

MULTIPLE MONOCLONAL B CELL EXPANSIONS AND
c-myc ONCOGENE REARRANGEMENTS IN
ACQUIRED IMMUNE DEFICIENCY SYNDROME-RELATED
LYMPHOPROLIFERATIVE DISORDERS

Implications for Lymphomagenesis

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One significant aspect of the acquired immune deficiency syndrome (AIDS)¹ epidemic is its association with an increased incidence of neoplasia, primarily Kaposi's sarcoma and malignant lymphoma (1). The occurrence of these neoplasms appears to be intimately connected with the condition of impaired immunity present in AIDS, because similar tumors are known to be associated with other types of immunodeficiency, including congenital, drug-induced, and age-related immune deficiency syndromes (1-3). Therefore, studies of the pathogenesis of these neoplasms may provide important information concerning the biology of AIDS and the relationship between immune surveillance and tumor development in general. In particular, the study of neoplasia in patients with AIDS represents a rare opportunity to follow the development of a human tumor from the onset of a strongly predisposing condition, i.e., AIDS, to its biological and clinical appearance.

Based on these considerations, we recently began a series of investigations into the molecular pathogenesis of AIDS-related neoplasms, focusing on the lymphoproliferative disorders that occur in association with AIDS and AIDS-related complex (ARC). This group includes malignant lymphomas, most commonly B cell non-Hodgkin's lymphomas (B-NHL) (4), and T cell leukemias and Hodgkins disease (HD), which recently have been identified in AIDS patients (Knowles, D. M., P.-G. Pelicci, M. Subar, R. Wieczorek, G. Chamulak, J. S. Burke, G.

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¹ *Abbreviations used in this paper:* AIDS, acquired immune deficiency syndrome; ARC, AIDS-related complex; ARV, AIDS-associated retrovirus; BL, Burkitt's lymphoma; B-NHL, B cell non-Hodgkin's lymphoma; HIV, human immunodeficiency virus; LAS, lymphadenopathy syndrome.

Wormser, Z. A. Arlin, and R. Dalla-Favera, manuscript submitted for publication). In addition, AIDS and ARC are characterized by enlarged but apparently benign lymph nodes, commonly referred to as the lymphadenopathy syndrome (LAS), that exhibit the histopathologic features of benign follicular hyperplasia (1). The relationship between LAS and the development of B-NHL is intriguing in terms of epidemiology, because a significant number of patients with LAS develop B-NHL (1), and in terms of pathogenesis, since several biological features of LAS could represent predisposing conditions leading to the development of B-NHL. In particular, polyclonal B lymphocyte activation, aberrant B cell immunoregulation, and massive Epstein-Barr virus (EBV) infection represent conditions present in LAS (1) that are also critically associated with some types of B-NHL, namely African Burkitt lymphomas (BL) (5).

In this study, the possible pathogenetic relationship between LAS and B-NHL was further explored by the comparative analysis of samples of lymph nodes from patients with LAS and B-NHL for the presence of clonal B cell populations, oncogene rearrangements, and human immunodeficiency virus (HIV) sequences. These are critical elements in elucidating a possible multistep process leading to B cell lymphomagenesis in AIDS. The results of our studies support a model in which the multiple clonal B cell expansions detectable in LAS increases the probability of rearrangements/translocations of the *c-myc* oncogene, which represents the consistent pathogenetic marker of AIDS-associated B-NHLs. While EBV is likely to be involved in the development of clonal B cell expansions, HIV sequences are not detectable in AIDS-associated B-NHLs and do not appear to play a direct role in lymphomagenesis.

Materials and Methods

Pathologic Samples and Diagnosis. Representative samples of lymph nodes, peripheral venous blood, and aspirated bone marrow were collected, after informed consent, from high-risk-group adults with AIDS or ARC who received medical care at the New York University Medical Center or at the Westchester County Medical Center. The diagnosis of AIDS or ARC was rendered according to established criteria (6). Similar samples also were obtained, during the course of standard diagnostic procedures, from adult patients who were not at risk for AIDS and who received medical care at the New York University Medical Center during the same time period.

Each lymph node biopsy was classified as benign lymphoid hyperplasia (LAS) or non-Hodgkin's lymphoma (NHL) using histopathologic and immunophenotypic criteria. Each NHL was subclassified histopathologically according to the Working Formulation of NHLs (7). Immunophenotypic analyses for the expression of surface immunoglobulin (sIg), cytoplasmic immunoglobulin (cIg), sheep erythrocyte (E) rosettes, and terminal deoxynucleotidyl transferase (TdT) were determined as previously described (8). B and T cell-associated and restricted differentiation antigen expression was determined with an extensive panel of monoclonal antibodies (anti-HLA-DR, J5, OKT3, OKT4, OKT6, OKT8, OKT10, OKT11, OKM1, OKB1, OKB2, OKB4, OKB7, B1, B2, B4, BA-1, BA-2, Leu-1, Leu-7, Leu-12, Leu-14, Leu-M1, BL1, BL2, BL3, BL4, and BL7), principal characteristics and distribution of reactivity of which have been previously reviewed (9).

Reactivity with each monoclonal antibody was tested on viable mononuclear cell suspensions by indirect immunofluorescence using an argon laser fluorescence-activated cell sorter (FACS 420, Becton-Dickinson Immunocytometry Systems, Mountain View, CA) or a Leitz Laborlux 12 immunofluorescent microscope (10). In the case of lymph node biopsies, these immunophenotypic analyses were supplemented by immunohistologic analyses of cryostat tissue sections using an indirect immunoperoxidase technique (11).

DNA Extraction and Southern Blot Analysis. DNA was prepared by cell lysis, digestion with proteinase K, extraction with phenol, and precipitation with ethanol (12). 20 μg of DNA were digested with the appropriate restriction endonuclease, electrophoresed in agarose gels, denatured, neutralized, transferred to a nitrocellulose filter, and hybridized to nick-translated probes according to established procedures (12). Hybridization was performed in 50% formamide, 3 \times SSC, at 37°C with washes in 0.2 \times SSC, 0.5% SDS, pH 7, at 60°C for 2 h. Analysis of Ig and T cell receptor β chain (T_β) gene rearrangements was performed by Southern blot hybridization, using various DNA clones representative of different portions of Ig and T_β gene loci as probes. Probes were ^{32}P -labelled by nick translation (12). The T_β gene locus was analyzed by hybridization of Eco RI-, Bam HI-, and Hind III-digested DNA to a T_β gene probe (a gift from Dr. Tak Mak, Ontario Cancer Institute, Toronto, Canada) that hybridizes to both T_β constant regions (13, 14). The IgH gene locus was studied by hybridization of Hind III- and Eco RI-digested DNA to a J_H -specific probe (a gift from Dr. Stanley Korsmeyer, National Institutes of Health, Bethesda, MD) (15). The organization of the *c-myc* gene was analyzed by hybridization of the human *c-myc* probe MC413RC to Eco RI- or Hind III-digested DNA (16). The presence of integrated HIV sequences was investigated by hybridization of a probe representative of the HIV genome (AIDS-associated retrovirus, ARV, clone $\lambda 7A/2$, [Luci et al., 17]) to Hind III- or Sst I-digested DNA. In all the Southern blot hybridization experiments, the sensitivity of the assay was estimated at 1–5%, i.e., a hybridization fragment was detectable when present at a single copy in at least 5% of the cells from which DNA was extracted. These levels of sensitivity were measured by dilution experiments using probes labelled at a specific activity of 1–2 $\times 10^8$ cpm/ μg and an autoradiographic exposure time of 5–7 d with intensifying screens (Quanta III, DuPont Co., Wilmington, DE).

Results

Multiple B Cell Clonal Expansions Are Present in B-NHL and LAS. We have used Ig and T_β gene rearrangement analysis to investigate the presence of clonal lymphoid populations within pathologic samples of lymph nodes obtained from patients with LAS and NHL and to determine their B or T cell lineage. Although the enlarged lymph nodes of LAS are commonly considered to be benign polyclonal lymphoid proliferations, gene rearrangement analysis represents the most accurate method to investigate the presence of clonal B and T cell populations, respectively. In fact, the clonal determination of B cell lineage based on κ or λ light chain expression sometimes may be inaccurate and lacks sensitivity, while there is no phenotypic marker of clonality for T cells (18). In the case of NHLs, the immunogenotypic determination of lineage can confirm the data obtained by cell surface marker analysis. The issue of clonality of these tumors is relevant in view of recent studies reporting the existence of biclonal tumors and their increased frequency in immunosuppressed patients (19–21).

The organization of the Ig and T_β loci was investigated in the panel of cases of B-NHL and LAS listed in Tables I and II (see Materials and Methods for diagnostic criteria). Southern blot hybridization analysis was performed using probes specific for the J_H locus (15) and for the constant region of the T_β ($T_\beta C$) (13, 14) locus, respectively. The results of this analysis are summarized in Tables I and II, and representative data are illustrated in Figs. 1 and 2. Consistent with the data from cell surface marker analysis, all of the cases of NHL that displayed B cell-specific cell surface markers displayed clonal rearrangements occurring both in the Ig heavy chain gene locus (Fig. 1) and in the light chain gene loci

TABLE I
Phenotypic and Genotypic Characteristics of B-NHL Biopsies

Patient/biopsy code	Histopathology*	Gene configurations [†]		Cytogenetics
		Ig locus [‡]	<i>c-myc</i>	
DK 348	Small cell, noncleaved	R 1, 2, 3	R	ND
DK 179	Small cell, noncleaved	R 1, 2	R	ND
DK 285	Small cell, noncleaved	R 1, 2	R	ND
DK 120	Small cell, noncleaved	R 1, 2, 3	R	8:14
DK 451	Large cell, noncleaved	R 1	G	8:22
DK 452	Large cell, noncleaved	R 1	R	8:14
DK 180	Large cell, noncleaved	R 1	G	ND
DK 181	Large cell, noncleaved	R 1, 2, 3	G	ND
DK 642	Small cell, noncleaved	R 1	R	ND
DK 644	Small cell, noncleaved	R 1, 2	R	ND
DK 645	Small cell, noncleaved	R 1	R	ND

All biopsies showed B cell-specific lineage markers; none showed T cell-specific markers. All had T_β rec loci in germline configuration, and none were found to contain HIV DNA sequences.

* Classified according to the working formulation of NHL (21).

[†] G, germline configuration; R, rearranged configuration.

[‡] Presence and the number of different rearrangement bands (see text) has been indicated by numbers.

TABLE II
Analysis for Ig and c-myc Gene Rearrangements and HIV-related Sequences in LAS Biopsies

Diagnosis	n	Ig gene rearrangements	
		Germline	Rearranged
AIDS/LAS	22	18	4
Non-AIDS controls*	11	11	0

None of the biopsies from either group showed *c-myc* gene rearrangements or contained HIV sequences.

* Biopsies of benign hyperplastic lymph nodes.

(data not shown). According, clonal T_β gene rearrangements were not found in these tumors (not shown, see Table I).

Upon DNA digestion with two different restriction enzymes, the majority (6 to 10) of the cases of B-NHL displayed two or more rearrangements bands (see Fig. 1 for representative data obtained with Eco RI). These bands appear to be of different intensity and are accompanied by the germline band. Although these multiple bands could theoretically be due to mutations within the Ig locus leading to changes in restriction patterns, the frequency of detection of this phenomenon in AIDS-NHL vs. non-AIDS-related NHL, together with the detection of analogous patterns in LAS (see below), indicate the existence of multiple clonal B cell expansions within the same tumor (19–21). In one single case (DK 180) the presence of two distinct clonal populations was directly demonstrated by the separate analysis of two tumor biopsies (DK180, DK181) derived from separate sites (Fig. 1). Thus, multiple clonal B cell expansions appear to be more frequently detectable in AIDS-associated B-NHLs (60% of cases) than in conventional B-NHLs (10% in our collection of 30 cases; 5% in the previously reported series

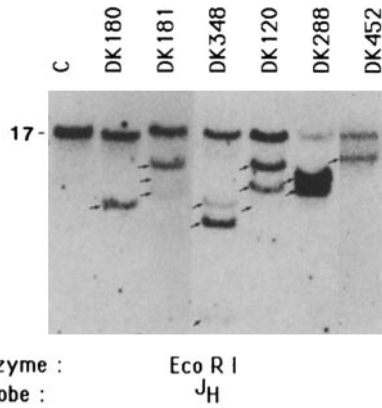


FIGURE 1. Immunoglobulin gene rearrangement analysis in AIDS-associated lymphomas. DNA (20 μ g) from the indicated cases (see Table I) and from normal human placenta (C) were digested with Eco RI and hybridized to a J_H probe (15) according to the method of Southern (12). Arrows indicate rearranged bands; the 17 kb reference is indicated at left.

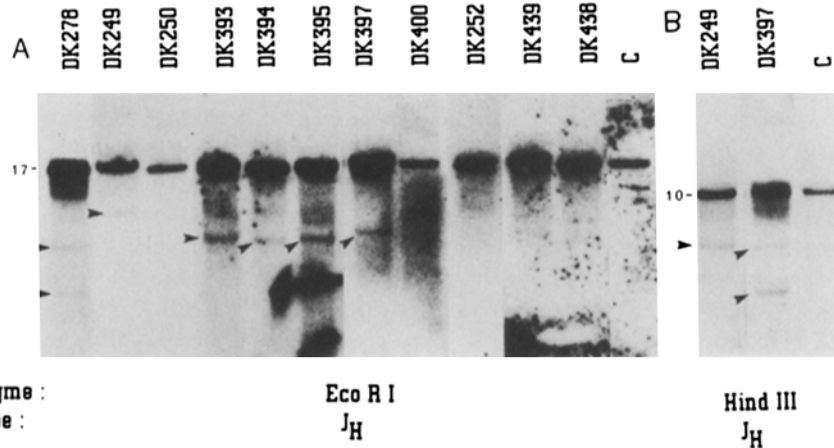


FIGURE 2. Immunoglobulin gene rearrangements analysis in cases of LAS. DNA (20 μ g) from the indicated cases and from normal human placenta (C) was digested with Eco RI (A) or Hind III (B) restriction enzymes and hybridized to a J_H probe (15). The hybridized filter was exposed to x-ray film for 10 d. Arrows indicate rearranged bands; the 17 and 10 kb references are indicated in A and B, respectively.

[20]). In this respect, AIDS-associated B lymphomas appear to be analogous to lymphoid neoplasms occurring in other forms of immunodeficiency (19–21).

Notably, clonal rearrangements of Ig genes, but not of T_β genes, were detectable in a significant fraction of LAS samples. Representative Eco RI and Hind III Southern blots of these cases are illustrated in Fig. 2, while a summary of the survey is reported in Table II. The rearranged bands commonly display a weak hybridization signal, with or without an accompanying hybridization smear. This indicates that individual B cell clones, present at low abundance (~5%, see Materials and Methods), are sometimes accompanied by other oligoclonal B cell populations. These findings appear to be specific for AIDS-related LAS, since comparable clonal B or T cell populations were not detectable in

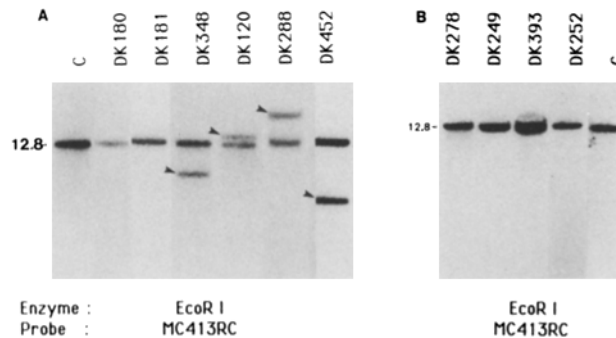


FIGURE 3. Analysis of *c-myc* gene rearrangements in AIDS-associated lymphomas and in cases of LAS. DNA (20 μ g) from the indicated B-NHL cases (see Table I) (A), from LAS cases (see Table II) (B), and from normal human placenta (C) was digested with EcoRI and hybridized to a probe (MC413RC) representative of the third exon of the human *c-myc* gene (16). Rearranged *c-myc* bands are shown by arrows; 12.8 kb reference is also shown.

analogous surveys of biopsies from non-AIDS-related lymphadenopathies (see Table II). Therefore, we conclude that the frequent presence of oligoclonal B cell populations represents a distinctive feature of AIDS vs. non-AIDS-associated B-NHL and LAS.

c-myc Oncogene Rearrangements Are Present in AIDS B-NHL But Not in LAS. The *c-myc* locus is involved in specific chromosomal translocations that are thought to play a role in Burkitt's lymphoma (16, 22, 23). A number of preliminary observations suggest that at least some AIDS-associated malignant lymphomas share some features with BL, including (a) a sometimes similar cytologic and histopathologic appearance (24–26); (b) an apparently frequent association with EBV infection (24–28); and (c) the presence of the same specific chromosomal translocations (26–28). The unexpected detection of clonal B cell populations within LAS biopsies prompted us to extend *c-myc* gene analysis to LAS biopsies.

The involvement of the *c-myc* locus in chromosomal translocations is suggested by cytogenetic analysis showing the presence of the (8:14), (8:22), or (2:8) translocations. *c-myc* involvement may also be directly demonstrated by restriction enzyme hybridization analysis using enzymes that can detect either mutations within or deletion of the 5' portion of the gene (29). Cytogenetic analysis was performed in 3 of the 10 cases of B-NHL and restriction enzyme analysis was performed in all 10 cases of B-NHL and in all LAS samples (see Tables I and II). DNA samples from AIDS-associated NHLs or LAS were digested with EcoRI restriction enzyme and hybridized to a human *c-myc* probe representative of the third exon of the human *c-myc* gene (16) (see Fig. 3). *c-myc* rearrangements were detectable in 8 out of 10 NHL cases. It is important to note that only one rearrangement band was detectable in all cases, including the ones displaying multiple Ig rearrangement bands. Because, based on our survey of ~40 BL cases, the probability of coincidental comigration of two or more rearranged *c-myc* alleles is extremely low, these data suggest that only one of the B cell clones contains a rearranged *c-myc* locus. In one of the two NHL cases where the rearrangements are not detectable, the involvement of the *c-myc* oncogene is demonstrated by the presence of a variant (8:22) chromosomal translocation, which has been shown (30) to involve chromosome breakpoints located at the 3'

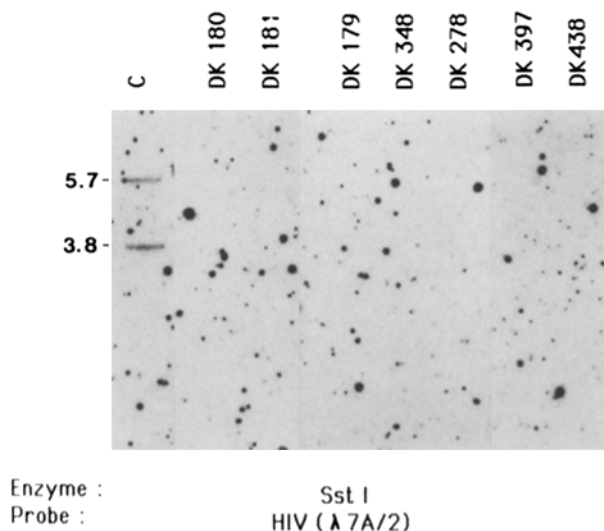


FIGURE 4. Lack of detectable HIV DNA sequences in AIDS-associated lymphomas and in cases of LAS. 20 μ g of DNA from the indicated cases and 5 μ g of DNA from an ARV-infected cell line (C) were digested with Sst I and hybridized to an HIV DNA probe representative of the A region of the HIV genome (17). The hybridized filter was exposed to x-ray film for 10 d. 3.8 and 5.7 kb references are shown.

side of the *c-myc* locus without apparent rearrangement of the gene. In the remaining case of B-NHL, no cytogenetic data is available and no *c-myc* rearrangement is detectable. This suggests either the presence of a variant translocation or that the *c-myc* gene is not involved in this one neoplasm. Conversely, rearrangements were not detectable in any of the samples of LAS (Fig. 3B and Table II).

In summary, *c-myc* oncogene translocations and/or rearrangements appear to be a virtually necessary pathogenetic feature of AIDS-associated B-NHLs. Most notably, only a single rearranged *c-myc* band is detectable in NHL, while none is detectable in LAS. These findings suggest that while both B-NHL and LAS biopsies contain multiple clonal B cell expansions, only one clone, and only in B-NHL, carries a rearranged *c-myc* gene.

HIV Sequences Are Not Present in NHL and LAS B Cell Clones. The possible retroviral etiology of AIDS prompted us to investigate the presence of HIV sequences in AIDS-associated NHL and LAS. The HIV DNA probe, representative of part of the viral genome, has been shown (17) to recognize HIV sequences in HIV-infected cell lines. DNA from both NHL and LAS cases was digested with Sst I (see Fig. 4) and Hind III (data not shown), both of which have sites within the viral genome, thus allowing the detection of viral sequences independent of the proviral integration site (17). HIV DNA sequences were not detectable in any of the cases of LAS or B-NHL (see Fig. 4 for experimental data and controls). Given the fact that in our experimental conditions this assay allows the detection of single-copy sequences present in at least 5% of the cell population and that our B-NHL samples contained at least 80% pathologic cells (by histopathologic examination), we conclude that the genomes of AIDS-associated B-

NHLs do not carry HIV or HIV-related sequences. Furthermore, HIV sequences present in at least a single copy per haploid genome should be detectable within those clones. Our data allow us to conclude also that HIV sequences are not present in any of the clonal B cell populations detected in LAS.

Discussion

This study indicates that the lymphoid organs in patients with AIDS who have an associated LAS or NHL are frequently infiltrated by oligoclonal B cell populations. The presence of B cell clones was not detected or suspected in LAS, based upon histopathologic or surface-marker analysis of involved lymph nodes, which had suggested the presence of a marked but apparently benign follicular hyperplasia (1). This alteration is generally indistinguishable from lymphadenopathy associated with acute viral illnesses or autoimmune conditions (1) where Ig gene rearrangements were not detected (1). Accordingly, while a single clonal B cell population is obviously expected to be present in B-NHL biopsies, the finding of additional B cell clones represents an extremely rare event in NHLs unassociated with AIDS. Taken together, these observations suggest that a common condition in AIDS and ARC favors the development of clonal B cell expansions. Indirect support for this notion can be derived from recent work reporting that oligoclonal immunoglobulins, presumably secreted by oligoclonal B cells, are present in the serum of patients with AIDS (31).

Among the possible causes that may lead to abnormal B cell proliferations in AIDS, the conditions of impaired immune functions and massive EBV infections, present in both LAS and NHL patients, are likely to represent important factors. Direct support for a combined role of immunodeficiency and EBV in favoring clonal B cell expansions derives from recent work by Birx et al. (32), suggesting that both a profound defect of T cell immunity to EBV and an abnormally high number of circulating EBV-infected B cells are present in AIDS patients. EBV-infected B cells are long-lived and replicate *in vivo* as well as *in vitro*, where they can be readily and efficiently established as cell lines (32). These observations suggest that these EBV-infected B cell clones can account for the monoclonal B cell populations detectable in patients with AIDS by Ig gene rearrangement analysis. The expansion of these pools of immortalized yet not fully transformed cells may increase the probability of additional genetic alterations leading to malignant transformation.

In 9 out of 10 biopsies, the presence of malignant B-NHL is associated with the presence of translocations or rearrangements of the *c-myc* oncogene locus. This genetic alteration is consistently found in BL and only occasionally in other undifferentiated B cell lymphomas and lymphoblastic leukemias (33). In the context of AIDS-associated NHLs, *c-myc* gene rearrangements provide a marker for the malignant clone, thereby allowing two important observations. First, in each one of the NHL biopsies displaying multiple clonal Ig gene rearrangements, only one rearranged *c-myc* allele is detectable, which suggests that only one clone carries the genetic abnormality associated with malignant lymphoma. We then presume that only one of the multiple B cell clones detected by Ig rearrangements represents the truly malignant lymphoma, whereas the remaining may be EBV-immortalized nonmalignant clones analogous to the ones found in LAS. Second,

it is important to note that despite their clinical and histopathologic heterogeneity, including both Burkitt and non-Burkitt types (see Table I), AIDS-associated NHLs usually appear homogeneous with respect to oncogene activation. Thus, the biological alterations present in AIDS may favor the development of lymphomas carrying activated oncogenes. In this respect, the association between EBV-infection and immunosuppression present in AIDS is reminiscent of analogous conditions present in endemic, African-type BL, which are also typically characterized by chromosomal translocations involving the *c-myc* locus (16).

Another issue addressed by our study is whether HIV sequences can be found in AIDS-associated NHLs, i.e., whether HIV can be directly involved in the pathogenesis of NHL. The relevance of this issue is underscored by recent reports suggesting that HIV can infect EBV⁺ B cells (34), and that HIV infection is endemic in Africa (35) in some of the regions where BL is also endemic (5). Our data showing the lack of HIV sequences in NHL samples indicate that HIV has no direct role in lymphomagenesis. However, in view of the specific cytopathic effect of HIV for T4⁺ lymphocytes (36) and of the suggested role of these cells in inhibition of EBV-activated B cells (37), it is possible that HIV infection, present in all the patients included in this study, may indirectly contribute to the clonal B cell expansion.

In conclusion, the data and the arguments presented here support a model for a multistep process of lymphomagenesis in AIDS in which LAS would represent a predisposing condition for NHL. Immunosuppression and EBV infection present in LAS can favor the expansion of B cell clones, which in turn may increase the probability of the occurrence of additional genetic alterations, namely translocations of the *c-myc* gene causing the tumorigenic conversion of these cells. In vitro experiments involving the transfer of activated/or rearranged *c-myc* genes into EBV-immortalized B lymphocytes from AIDS/LAS patients represent an obvious experimental approach for testing this model.

Summary

AIDS (acquired immune deficiency syndrome) and ARC (AIDS-related complex) are associated with a spectrum of lymphoproliferative disorders ranging from lymphadenopathy syndrome (LAS), an apparently benign polyclonal lymphoid hyperplasia, to B cell non-Hodgkin's lymphoma (B-NHL), i.e., malignant, presumably monoclonal B cell proliferations. To gain insight into the process of lymphomagenesis in AIDS and to investigate a possible pathogenetic relationship between LAS and NHL, we investigated (a) the clonality of the B or T lymphoid populations by Ig or T_β gene rearrangement analysis, (b) the presence of rearrangements involving the *c-myc* oncogene locus, and (c) the presence of human immunodeficiency virus (HIV) sequences in both LAS and B-NHL biopsies. Our data indicate that multiple clonal B cell expansions are present in a significant percentage of LAS (~20%) and B-NHL (60%) biopsies. *c-myc* rearrangements/translocations are detectable in 9 of our 10 NHLs, but not in any of the LAS cases. However, only one of the B cell clones, identified by Ig gene rearrangements carries a *c-myc* gene rearrangement, suggesting that only one clone carries the genetic abnormality associated with malignant B cell lymphoma. Furthermore, the frequency of detection of *c-myc* rearrangements in

AIDS-associated NHLs of both Burkitt and non-Burkitt type suggest that the biological alterations present in AIDS favor the development of lymphomas carrying activated *c-myc* oncogenes. Finally, our data show that HIV DNA sequences are not detectable in LAS nor in NHL B cell clones, suggesting that HIV does not play a direct role in NHL development. Taken together, these observations suggest a model of multistep lymphomagenesis in AIDS in which LAS would represent a predisposing condition to NHL. Immunosuppression and EBV infection present in LAS can favor the expansion of B cell clones, which in turn may increase the probability of occurrence of *c-myc* rearrangements leading to malignant transformation.

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