Barr virus (EBV) infection, activation of the c-MYC and RAS oncogenes, as well as inactivation of the p53 tumor suppressor gene at variable frequencies. On the contrary, the molecular pathogenesis of AIDS-DLCL is largely obscure, because no genetic lesion other than EBV infection has been specifically identified in this group. In this study, we have tested a panel of 40 AIDS-NHL for structural alterations of BCL-6, a putative proto-oncogene that is frequently altered in DLCL in the immunocompetent host. Our results show that rearrangements of BCL-6 are present in 20% of AIDS-DLCL (6 of 24), including 2 of 8 LNCLL and 3 of 16 LC-IBPL, but in no case of AIDS-SNCCL. BCL-6 rearrangements were detected both in the presence and in the absence of EBV infection of the tumor clone, but in no case were associated with activation of c-MYC or mutations of p53. These data identify a novel genetic lesion in AIDS-DLCL and corroborate the notion that lymphomagenesis in AIDS follows two distinct molecular pathways that are associated with the development of histologically distinct types of AIDS-NHL.

From the Divisions of Oncology and Surgical Pathology, Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY, and the Departments of Pathology and Hematology, University of Southern California School of Medicine, Los Angeles, CA.

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Address reprint requests to Riccardo Dalla-Favera, MD, Division of Oncology, Department of Pathology, College of Physicians and Surgeons, Columbia University, 630 W 168th St, New York, NY 10032.

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arrangements in a panel of AIDS-NHL representative of both the SNCCL and DLCL subtypes. We wished to define the frequency of BCL-6 involvement in AIDS-NHL and to correlate its presence with other genetic lesions that have been described in the pathogenesis of AIDS-NHL.

MATERIALS AND METHODS

Pathologic samples. Biopsy samples of lymph node, bone marrow, peripheral blood, or other involved organs from 40 patients with AIDS were collected during the course of standard diagnostic procedures. Thirty-two samples were derived from patients referred to the Department of Pathology, New York University (New York, NY) or to the Department of Pathology, Columbia University (New York, NY) and their histology was reviewed by D.M.K. Eight samples were derived from patients referred to the Departments of Hematology and Pathology, University of Southern California School of Medicine (Los Angeles, CA) and their histology was reviewed by D.S. Diagnosis was based on analysis of histopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of Ig gene rearrangement. In most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytotoxicometric or tissue section immunohistochemical analysis of cell surface markers and by Ig gene rearrangement analysis.

DNA extraction and Southern blot analysis. DNA was purified by digestion with proteinase K, "salting out" extraction, and precipitation by ethanol. For Southern blot analysis, 6 μg of DNA was digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to a nitrocellulose filter, hybridized overnight at 60°C, washed twice, and autoradiographed using intensifying screens (Quanta III; Dupont, Boston, MA).

DNA probes. Ig gene rearrangement analysis was performed using a JH probe (a gift of Dr S.J. Korsmeyer) on HindIII, EcoRI, and BamHI digests. The organization of the BCL-6 locus was investigated by hybridization of Xba I, BamHI-, and Bgl II-digested DNA to the human BCL-6 probe Sac4.0.36 In selected cases, a second probe representative of the BCL-6 locus, Sac6.8, was also used. 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.44 The presence of the EBV genome was investigated with a probe specific for the EBV termini (5.2-kb BamHI-EcoRI fragment isolated from the fused BamHI terminal fragment Nl-het).45

RESULTS

Forty cases of systemic AIDS-NHL were studied, including 13 SNCCL and 24 DLCL (8 LNCLCL and 16 LC-IBPL). In addition, 3 cases of CD30+ lymphomas, which have been sporadically reported in AIDS,45 were also included. All cases displayed a predominant monoclonal B-cell population as determined by Ig gene rearrangement analysis (data not shown).

Analysis of BCL-6 rearrangements. The BCL-6 gene contains at least 9 exons spanning approximately 26 kb of genomic DNA.28 Sequence analysis has shown that the first exon is noncoding and that the translation initiation codon is located within the third exon.28 Rearrangements of BCL-6 can be detected by Southern blot analysis using a probe (Sac4.0) and restriction enzymes (BamHI and Xba I) that, in combination, explore a region of 15.2 kb containing the 5' portion of the BCL-6 gene28,36 (Fig 1). This same region was previously shown to contain the cluster of chromosomal breakpoints detected in NHL of the immunocompetent host.28,31 Cases showing an abnormally migrating band in only one digest were further studied by hybridizing the Sac4.0 probe to additional digests (Bgl II) or, alternatively, by hybridizing BamHI and Xba I digests to a probe (Sac6.8) derived from the BCL-6 first intron, which, being located 3' of the breakpoint cluster, explores the reciprocal chromosome 3 (Fig 1). Only cases showing abnormally migrating bands with two restriction enzymes and/or two probes were scored as rearranged.

Rearrangements of BCL-6 were detected in 5 of 24 AIDS-DLCL (20.8%), both in the LNCLCL (2 of 8; 25%) and in the LC-IBPL (3 of 16; 18.7%) variants (Table 1 and Fig 1). All cases of AIDS-SNCLCL and CD30+ lymphomas displayed a germline BCL-6 locus (Table 1 and Fig 1). The location of the breakpoints detected in AIDS-NHL corresponds to the pattern most commonly observed in DLCL of the immunocompetent host.28,31 (Fig 1).

Other genetic lesions. The other genetic lesions investigated in the panel of AIDS-NHL included infection by EBV of the tumor clone, activation of the c-MYC and RAS proto-oncogenes, and inactivation of the p53 tumor suppressor gene. The experimental strategies used to investigate these lesions have been described in detail elsewhere.14,15,44 For some of the cases, the molecular characterization of these genetic lesions had been previously reported14,15,45; for the other cases, it has been assessed in the course of this study.

EBV infection was assessed by Southern blot hybridization using a probe representative of the EBV termini46 that allows us to analyze clonality in EBV-infected tissues28 (Fig 2). A monoclonal infection was detected in 5 of 13 (38%) SNCLCL, 17 of 24 DLCL (71%) (3 of 8 [37.5%] LNCLCL and 14 of 16 [87.5%] LC-IBPL), and 3 of 3 (100%) CD30+ cases.

Rearrangements of c-MYC were tested by hybridizing HindIII- and EcoRI-digested DNAs with a probe representative of c-MYC exon 34 (Fig 2). Rearrangements were present in 13 of 13 SNCLCL (100%), 5 of 24 (20.8%) DLCL (2 of 8 [25%] LNCLCL and 3 of 16 [18.7%] LC-IBPL), and 2 of 3 CD30+ cases.

Mutations of p53 and RAS were analyzed by a two-step strategy. Single-strand conformation polymorphism (SSCP) analysis was applied to p53 exons 5 through 9 (in 29 cases) or p53 exons 5 through 8 (in 6 cases) (Fig 2) and to N-, K-, and H-RAS exons 1 and 2 (in 29 cases); cases displaying an altered electrophoretic pattern by SSCP were further studied by DNA direct sequencing of the PCR product. p53 mutations were scored in 8 of 13 (61.5%) SNCLCL, in none of the DLCL tested (0 of 22). Finally, RAS activation by point mutation was positive in 3 of 13 (23%) SNCLCL and in l of 16 (6%) DLCL tested.

The molecular features of the cases displaying BCL-6 rearrangements are listed in Table 2. Overall, BCL-6 rearrangements were detected both in the presence and in the absence of clonal EBV infection of the tumor, whereas c-
BCL-6 rearrangements are involved in approximately 20% of AIDS-DLCL, whereas they are consistently negative in AIDS-SNCCCL. In this respect, BCL-6 rearrangements may be considered the first identified genetic lesion specific for the DLCL type among AIDS-NHL. BCL-6 rearrangements are present in both subgroups of DLCL, i.e., LNCCL and LC-IBPL, and occur both in the absence and in the presence of EBV infection of the tumor clone (Table 2). On the other hand, BCL-6 rearrangements were never detected in AIDS-DLCL carrying c-MYC alterations (Table 2). Future studies of larger series of cases will clarify whether these two genetic
lesions represent mutually exclusive events in the pathogenesis of AIDS-DLCL.

These data are consistent with a model of AIDS-lymphomagenesis that suggests that genetically distinct pathways are specifically associated with different histologic types of AIDS-NHL. The molecular pathway leading to AIDS-SNCCL involves c-MYC rearrangements, p53 mutations, and EBV infection in 100%, 60%, and 40% of the cases, respectively. The presence of somatic hypermutation in the variable regions used by AIDS-SNCCL points to chronic antigen stimulation as an additional mechanism in the development of these tumors. The second genetic pathway is associated with AIDS-DLCL and involves EBV in the large majority of cases, as well as c-MYC and/or BCL-6 rearrangements in a fraction of cases. These distinct pathogenetic mechanisms correlate with a number of clinical features that distinguish AIDS-SNCCL from AIDS-DLCL, including different age at onset and different CD4 counts at the time of lymphoma development.

Although the number of AIDS-DLCL studied is presently limited,3,14-26 the presence of somatic hypermutation in Ig variable regions used by AIDS-SNCCL points to chronic antigen stimulation as an additional mechanism in the development of these tumors. The second genetic pathway is associated with AIDS-DLCL and involves EBV in the large majority of cases, as well as c-MYC and/or BCL-6 rearrangements in a fraction of cases. These distinct pathogenetic mechanisms correlate with a number of clinical features that distinguish AIDS-SNCCL from AIDS-DLCL, including different age at onset and different CD4 counts at the time of lymphoma development.

### Table 1. Frequency of BCL-6 Rearrangements in AIDS-NHL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Clonality</th>
<th>BCL-6</th>
<th>EBV</th>
<th>c-MYC</th>
<th>p53</th>
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<td>LNCCL</td>
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* The DLCL included in the panel can be further distinguished into two subgroups (LNCCL and LC-IBPL), as previously reported.1,2
† NHL expressing the CD30 cell surface antigen.

### Table 2. Molecular Features of AIDS-DLCL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Clonality</th>
<th>BCL-6</th>
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<th>c-MYC</th>
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The results of the analysis of EBV, c-MYC, p53, and RAS of some of these cases have been previously reported.1,4,15,45

Abbreviation: ND, not done.

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**Fig 2.** Analysis of EBV infection (A), c-MYC rearrangements (B), and p53 mutations (C) in AIDS-NHL. (A) Analysis of EBV termini heterogeneity in AIDS-NHL. DNAs were digested with BanHI and subjected to Southern hybridization using a DNA probe specific for the fused termini of the EBV genome. U937, a monocytic leukemia cell line, is used as a negative control. A lymphoblastoid cell line derived from normal polyclonal B cells (NC2) is used as control for polymorphic EBV termini. Representative samples of AIDS-NHL, both positive (DK3794, DK4338, DK2814, and DK3973) and negative (DK3479), are shown. (B) Southern blot analysis of c-MYC rearrangements in AIDS-NHL. Genomic DNAs from the cases shown were digested with HindIII and probed with clone MC413RC, a representative of c-MYC exon 3. A lymphoblastoid cell line (NC2) was used as control for c-MYC germline configuration. Among the cases shown, two cases of AIDS-DLCL (DK3537 and DK1446) display a c-MYC rearrangement. (C) Analysis by PCR-SSCP of the p53 gene in AIDS-NHL. Representative examples are shown for p53 exon 5. Samples were scored as abnormal when differing from the normal control (N). A sample known to harbor a p53 mutation was used as positive control (POS). Among the cases shown, DK1171, a case of AIDS-SNCCL, shows a p53 mutation that was further characterized by direct sequencing of the PCR product.
limited, our results suggest that the frequency of BCL-6 rearrangements in AIDS-DLCL is significantly lower than that in DLCL in the immunocompetent host, where BCL-6 rearrangements occur in more than 40% of the cases. It is possible that the genetic pathogenesis of these two groups of tumors is different, and that the molecular mechanisms active in AIDS-DLCL are characterized by a higher degree of heterogeneity. Among DLCL in the immunocompetent host, BCL-6 rearrangements are associated with distinct clinical features, including the extranodal origin of the lymphoma and the lack of bone marrow involvement. In addition, the presence of this rearrangement appears to represent a favorable prognostic marker. Future studies are warranted to determine whether BCL-6 rearrangements are associated with distinctive clinical features in AIDS-NHL as well.

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