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 Ricardo 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-SNCLL 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 (5 of 24), including 2 of 8 LNCLL and 3 of 16 LC-IBPL, but in no case of AIDS-SNCLL. 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.

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NON-HODGKIN'S lymphomas (NHL) represent one of the most common malignancies associated with human immunodeficiency virus (HIV) infection, and are recognized as an acquired immunodeficiency syndrome (AIDS)-defining condition.1-13 Since their initial observation in 1982,4 the incidence of AIDS-associated NHL (AIDS-NHL) has been consistently increasing,1,3 and they now represent the most frequent HIV-associated malignancy in some AIDS risk groups, namely, the hemophiliacs.3 Indeed, some estimates project that 10% to 20% of all new NHL cases in the United States may eventually be related to AIDS.6 AIDS-NHL are almost invariably B-cell-derived NHL.1,2,7-12 When compared with NHL of similar histology arising in the immunocompetent host, AIDS-NHL display distinctive clinical features, including late stage at presentation, poor prognosis, and the frequent involvement of extranodal sites.1,2,7-12 Systemic AIDS-NHL are histologically heterogeneous, and have been initially classified into three distinct categories, including small noncleaved cell lymphoma (SNCLL), large noncleaved cell lymphoma (LNCLL), and large cell-immunoblastic plasmacytoid lymphoma (LC-IBPL),7,9 Subsequently, most investigators have agreed to classify LNCLL and LC-IBPL as a single category under the term of diffuse large-cell lymphoma (DLCL).13 Some progress has been made in elucidating the molecular pathogenesis of AIDS-SNCLL.1-3 We and others have shown that AIDS-SNCLL is associated at variable frequency with multiple genetic lesions, including Epstein-Barr virus (EBV) infection, c-MYC translocation, RAS gene family mutation, and p53 inactivation by point mutation and allelic loss.1,3,14-26 On the other hand, the pathogenesis of AIDS-DLCL is relatively less defined. EBV infection appears to be the only genetic lesion associated with a significant fraction of these tumors, particularly with the subset displaying plasmacytoid features; p53 lesions have not been found and c-MYC activation is restricted to a small minority of cases.1,3,14-26 Recently, rearrangements of the BCL-6 gene, located on 3q27 and coding for a putative zinc finger transcription factor, have been identified as a recurrent genetic lesion of DLCL in the immunocompetent host.27,28 The same gene has also been called LAZ-30,32 or BCL-5,13 although the name BCL-5 had already been assigned to a locus on 17q2229 and the name BCL-6 has been officially adopted.34 Rearrangements of BCL-6 result in truncation within 5' noncoding regulatory sequences and occur in 30% to 40% of DLCL.28 Cytogenetic analysis has suggested that breakpoints at 3q27, the site of BCL-6, may occur also in AIDS-DLCL,29 although the exact frequency has not been determined and the involvement of BCL-6 has not been documented. These observations have prompted our analysis of BCL-6 re-
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.36 In most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytofluorometric 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.37 For Southern blot analysis,38 6 µg of DNA was digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to Duralon filters (Stratagene, La Jolla, CA), and hybridized to probes that had been39 P-labeled by the random primer extension method.38 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.36 In most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytofluorometric or tissue section immunohistochemical analysis of cell surface markers and by Ig gene rearrangement analysis.

DNA probes. Ig gene rearrangement analysis was performed using an IgH probe40 (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 Sac4.0.73 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.41 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 NJ-het).42

RESULTS

Forty cases of systemic AIDS-NHL were studied, including 13 SNCCL and 24 DLCL (8 LNCCCL and 16 LC-IBPL). In addition, 3 cases of CD30+ lymphomas, which have been described in detail elsewhere, have been previously reported.44 45 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 termini that allows us to analyze clonality in EBV-infected tissues (Fig 2). A monoclonal infection was detected in 5 of 13 (38%) SNCCL, 17 of 24 DLCL (71%) (3 of 8 [37.5%] LNCCCL and 14 of 16 [87.5%] LC-IBPL), and 3 of 3 (100%) CD30+ cases.

Rearrangements of BCL-6 were detected in 5 of 24 AIDS-DLCL (20.8%), both in the LNCCCL (2 of 8; 25%) and in the LC-IBPL (3 of 16; 18.7%) variants (Table 1 and Fig 1). All cases of AIDS-SNCCL 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 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 (Sac0.8) 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.

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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 protooncogenes, and inactivation of the p53 tumor suppressor gene. The experimental strategies used to investigate these lesions have been described in detail elsewhere. For some of the cases, the molecular characterization of these genetic lesions had been previously reported; 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 termini that allows us to analyze clonality in EBV-infected tissues (Fig 2). A monoclonal infection was detected in 5 of 13 (38%) SNCCL, 17 of 24 DLCL (71%) (3 of 8 [37.5%] LNCCCL 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 3′ (Fig 2). Rearrangements were present in 13 of 13 SNCCL (100%), 5 of 24 (20.8%) DLCL (2 of 8 [25%] LNCCCL 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%) SNCCL, but in none of the DLCL tested (0 of 22). Finally, RAS activation by point mutation was positive in 3 of 13 (23%) SNCCL and in 1 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 in AIDS-Associated NHL**

**Fig 1.** Analysis of BCL-6 rearrangements in AIDS-NHL (A, B, and C) and restriction map of the germline BCL-6 locus (D). (A, B, and C) DNAs were digested with BamHI (A) or XbaI (B and C) and hybridized to probes Sac4.0 (A and B) or Sac0.8 (C). The BCL-6 germline bands detected by BamHI (11.4 kb) and XbaI (14 kb) are indicated. U937 was used as a BCL-6 germline control. Among the cases shown, rearrangements were detected in cases DK782, DK827, and DS16, represented by AIDS-DLCL. (D) Exon-intron organization of the BCL-6 gene. Coding and noncoding exons are represented by solid and open boxes, respectively. The transcription initiation site has not been mapped (shaded box on 5' side of first exon). The breakpoints detected in AIDS-NHL are indicated by arrows. Restriction enzyme symbols are S, SacI; B, BamHI; X, XbaI; R, EcoRI. RE, restriction enzyme.

**MYC** alterations and p53 mutations were consistently absent in the cases displaying BCL-6 rearrangements.

**DISCUSSION**

DLCL represents the most frequent type of AIDS-NHL in the HIV-infected adult.6 Despite its epidemiologic relevance, the molecular pathogenesis of these tumors is largely unclarified.3 In this study, we report a novel genetic lesion in AIDS-NHL that appears to be restricted to the AIDS-DLCL histologic type. Our analysis of the genomic configuration of BCL-6 in a panel of AIDS-NHL indicates that BCL-6 rearrangements are involved in approximately 20% of AIDS-DLCL, whereas they are consistently negative in AIDS-SNCLL. 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 the variable regions used by AIDS-SNCCL points to chronic antigen stimulation as an additional mechanism in the development of these tumors.26 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.1,2,8

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

<table>
<thead>
<tr>
<th>DLCL*</th>
<th>SNCCL</th>
<th>LNCCL</th>
<th>LC-IBPL</th>
<th>CD30+ NHL†</th>
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<tr>
<td>2/8</td>
<td>3/16</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.† NHL expressing the CD30 cell surface antigen.43

Table 2. Molecular Features of AIDS-DLCL

<table>
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<tr>
<th>Patient No.</th>
<th>Histology</th>
<th>Clonality</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.14,15,45

Abbreviation: ND, not done.
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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