NODDOKGIN’S lymphoma (NHL) represents a major clinical manifestation of AIDS. By examining a panel of 16 AIDS-NHLs, the pathogenesis of AIDS-associated NHL (AIDS-NHL) and, in particular, their possible linkage to the condition of impaired immunity present in AIDS is not understood. Preliminary studies involving a few cases of AIDS-NHL have noted the presence of specific chromosomal translocations and rearrangements of the \textit{c-myc} gene. In addition, a possible role for Epstein-Barr virus (EBV) has been proposed based on (a) the defective immune regulation of EBV infection in AIDS and the AIDS-related complex (ARC), and (b) the involvement of this virus in B lymphomas associated with different inherited or acquired deficiencies of the cellular immune system. However, no conclusion can be drawn concerning the possible combined pathogenetic role of \textit{c-myc} oncogene activation and EBV infection because of the small number of cases studied and the lack of studies providing direct evidence for the presence of EBV sequences in most AIDS-NHL cell genomes. In addition, the possible role of other oncogenes, such as \textit{bcl-2}, and viruses, such as HTLV-I, which are involved in the pathogenesis of different types of NHL, has not been investigated. Therefore, we directly addressed these points by examining a panel of 16 AIDS-NHLs.

**MATERIALS AND METHODS**

\textit{Pathologic samples and diagnosis}. Biopsies obtained from 16 patients with AIDS were diagnosed by standard histopathologic criteria as NHL. Each case was subclassified histopathologically according to the working formulation of NHL. Immunophenotypic analyses for the expression of surface immunoglobulin (Slg), sheep erythrocyte (E) rosettes, terminal deoxynucleotidyl transferase (TdT), and the B-cell-associated antigens HLA-DR, CALLA, B1, and B2 were performed as previously described. DNA extraction and Southern blot analysis. DNA was prepared by cell lysis, proteinase K digestion, phenol extraction, and ethanol precipitation. Fifteen micrograms of DNA were digested with appropriate restriction endonucleases, electrophoresed in an agarose gel, denatured, neutralized, transferred to nitrocellulose filters, and hybridized according to the method of Southern. DNA probes were \(^{32}\)P-labeled by nick translation. Filters were washed in 0.2 \(\times\) SSC/0.5% sodium dodecyl sulfate (SDS) for two hours at 60\(^\circ\)C and then autoradiographed using intensifying screens (Quanta III, Dupont).

**DNA probes.** The organization of the \(\text{IgH}\) locus was studied by hybridization of \textit{EcoRI-}, \textit{HindIII-}, or \textit{BamH}-digested DNA to joining region (\(\text{JH}\)) and constant region (\(\text{C}\)) specific probes (gifts of Stanley Korsmeyer). The organization of the \(\text{c-myc}\) gene was analyzed by hybridization of the human \(\text{c-myc}\) probe MC413RC, representative of the third exon of the \(\text{c-myc}\) locus to \textit{EcoRI-}, \textit{HindIII-}, or \textit{BamH}-digested DNA. Rearrangements of the \(\text{bcl-2}\) locus were studied using the previously described \textit{pF1}-1 and \textit{pF2}-2 probes, (gifts of Michael Cleary). The presence of the EBV genome was analyzed using a probe containing sequences of the \textit{EBNA-1}\) gene and the \(\text{EBV}\) origin of replication (\textit{OriP}) (gift of Bill Sudgen). This probe hybridizes to the \(\text{BamH}\) and \(\text{BamH}\) fragments of the EBV genome. The presence of \(\text{HTLV-1}\) sequences was studied by hybridization to an HTLV-env probe (gift of B. Poiesz).

**Immunofluorescence studies.** The isolated tumor cells were applied to glass slides by cytocentrifugation (2.5 \(\times\) 10\(^6\) cells/slide) after double washing in phosphate-buffered saline (PBS, pH 7.4). The slides were air-dried and then fixed with cold methanol/acetone (1:2) for at least five minutes. After three 10-minute washes with PBS, the slides were incubated with positive anti-\(\text{EBNA}\) human serum (1:10) at 37\(^\circ\)C for 15 minutes in a humidified atmosphere. After a ten-minute PBS wash, the slides were incubated with human complement (a\(\text{EBNA}\) negative, 1:10) for 15 minutes at 37\(^\circ\)C. After another ten-minute wash, the slides were incubated with F(ab')

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**RESULTS**

**Frequency of c-myc gene rearrangements.** Chromosomal translocations involving the c-myc locus in Burkitt lymphoma (BL) have been shown to lead to (a) truncations of the c-myc gene within its first intron, first exon, or flanking sequences in (8:14) translocations in sporadic BL (sBL); or (b) mutations of sequences within the first c-myc exon in (8:14) translocations in endemic BL (eBL) as well as in (8:22) and (2:8) translocations in both eBL and sBL. The truncations can be detected by Southern blot hybridization using restriction enzymes cutting outside c-myc sequences (eg, EcoRI and HindIII), whereas most (60%) of the mutations are detectable as polymorphisms of a PvuII restriction site located at the 3’ side of exon 1.

With these assays, rearrangements of the c-myc locus were detectable by Southern blot hybridization of EcoRI-digested DNA in 7 of 10 SNCC, 3 of 4 LNCC, and 1 of 2 LC-IBP NHLs (Fig 1 shows representative results). The cases lacking detectable rearrangement were analyzed for PvuII site mutations, and a single SNCC case (not shown; Table 1) scored positive. Thus, 12 of 16 AIDS NHLs displayed patterns of c-myc oncogene activation generally consistent with those observed in sBL. This may represent a minimal estimate, since additional cases carrying mutations in the first exon may exist and not be detected by the PvuII analysis.

**Characterization of chromosomal recombinations.** The demonstration of c-myc gene rearrangements in most AIDS-NHLs led us to examine whether these rearrangements corresponded to the juxtapositions of c-myc on chromosome 8 with immunoglobulin heavy-chain (IgH) loci on chromosome 14 characteristic of (8:14) translocations in BL. In particular, two distinct patterns of (8:14) translocations have been described in BL. The first involves recombination between the JH region of IgH with sequences >100 kilobases 5’ to c-myc and is typical of eBL. The second involves recombination between the Switch (S\(_s\)) region of IgH and sequences within the c-myc locus and is typical of sBL. We attempted to identify and map chromosomal breakpoints in our panel of AIDS-NHLs by studying the linkage between c-myc and various IgH regions, namely J\(_{H}\), S\(_s\), and C\(_\gamma\) (Fig 2C) in Southern blot hybridization experiments. In the AIDS-NHL cases that carry rearranged c-myc alleles, breakpoints were found within or in close proximity to the S\(_s\) region, as shown by the linkage between c-myc and C\(_\gamma\) (but not J\(_{H}\)) sequences in BamHI and HindIII digests and, indirectly, by the lack of linkage between J\(_{H}\) and c-myc sequences in EcoRI digests (Fig 2). We conclude that 11 of 16 AIDS-NHLs carry IgH/c-myc recombinations analogous to sBL.

**Infrequent presence of EBV sequences.** We investigated the presence of EBV sequences by Southern blot hybridization using an EBV probe for the EBNA-1 gene and the EBV OriP, since these regions are consistently present in EBV-infected cells. EBV sequences were not detectable in 10 of 16 AIDS-NHLs, whereas hybridization bands were detected in six cases, either in the EBV genomic configuration or as rearranged or partially deleted fragments (Fig 3). Although examination of the intensity of the hybridization bands suggests that EBV sequences are present within the malignant cells representing >90% of the biopsy, the same result could theoretically be obtained if EBV sequences are present at high copy numbers in minor B-cell populations. We
C-MYC ACTIVATION AND EBV SEQUENCES IN NHL

from control DNAs run on the same gel; their sizes are indicated in

and B). DNA from

tation (A

HindIII

kilobases (kb). The two germline

between lanes. Arrows indicate positions of germline fragments

lose filters. Comigrating fragments are indicated by dashes

Fig 2. Southern blot analysis of Ig and c-myc gene organiza-
tion (A and B). DNA from two representative cases (4,15) in Table
1 was digested with the indicated restriction endonucleases,
size-separated on 1% agarose gels and transferred to nitrocellu-
lose filters. Comigrating fragments are indicated by dashes
between lanes. Arrows indicate positions of germline fragments
from control DNAs run on the same gel; their sizes are indicated in
kilobases (kb). The two germline HindIII fragments containing Jb
and Cμ display similar sizes under our experimental conditions. (C)
Schematic representations of the human IgH and c-myc loci
together with the IgH and c-myc probes used. Restriction sites in
the map: (R) EcoRI; (B) BamHI; (H) HindIII.

then directly investigated the presence of EBV proteins
within the malignant cells by indirect immunofluorescence
using a human α-EBNA positive sera. Typical patchy
nuclear fluorescence was detectable in the majority of
the malignant cells (80%) in the cases that contained EBV
sequences, but not in cases negative by Southern blot hybrid-
ization (Table 1).

Lack of bcl-2 rearrangements and HTLV-1 se-
quencies. Most low-grade follicular B-NHLs, 40% of
diffuse large cell B-NHLs, and 30% of undifferentiated BL
have been reported to carry rearrangements involving the
bcl-2 locus on chromosome 18.28 To determine if bcl-2
rearrangements were present in our AIDS-NHL cases,
Southern blot hybridization analysis of the tumor DNAs was
performed with probes from both the major15 and minor22
bcl-2 gene breakpoint regions. No rearrangement was
detected by any of these probes in the 16 AIDS-NHLs (data
not shown). All of the cases were also negative for HTLV-1
and HIV sequences (data not shown).

DISCUSSION

The major findings of this report are the frequency of
c-myc gene rearrangements in AIDS-NHLs of different
histologies and, concomitantly, the absence of EBV
sequences in most of these tumors. Although c-myc oncogene
activation and EBV infection in the context of immunosup-
pression has been repeatedly suggested as the main steps of
lymphomagenesis in AIDS,6,9 no direct evidence for this has
been reported in sizable panels of cases. Our survey of 16
cases clearly points to a role for c-myc activation in most
AIDS-NHLs, whereas the surprising finding of the absence
of EBV sequences in most of these cases suggests a general
reevaluation of the problem of AIDS-NHL pathogenesis.

Specific chromosomal recombinations [(8:14), (8:22),
and (2:8)] involving the c-myc and Ig loci represent features
of both the endemic and sporadic form of BL and of the Lc-type
of acute lymphocytic leukemia. A role for these alterations in
malignant B-cell transformation is strongly suggested by (a)
the evidence of c-myc/Ig juxtapositions in B-cell malignan-
cies in mice and rats29; (b) the increased incidence of B-cell
tumors in transgenic mice carrying Ig-myc chimeric con-
structs,30 and (c) the in vitro transforming activity of activ-
ated c-myc genes on human B-lymphoblastoid cells.22 It is
therefore likely that, by analogy with non-AIDS–associated
BL, the c-myc rearrangements shown in 12 of 16 AIDS-
NHLs contribute to the pathogenesis of these tumors by
disrupting the normal control of c-myc gene expression. Our
data indicate an association between c-myc rearrangements
and a variety of NHL types, including high-grade (Burkitt
and non–Burkitt-type) and intermediate-grade lymphomas,
and indicate a specific association between immunosuppres-

Fig 3. EBV DNA hybridization. The presence of EBV
sequences was detected by BamHI digestion and hybridization to a
probe containing the genes EBNA-1 and OriP corresponding to the
9.0-kb BamHI C and the 4.5-kb BamHI K fragments (described in
text). Control lane (C) represents hybridization to DNA from the
EBV + eBL cell line Daudi. Lane numbers correspond to cases in
Table 1.
sion and B-cell tumors involving c-myc oncogene activation. In this context, one or more of the biologic alterations present in AIDS theoretically may favor the occurrence of chromosomal translocations involving c-myc. Alternatively, and perhaps more likely, cells in which these translocations have occurred may acquire specific biologic modifications that make them particularly suited to expand and progress toward malignancy in the context of immunodeficiency states. BL cells were recently reported to lack specific cell-surface molecules involved in immunorecognition by T cells and are unable to elicit either autologous or allogeneic T-cell responses and are unable to elicit either autologous or allogeneic T-cell responses in vitro.31,32 The effects of these alterations would obviously be amplified in AIDS.

A suggested role for EBV in AIDS lymphomagenesis was prompted by the association of this virus with B-cell lymphomas occurring in a variety of inherited and acquired immunodeficiencies10-13 and by the observation that the immunosurveillance of EBV-infected B cells is defective in AIDS and ARC.6 In addition, multiple B-cell clonal expansions, presumably carrying EBV, have been detected in the lymphadenopathy syndrome (LAS) or AIDS-NHL nodal biopsies,6 and EBV-positive B-lymphoblastoid cell lines can be readily established in vitro from the peripheral blood of AIDS patients.9 These observations had previously led us1 and other researchers8,9 to propose that AIDS-associated immunosuppression and EBV infection may favor the clonal expansion of EBV-infected B cell populations, increasing the probability of the occurrence of genetic alterations, namely translocations of the c-myc gene, which can lead or contribute to NHL development. This model was supported by in vitro data showing that the expression of an activated c-myc oncogene can cause the tumorigenic conversion of EBV-infected lymphoblasts from an AIDS patient.25 Despite these observations, it is now clear that most AIDS-NHLs do not carry EBV sequences in their genome; thus, EBV is not directly involved in cell transformation in these cases. Models involving EBV remain valid for the approximate one-third of cases that do contain viral sequences. Further studies are required, however, to establish whether in these cases EBV has a primary role in lymphomagenesis or secondarily infects the malignant cells.

In general, the concurrence of c-myc gene rearrangements with the relatively low frequency of EBV sequences suggest a similarity between AIDS-NHL and the sporadic rather than the endemic form of BL, as previously suggested.6,25 This similarity is enhanced by the observation that AIDS-NHLs carry truncations of the c-myc gene and recombinations with the Switch region of the IgH locus which are typical of sBL and are likely to occur at the time of IgH isotype switching, ie, relatively late during B-cell development.27 As is the case for sBL, additional genetic alterations are probably necessary to complement c-myc activation. We showed that these alterations are not represented by bcl-2 rearrangements or HIV or HTLV-1 infection. Other, possibly unknown oncogenes or viruses may be involved in the pathogenesis of both the large portion of AIDS-NHLs carrying c-myc rearrangements and in the few cases in which no alteration of c-myc was detected in this study.

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Frequent c-myc oncogene activation and infrequent presence of Epstein-Barr virus genome in AIDS-associated lymphoma

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