Molecular Epidemiology of Burkitt's Lymphoma From South America: Differences in Breakpoint Location and Epstein-Barr Virus Association From Tumors in Other World Regions

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We have previously shown that the endemic (African) and sporadic (North American) forms of Burkitt's lymphoma (BL) differ at a molecular level. We have now extended our studies to the molecular epidemiology of BL in South America, specifically to two climatic regions: temperate (Argentina and Chile) and tropical (Brazil). We have examined the patterns of chromosomal breakpoint locations in 39 tumors with respect to geography and Epstein-Barr virus (EBV) association. The result of these analyses provide further support for the existence of pathogenetically distinct subtypes of BL in different world regions. The majority of breakpoints on chromosome 8 in South American BL (41%) occurred in the immediate flanking region of c-myc, i.e., further 5' of the "typical" sporadic breakpoints, in the first exon/intron region, and further 3' of the "typical" endemic breakpoints, which are usually distant from c-myc. However, the distribution of breakpoints on chromosome 14 in tumors from the temperate and tropical regions of South America is similar to that observed in sporadic and endemic tumors.

Interestingly, only one tumor with an unrearranged c-myc gene joined to the 5' region of chromosome 14 was observed. This combination was also rarely observed in our earlier series and presumably is either less readily generated by the mechanism that mediates 8;14 translocation or requires other, infrequent genetic changes to provide the necessary selective advantage for lymphomagenesis. The frequency of EBV association in South American BL (51%) is also intermediate with respect to tumors from the United States (30%) and Africa (100%). No correlation with the breakpoint location on chromosome 8 was discernable. Surprisingly, only 54% of tumors with breakpoint outside c-myc were EBV positive. This is in contrast to endemic tumors and suggests that any pathogenetic contribution of EBV is not dependent on breakpoint location, but is more likely to complement additional pathogenetic elements that differ in different world regions.

B URKITT'S LYMPHOMA (BL) is a B-cell neoplasm, classified in the National Cancer Institute (NCI) Working Formulation as a small, noncleaved cell lymphoma (SNCL), that occurs in children and young adults throughout the world.1 The high incidence of this disease in Equatorial Africa has led to the African form being called "endemic" (eBL), while cases occurring in other regions are referred to as "sporadic" (sBL). Epstein-Barr viral (EBV)2,3 DNA is found in virtually all cases of eBL, but in a much lower percentage of sBL from the United States.4,5 Both eBL and sBL, however, contain the same nonrandom chromosomal translocations6 that result in the juxtaposition of c-myc to an Ig chain locus10 and provide a critical element (c-myc deregulation)8 to the process of malignant transformation.

The position of the breakpoint on chromosome 8 is clearly of critical importance, because it determines whether the translocation separates regulatory regions (including the major promoters, P1 and P2) from the gene or causes damage to the regulatory elements themselves, which are located both within the gene and in its 5' flanking sequences.9,10 The breakpoint on chromosome 14 determines which of the regulatory regions of the Ig heavy chain locus are juxtaposed to c-myc, and it is probable that tumorigenic deregulation of c-myc ultimately depends on both breakpoint locations. We have recently shown that the distribution of breakpoints on chromosome 8 in 8;14 translocations differs in different world regions.4,5,11 In eBL, the breakpoint is usually (in 74%) far upstream of c-myc, whereas in sBL most breakpoints are within the gene, in the first exon/intron (in 56%). Moreover, EBV was more often associated with tumors that had breakpoints far 5' of c-myc.4,5 These findings suggested that there are at least two pathogenetically distinct subtypes of BL: (1) those with chromosome 8 breakpoints far upstream of c-myc (> 5 kb), in which EBV may play a critical role in the production or maintenance of the malignant phenotype; and (2) those with a breakpoint within or close to c-myc in which EBV may have a pathogenetic role in a much smaller proportion of cases. The former occurs predominantly in Equatorial Africa, the latter in the United States. It is important to bear in mind that the incidence of SNCL in Equatorial Africa is some 50-fold higher than in the United States.10 Molecular subtypes that are frequent in Africa, but much less frequent in other world regions, eg, far 5' breakpoints on chromosome 8, may thus have a several hundred-fold higher incidence in Africa than elsewhere. Conversely, molecular subtypes that we have rarely found in Africa, eg, intron breakpoints, appear to have more closely similar incidence rates in all world regions.

These data suggest that the geographic origin of the tumor is a determining factor with respect to the pathoge-
nomic mechanism. We decided, therefore, to study BL from other world regions to determine whether the patterns of chromosomal breakpoints are similar to either eBL, or sBL, or have a unique pattern. South America is of particular interest in this regard, because it encompasses both tropical and temperate regions, reproducing the climatic conditions in which eBL and sBL are found, although differing in other important aspects, eg, socioeconomically and ethnically, from both Africa and the United States. We report here the results of an examination of the molecular characteristics and EBV association of 39 tumors from Argentina, Chile, and Brazil.

MATERIALS AND METHODS

We have studied 39 SNCLs from South America (Table 1). The biopsies were obtained from a number of institutions: the Hospital de Niños “Ricardo Gutiérrez” and Hospital Nacional de Pediatría “Juan Garrahan,” both in Buenos Aires, Argentina (17 tumors); the Hospital de Niños Roberto del Río and Hospital Infantil Luis Calvo Mackenna in Santiago, Chile (10 tumors); the Hospital AC Camargo, Sao Paulo, Brazil, and CIHH Domingos Boldrini, Campinas, Brazil (12 tumors). The samples were frozen at −70°C immediately after surgery. A diagnosis was made in each center according to standard histopathologic criteria.

Molecular analysis. High molecular weight DNA was prepared from each tumor by cell lysis, proteinase K digestion, phenol-chloroform extractions, and ethanol precipitation as described by Maniatis et al. DNA samples were digested with appropriate restriction enzymes (BamHI, EcoRI, HindIII, PstI, PvuII, and SmaI) fractionated in 0.8% agarose gels and transferred to nylon membranes according to standard Southern blotting procedures. The blots were hybridized to radiolabeled 32P-probes, using methods previously described.12

Table 1. Epidemiologic and Molecular Features of South American SNCLs

| Sample | Origin | Age/Sex | Site | EBV | Chromosome 8 | Chromosome 14*
|--------|--------|---------|------|-----|-------------|----------------|
| FNR    | Bra    | 6/M     | Abdomen | +   | Far 5'†  | NSp
| CCH    | Bra    | 4/M     | Abdomen, CNS | +   | Far 5’ | NSp
| RPF    | Bra    | 11/M    | Abdomen | +  | Far 5’ | NSp
| FB     | Bra    | 4/M     | Jaw, abdomen | +  | Far 5’ | NSp
| SRC    | Bra    | 11/M    | Abdomen, BM | – | Far 5’† | NSp
| DGG    | Chi    | 7/M     | Abdomen | +  | Far 5’† | NSp
| CAB    | Chi    | 5/M     | Abdomen | +  | Far 5’† | NSp
| AE     | Chi    | 5/M     | Abdomen | +  | Far 5’† | NSp
| GS     | Chi    | 4/F     | Abdomen, BM, CNS | – | Far 5’ | NSp
| FL     | Chi    | 11/M    | Abdomen, BM, CNS | – | Far 5’ | Sp
| CV     | Arg    | 7/F     | Abdomen | +  | Far 5’† | NSp, Jh
| 135    | Arg    | 22/M    | Abdomen, BM | – | Far 5’ | NSp
| RJ     | Arg    | 8/M     | Abdomen | –  | Far 5’ | NSp
| VGO    | Bra    | 5/M     | Abdomen | +  | H3-Pst | Sp
| MP     | Bra    | 4/M     | Abdomen | +  | H3-Pst | Sp
| In     | Bra    | 5/M     | Abdomen, pleura | – | H3-Pst | NSp
| PM     | Chi    | 7/M     | Abdomen | +  | H3-Pst | NSp
| VA     | Arg    | 4/M     | Abdomen | +  | H3-Pst** | Sp
| SH     | Chi    | 5/M     | Abdomen | +  | Pst-Pvu | NSp
| HA     | Chi    | 3/F     | Abdomen | –  | Pst-Pvu | NSp
| HD     | Arg    | 9/M     | Abdomen | –  | Pst-Pvu | NSp, Jh
| ZJ     | Arg    | 7/M     | BM, maxilla | – | Pst-Pvu | Cj
| HQ     | Chi    | 11/M    | Abdomen, BM | – | Pvu-Sma | NSp
| SG     | Arg    | 3/M     | Abdomen, nodes | +  | Pvu-Sma | Sp
| MD     | Arg    | 5/M     | Abdomen | +  | Pvu-Sma | Sp
| OJ     | Arg    | 7/M     | Abdomen | +  | Pvu-Sma | Sp
| AF†    | Arg    | 6/F     | Intestine | +  | Pvu-Sma | NSp, Sp
| AS     | Arg    | 28/M    | Stomach, BM | –  | Pvu-Sma | NSp, Jh
| JR     | Arg    | 1/M     | Abdomen | –  | Pvu-Sma | NSp
| SCL    | Bra    | 6/F     | Abdomen | +  | Sma-Pvu | Sp
| ME     | Bra    | 5/M     | Abdomen, BM | –  | Sma-Pvu | NSp
| TJ     | Arg    | 13/M    | Abdomen, BM | +  | Sma-Pvu | ND
| RJR    | Arg    | 3/M     | Abdomen, nodes | – | Sma-Pvu | Sp
| BD     | Arg    | 12/M    | Abdomen | –  | Sma-Pvu | Sp
| AG     | Arg    | 13/F    | Abdomen | –  | Sma-Pvu | NSp
| BE     | Bra    | 9/M     | Abdomen | –  | Pvu-Pst | NSp, Jh
| ND     | Bra    | 5/M     | Abdomen | –  | Pvu-Pst | NSp, Jh
| ROC    | Chi    | 3/F     | Abdomen | +  | Pvu-Pst | Sp
| AA     | Arg    | 8/M     | Abdomen, nodes | +  | Pvu-Pst | NSp, Cj

Abbreviations: Bra, Brazil; Chi, Chile; Arg, Argentina; BM, bone marrow; CNS, central nervous system; ND, not determined.

*Breakpoint location on chromosome 14. Breakpoints are listed as Sp (within the HindIII-EcoRI fragment encompassing the Sp region) or NSp (outside this region); Jh indicates a breakpoint in the joining region; Cj, breakpoint in the constant region.

†The presence of a Pvu II mutation in the first exon. Breakpoints are indicated as being within a DNA fragment bounded by the enzyme sites shown.

‡Large noncleaved cell lymphoma.
The **myc** (first and third exon), **IgH** (JH, Sμ, and Cμ) (Fig 1), and EBV (terminal repeats) probes used, as well as the Southern blotting strategy used to determine breakpoint locations, have also been previously reported by us.5,13

All tumor samples were examined for mutations in the first exon by restriction analysis. Thus, only mutations resulting in a loss of the Pvu II site in its 3' region would be detected.

**RESULTS**

Twenty-seven of the tumors analyzed were from the temperate climatic region (17 from Argentina and 10 from Chile) while 12 were from the tropical region (specifically Sao Paulo, Brazil). The origin, age, and sex of the patients as well as the anatomical site and a summary of the molecular characterization of their tumors are shown in Table 1. The age range was 1 to 28 years and all patients presented with abdominal lymphoma, except for two who presented with jaw tumors.

Because both Argentina and Chile are climatically similar, for the purposes of the present analysis we have combined the tumors from these countries into a single group.

Although there are no cytogenetic data available, 32 of 39 tumors (82%) showed structural rearrangements that have been previously described in t(8;14) and 20 of these had definite t(8;14) at a molecular level. However, we cannot rule out the possibility of variant translocations in the remaining seven tumors that showed a non-Sμ non-JH breakpoint on chromosome 14 and an unaltered c-myc gene on chromosome 8.

**Chromosome 8 breakpoints.** The pattern of chromosomal breakpoint locations differed with the geographic origin of the tumor (Fig 2). Forty-one percent (11 of 27) of tumors from the temperate region (Argentina and Chile) had breakpoints in the immediate 5' region of c-myc in a Pst I-Sma I fragment (Figs 1 and 2). In contrast, no tumors from the tropical region displayed chromosomal breakpoints within this fragment; the majority (5 of 12 [67%]) had breakpoints further 5'.

Among the 13 tumors with a breakpoint outside the HindIII fragment encompassing c-myc, all except one were also rearranged outside the 5' EcoRI site encompassing c-myc. The exceptional tumor (RPF, from Brazil) had a breakpoint between the 5' EcoRI and HindIII sites, only a short distance upstream of myc.

Because tumors in which c-myc is not rearranged often show mutations within the c-myc locus, sometimes observed as a loss of the Pvu II site in the 3' region of the first exon, we examined, by restriction enzyme analysis, the South American lymphomas for the presence of such a mutation. Five of the 13 tumors (38%) with breakpoints outside HindIII carried a Pvu II mutation characteristic of many cBL. One additional case (VA) showed this mutation in association with a breakpoint between HindIII and Pst I.

Of the 26 tumors that had breakpoints within the HindIII

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**Fig 1.** Restriction maps of the human c-myc and Ig heavy chain genes. Restriction endonuclease sites are shown. H, HindIII; Ps, PstI; P, Pvu II; S, Sma I; E, EcoRI; B, BamHI.
fragment encompassing the c-myc gene, 62% (16 of 26) had breakpoints in the 5' flanking region (between the HindIII and the Smal I sites) (Fig 2). Thus, these tumors retained the capacity to transcribe c-myc messenger RNA (mRNA) from either the P1 or P2 promoter.

**Chromosome 14 breakpoints.** Determination of the location of breakpoints on chromosome 14 (Fig 1) was possible in all but one tumor (Table 1). Breakpoints outside the Sp μ region are almost twice as frequent as Sp μ breaks (in the HindIII-EcoRI segment). The proportions of tumors with Sp μ breakpoints from the temperate and tropical regions were 38% and 25%, respectively.

**Breakpoint combinations.** Analyses of the combination of breakpoint locations on chromosomes 8 and 14 are shown in Fig 3 and Table 2. Of the four main breakpoint combinations, R/Sp μ, R/NSp μ, and U/NSp were equally frequently observed. Only a single tumor with a translocation that joined an unrearranged c-myc with a Sp μ region (U/Sp μ) was observed.

**EBV association.** Overall, the frequency of EBV-positive lymphomas in South America was 51%. Tumors from either the temperate or the tropical region did not significantly differ in their association with EBV (48% and 58%, respectively), although the numbers are too small for a valid comparison between these groups to be made. As shown in Fig 4, the EBV genomes present in these tumors appeared to be monoclonal when probed with the EBV terminal repeat region. This result clearly shows that the virus was associated with the tumor cells rather than with normal cells present in the tumor sample.

### Table 2. Distribution of Different Combinations of Chromosome 8 and 14 Breakpoints in Various World Regions

<table>
<thead>
<tr>
<th>Region</th>
<th>R/Sp μ</th>
<th>R/non-Sp μ</th>
<th>U/Sp μ</th>
<th>U/non-Sp μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina + Chile</td>
<td>9 (35)</td>
<td>9 (35)</td>
<td>1 (3)</td>
<td>7 (27)</td>
</tr>
<tr>
<td>Brazil</td>
<td>3 (25)</td>
<td>4 (33)</td>
<td>0</td>
<td>5 (42)</td>
</tr>
<tr>
<td>United States</td>
<td>9 (28)</td>
<td>20 (63)</td>
<td>1 (3)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Equatorial Africa</td>
<td>3 (13)</td>
<td>3 (13)</td>
<td>2 (9)</td>
<td>15 (65)</td>
</tr>
</tbody>
</table>

Percentages in parentheses.

Abbreviations: R, rearranged c-myc (within HindIII); U, unrearranged c-myc (outside HindIII); Sp μ, breakpoint within the HindIII-EcoRI fragment encompassing the Sp μ region; non-Sp μ, break outside the Sp μ region (Cp μ was considered as an Sp μ).

We compared EBV association with the breakpoint location on chromosomes 8 and 14. There was no evident correlation of the presence or absence of EBV with any chromosome 8 breakpoint region. Figure 2 shows that there is an equal distribution of EBV-positive and EBV-negative tumors in each region of c-myc. The majority of the tumors with an Sp μ breakpoint (62%) were EBV-positive, whereas most of the EBV-negative tumors (74%) had non-Sp μ breakpoints.

**DISCUSSION**

We have previously shown that in the SNCLs there is an association between the breakpoint location on chromosome 8 and the geographic origin of the tumor. A significantly higher proportion of tumors from Equatorial Africa (Ghana) had breakpoints outside the HindIII fragment encompassing c-myc than tumors from the United States. This, coupled to clinical and epidemiologic differences, suggests that SNCLs with breakpoints far 5' of the c-myc gene (commonly found in Africa) and those with breakpoints that interrupt the c-myc gene (commonly found in the United States) are pathogenetically different, perhaps with respect to the type of B cell in which the translocation develops. While ethnic factors cannot be completely excluded, the low incidence of SNCL in US blacks, which contrasts dramatically with the high incidence of the tumor in black Africans, suggests that the environment is the primary determinant of both the incidence and molecular subtype of SNCL. This notion is further supported by our present finding that, in South American tumors, the predominant breakpoint differs from both African and North American tumors.

Whereas 60% (18 of 30) of sporadic tumors from the United States had breakpoints in the first exon or intron of c-myc, only 26% (10 of 39) of tumors from South America had similar breakpoints. On the other hand, 16 of 39 (41%) of South American tumors had a breakpoint in the immediate 5' region of c-myc, whereas only 28% of tumors from the United States had a breakpoint in this region. Immediate 5' breakpoints were even more uncommon in Ghanaian tumors (9%).

These differences have important implications, because in tumors in which the breakpoint is in the first exon or intron of c-myc the normal c-myc promoters P1 and P2 are separated from the remainder of the gene and, hence, not available as transcription initiation sites. In this circumstance, transcripts are initiated from sites within the first.
intron. Moreover, in such tumors, the bulk of the regulatory region of the gene is deleted by the translocation. As many as 60% of North American SNCLs fall into this molecular category. In contrast, our analysis shows that the majority of tumors from South America (74%) have retained the normal P1 and P2 promoters. Breakpoints outside the HindIII fragment that encompasses c-myc (and, hence, outside the known regulatory region of c-myc) were observed primarily in Africa (74%), but also quite frequently in South America (33%). Such breakpoints were uncommonly observed in tumors from the United States (9%). These data suggest that there may be three main molecular subtypes of SNCL, and that it is probable that each of the major breakpoint regions (far S’, immediate S’, and first exon/intron, which predominate in Ghana, South America, and the United States, respectively) results in a different mechanism of c-myc deregulation. Presumably, the environmental conditions in each region are particularly conducive to the development of the molecular subtype of SNCL that predominates there.

The distribution of breakpoints on chromosome 14 observed in South American lymphomas is similar to that previously described for sporadic tumors: 30% of breaks are within the Sμ region. The breakpoint on chromosome 14 may be of considerable importance to an understanding of the mechanism of deregulation of c-myc, because breakpoints are either upstream or downstream of the 5’ intronic enhancer within the heavy chain locus, such that this regulatory sequence may or may not be juxtaposed to c-myc. Indeed, it is likely that it is the combination of breakpoint locations on chromosomes 8 and 14 that is crucial to the deregulation of c-myc.

SNCL can be subdivided on the basis of the four simplest combinations of breakpoints on chromosomes 8 and 14 (Table 2). From our data it appears that the combination of an unrearranged myc gene (within HindIII) with a switch breakpoint on chromosome 14 is very uncommon, occurring in only 4 tumors (2 African, 1 North American and 1 Chilean) among 93 tumors (4%) that we have examined to date. This may result from concomitant structural constraints with respect to the occurrence of this combination, or the necessity of other, rare genetic changes, to cooperate with this combination in providing the necessary growth advantage to the cell. Two of the other combinations examined (U/non-Sμ and R/non-Sμ) appear to occur at high frequency in African and North American tumors, respectively. However, in the South American lymphomas, these breakpoint combinations appear equally distributed.

The reason for the association of breakpoint locations, and particularly of specific breakpoint combinations, with geographic regions is unknown. It seems most probable that environmental factors, acting via the immune system, are responsible for causing expansions of slightly different B-cell populations (presumably with respect to the stage of differentiation), each more or less susceptible to the development of chromosome breaks at specific locations. However, an alternative explanation is that different c-myc-Ig translocations must cooperate with different genetic abnormalities, which are environmentally determined in bringing about deregulated growth.

In addition to differences in chromosomal breakpoint location among the regions examined, the proportion of tumors associated with EBV also differs. As is the case for breakpoint location, EBV association is also intermediate in frequency in South America. We have previously drawn attention to an apparent pattern in the breakpoint location on chromosome 8 and EBV association. Tumors with breakpoint locations outside the HindIII fragment are nearly always EBV associated, and such tumors are more frequent in Equatorial Africa. Breakpoints within the transcriptional unit of the gene, however, which occur predominantly in the United States, are equally likely to be EBV negative or positive. In South America, we have not found an association between EBV and a specific breakpoint location (Fig 2). Interestingly, a much higher proportion of tumors with a breakpoint far S’ of c-myc are EBV negative in South America (6 of 13) than in Africa (0 of 17). This raises the possibility that there are two types of SNCL with breakpoints far outside the c-myc gene, distinguished on the basis of EBV association. Moreover, South American tumors with a breakpoint in the small region immediately S’ of the c-myc promoters can be subdivided into those associated with EBV and EBV-negative tumors, which contrasts with the North American tumors, in which immediate S’ breaks (Pst I-Sma I) were invariably EBV negative. These findings indicate that, if there is an association of EBV with a breakpoint region, it is not a simple relationship. Perhaps the function subserved by EBV can be replaced by another factor or perhaps the precise breakpoint on chromosome 14 is very important; most of the EBV-negative tumors in South America (4 of 19 [76%]) and in the United States (13 of 20 [65%]) have non-Sμ breakpoints and probably, therefore, retain the intronic enhancer.

However, there does appear to be a gradient of EBV association extending from Africa through South America to the United States—a gradient that also corresponds to socioeconomic level and such factors as the frequency of infections in children.
Our findings strongly support the likelihood that there are several molecular subtypes of SNCL, each of which is more or less likely to occur in the geographic regions studied to date. It is interesting that the increased incidence of Ghanaian SNCL compared with SNCL in sporadic regions is largely, if not entirely, accounted for by tumors with far 5' breakpoints on chromosome 8. It will be important to determine the incidence of SNCL in various regions of South America, because this will permit the incidence of the molecular subtypes to be determined.

ACKNOWLEDGMENT

We thank the Fogarty International Center for developing this collaboration.

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