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ANALYSIS OF STEPWISE GENETIC CHANGES IN AN AIDS-RELATED BURKITT'S LYMPHOMA

Franco FAIS[1,2]*, Gilberto FRONZA[3], Silvio RONCELLA[4], Alberto INGA[3], Paola CAMPOMENOSI[3], Giovanna CUTRONA[1,2], Annalisa PEZZOLO[5], Franco FEDELI[4], Angelo ABBONDANDOLO[3], Nicholas CHIORAZZI[6], Vito PISTOIA[5] and Manlio FERRARINI[1,2],

[1]*Clinical Immunology Division, National Cancer Institute, Genoa, Italy*
[2]*Department of Oncology, Biology and Genetics, University of Genoa, Genoa, Italy*
[3]*Mutagenesis Laboratory, National Cancer Institute, Genoa, Italy*
[4]*Division of Pathology, St. Andrea Hospital, ASL 5, La Spezia, Italy*
[5]*Laboratory of Oncology, Institute Giannia Gaslini, Genoa, Italy*
[6]*Department of Medicine, North Shore University Hospital and New York University School of Medicine, Manhasset, NY, USA*

In this study, immunoglobulin variable (Ig V) region genes, *c-myc* re-arrangement and sequence and *p53* status were analyzed in clones derived from a Burkitt's lymphoma cell line (LAM) in which it was previously demonstrated that Epstein-Barr virus (EBV) infection occurred late during lymphomagenesis. Such evidence was based on the finding that 2 groups of cellular clones, characterized by the same *c-myc* re-arrangement but different EBV-fused termini, were obtained from the LAM cell line. The Ig V gene sequences were identical for the 2 groups of clones with different EBV-fused termini. The Ig variable heavy (V_H) gene sequence displayed a substantial accumulation of point mutations (but no intraclonal diversification), whereas the productive Ig V lambda (V_λ) gene sequence was virtually unmutated. Studies on the Ig V kappa (V_κ) locus suggested a receptor revision event (with a switch from κ to λ chain production) prior to EBV infection. Likewise, it was determined that the mutations observed in both p53 alleles and in the re-arranged *c-myc* gene occurred before EBV infection. Based on these findings, we present a model for the various steps of lymphomagenesis. It is proposed that stimulation by an antigen or a superantigen initially favored the clonal expansion and accumulation of other cytogenetic changes, including those involved in receptor editing. These events occurred prior to or during the germinal center (GC) phase of B-cell maturation. Thereafter, possibly upon exit of the cells from the GC, EBV infection occurred, further promoting lymphomagenesis. *Int. J. Cancer* 88:744–750, 2000.

Burkitt's lymphoma (BL) is a high-grade lymphoma of which there are 2 main variants, known as endemic (e) BL and sporadic (s) BL (Magrath, 1990). Although they share a number of cytogenetic features, eBL and sBL are distinguished by their clinical presentation and the presence of the Epstein-Barr virus (EBV) genome, which is harbored in the vast majority of eBL and absent in most sBL cases. In addition, eBL primarily affects children and young adults of particular geographical areas (Magrath, 1990). Patients with AIDS have a high frequency of BL, and in this setting, it resembles sBL except that the EBV genome is found more frequently (in up to 50% of cases, depending on different reports) (Gaidano and Dalla-Favera, 1995).

All BL cases are characterized by chromosomal translocation of the *c-myc* oncogene, which moves from its normal location on chromosome 8 to chromosome 14 (Dalla-Favera *et al.,* 1982) or, less frequently, to chromosome 2 or 22 (Croce *et al.,* 1983). Because of this translocation, *c-myc* becomes juxtaposed with the regulatory regions of Ig genes and is generally over-expressed. However, although this event must be considered a *conditio sine qua non,* the sole *c-myc* translocation appears to be insufficient to cause fully neoplastic transformation of the cells *in vivo.* Other cytogenetic changes commonly found in BL include mutations of *c-myc* (Bhatia *et al.,* 1994) and *p53* (Gaidano *et al.,* 1991) and changes in the 5′-untranslated region of *bcl-6* (Capello *et al.,* 1997). Moreover, the presence of the EBV genome may contribute to malignant transformation. Although the relative pathogenetic role of these factors is not fully understood, some information could be obtained by ascertaining the relative timing of these events. Our study deals with the timing of these transforming events in a special case of an AIDS-related BL (Roncella *et al.,* 1993).

We previously showed that primary samples obtained from the bone marrow and the peripheral blood of this BL as well as the cell lines and cell clones derived from them displayed 2 different EBV-fused termini. The 2 groups of malignant cells, however, had the same VDJ and *c-myc* re-arrangements. Based on this observation, we suggested that EBV infection was a late event in lymphomagenesis. Here, we investigated the timing and the features of other genetic changes that characterized the malignant cells. Our analysis disclosed a complex series of re-arrangements at Ig loci that might have occurred in the germinal center (GC) during antigen stimulation. Our findings suggest that some of the genetic alterations seen in BL may be generated during the GC reaction.

MATERIAL AND METHODS

Patients and cell lines

The patient LAM has been described previously (Roncella *et al.,* 1993). Spontaneous cell lines were obtained, after *in vitro* culture, from both the peripheral blood mononuclear cells (PB-LAM) and from the bone marrow (BM-LAM). These cell lines were cloned by limiting dilution, and the following clones were obtained and studied: BM-C4, BM-D4, BM-C4S1, BM-C4S2, BM-H4 (from BM-LAM), PB-C3S4, PB-F8, PB-F3, PB-D8 and PB-B2.1 (from PB-LAM). These clones expressed surface IgM/λ and showed the typical phenotype of BL with expression of CD10 and CD38 and lack of CD39 and CD23. LAM-K was obtained after rosetting the BM-LAM cell line using an anti-human κ antibody (Becton-Dickinson, Milan, Italy) and Dynabeads (Dynal, Milan, Italy), following the manufacturers' instructions. This cell line did not have *c-myc* re-arrangements and therefore was considered a normal lymphoblastoid, EBV^+ cell line.

Detection of EBV-fused terminal fragments by Southern blot

Southern blot analysis for the EBV-fused termini was performed on high m.w. DNA (20 μg) after digestion with the BamHI restriction enzyme (Boehringer-Mannheim, Mannheim, Germany). The XhoI 1.9 probe, which detects a DNA sequence adjacent to right terminal repeat sequences of the viral genome (Raab-Traub

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*Correspondence to: Servizio di Immunologia Clinica, Istituto Nazionale per la Ricerca sul Cancro, IST, L.go R. Benzi 10, 16132 Genoa, Italy. Fax: +39-010-5600.264. E-mail: ffais@hp380.ist.unige.it

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and Flynn, 1986), was labeled with digoxigenin-11-dUTP by nick translation following the instructions of the manufacturer (Boehringer-Mannheim). Filters were hybridized to the digoxigenin-labeled probe overnight at 42°C in 50% formamide solution and washed at high stringency (0.1 × SSC/0.1% SDS, 65°C). The chemiluminescent signal produced by the hybridized probe was detected as suggested by the manufacturer. Briefly, after hybridization, the filter was subjected to immunological detection using an alkaline phosphatase (AP)–conjugated anti-digoxigenin antibody, diluted 1:5,000, and then incubated in freshly prepared chemiluminescent AP substrate solution at 0.25 mM final concentration. The filter was exposed to record the chemiluminescent signals.

Preparation of templates for PCR

Total RNA was isolated from the cell lines using RNA clean (TIB Molecular Biology, Genoa, Italy) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed to cDNA using M-MLV reverse transcriptase (GIBCO BRL Life Technologies, Milan, Italy) and an oligo-dT (16 mer) primer. This reaction were carried out in 25 μl using 10 pmole of the primer at 42°C for 1 hr. Genomic DNA was prepared from cell lines using the QIAamp blood kit (Qiagen, Basel, Switzerland), following the manufacturer's instructions.

Conditions for PCR and cDNA sequencing

To determine the Ig V_H and V_L gene nucleic acid sequences of the cell lines, 2 μl of cDNA were amplified using a sense (V_H or V_L) leader family-specific primer in conjunction with an anti-sense Cμ, Cκ or Cλ primer (Fais *et al.*, 1999). PCR conditions have been previously reported (Fais *et al.*, 1999). PCR products were cloned into TA vector (Invitrogen, San Diego, CA), and at least 4 different molecular clones were sequenced for every LAM cellular clone using an automated sequenator (Applied Biosystems, Milan, Italy).

The *c-myc* re-arrangement was amplified using 0.5 μg DNA. The JHC primer CTG AGG AGA CGG TGA CCG TGG T was used in conjunction with the *c-myc* 4839R primer CCA GCA GCT CGG TCA CCA TCT C under the following conditions: denaturation at 94°C for 20 sec, annealing and extension at 68°C for 2 min; after 30 cycles, extension was continued at 72°C for an additional 10 min. rTth DNA polymerase (Perkin-Elmer, Milan, Italy) was used. PCR products, obtained from 2 independent tests for each cellular clone, were directly sequenced.

Analyses of Ig V_H and V_L sequences

Ig V_H and V_L sequences were compared with the V BASE sequence directory (MRC Centre for Protein Engineering, Cambridge, UK) using MacVector 6.0.1 software (Oxford Molecular Group, Oxford, UK). To evaluate the distribution of mutations among complementary determining region (CDR) and framework (FR) gene segments, the Chang-Casali binomial distribution model was used (Chang and Casali, 1994; details reported in Fais *et al.*, 1999).

Functional assay for p53 mutations

We evaluated the presence of functional *p53* mutations in the different cell lines by the yeast-based *p53* functional assay developed by Flaman *et al.* (1995). In this assay, yeast colonies that contain wild-type *p53* are white, while colonies that contain mutated *p53* are red. Briefly, cDNA was synthesized from LAM-K, BM-C4 and PB-D8 cell lines as reported above. The *p53* open reading frame between nucleotide positions 125 and 1122, including all sequence coding for the DNA-binding domain of the protein, was PCR-amplified using P3 and P4 primers and *Vent* DNA polymerase (New England Biolabs, Milan, Italy) and 2 μl of the cDNA reaction product for 35 cycles of 94°C for 30 sec, 65°C for 60 sec and 78°C for 80 sec. To test the cDNA *p53* status, PCR products and HindIII-StuI–linearized pRDI-22, lacking the sequences coding for the entire *p53* DNA-binding domain, were co-transformed by electroporation into the haploid strain yIG397 (MATa ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100, ura3-1 URA3 3xRGC::pCYC1::ADE2) containing the *ADE2* reporter gene under *p53* control. pRDI-22 was obtained from the yeast expression vector of human wild-type *p53* pLS76 (Ishioka *et al.*, 1993), containing LEU2 as the selectable marker in yeast cells, by BsmI(4432)/StuI(3589) digestion. The HindIII-StuI–digested, pRDI-22–gapped plasmid has 2 regions homologous to the terminal regions of the PCR products obtained using P3 and P4. After co-transfection followed by homologous recombination *in vivo* (gap repair), a p53 expression vector having the wild-type promoter region (derived from pRDI-22) and the core region of the p53 open reading frame (derived from the P3/P4 PCR product) was reconstituted. If the clone was initially ade- due to a mutation in the promoter region, the gap-repaired transformants result in almost exclusively white, normally sized colonies on limiting adenine plates (no small red colonies) since the promoter in the gap-repaired plasmid, derived from pRDI-22, is wild-type (gap repair-negative). On the contrary, if the PCR product contained a single mutation in the core region, approximately 100% of the derived transformed clones gave rise to small red colonies (gap repair-positive) (Flaman *et al.*, 1995). p53 mutants, giving about 100% red colonies (gap repair-positive), were re-amplified and sequenced as previously described (Inga *et al.*, 1997).

Gap-repaired transformants were selected on minimal plates lacking leucine but containing sufficient adenine (5 μg/ml) for adenine auxotrophs to grow and turn red. The percentage of small red colonies was determined. Eleven independent red clones (6 LAM-C4, 5 LAM-D8) were picked, grown overnight in synthetic minimal medium lacking leucine but containing an excess of adenine (200 μg/ml). pLS76 plasmids were recovered and the *p53* cDNA were re-amplified with P3 and P4 primers and Taq DNA polymerase (Promega, Florence, Italy). PCR products were purified by Microcon 100 (Amicon, Rho, Italy) and directly sequenced. In addition to P3 and P4, 2 internal primers were used for sequencing: P5 5′-TggCCATCTACAAgCAgTCA-3′, nucleotides 479–497, and P6 5′-gggCACCACCACACTATgTC-3′, nucleotides 638–657, of the *p53* cDNA.

RESULTS

Ig V_H gene-sequence analyses

Five clones from the PB-LAM cell line and 5 clones from the BM-LAM cell line were utilized. These 2 groups of clones had the same VDJ and *c-myc* re-arrangements but different EBV-fused termini (Fig. 1) (Roncella *et al.*, 1993). Re-arranged V_H gene

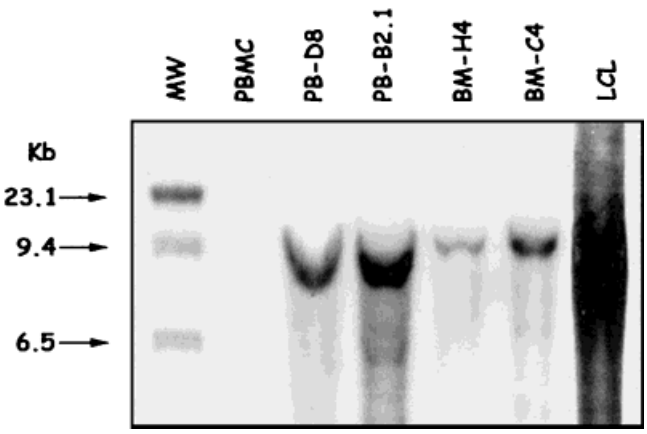

FIGURE 1 – Southern blot of EBV-fused termini of LAM-derived cell clones. BM-H4 and BM-C4 are 2 representative clones derived from the bone marrow; PB-D8 and PB-2.1 are 2 representative clones derived from the peripheral blood of the patient LAM. Peripheral blood mononuclear cells (PBMCs) were used as negative control, and a cell line originated following infection with B95.8 supernatant was used as polyclonal control. MW, molecular weight.

segments were amplified from each clone and sequenced. All clones expressed the V_H4–34 gene, the 3–9 D segment (also known as DXP1) and the J_H4 segment. However, despite extensive analysis (40 molecular clones were analyzed), intra-clonal diversification was never observed. The V_H4–34 gene expressed by LAM clones was extensively mutated (n = 24, 8%; Fig. 2); there was no statistical evidence for the accumulation of replacement mutations in the CDR based on Chang-Casali criteria ($p > 0.05$), though there was evidence for preservation of FR regions according to the same criteria ($p < 0.05$).

Ig V_L gene-sequence analysis

LAM cell lines and clones invariably expressed monotypic IgM/λ molecules when analyzed by immunofluorescence (Roncella *et al.,* 1993). When λ light-chain cDNA was amplified from 4 clones (BM-D4, BM-H4, PB-D8 and PB-B2.1) and analyzed, all clones invariably expressed the *lv318* gene of the V λ 3 family. This gene was virtually germline (Fig. 2).

Previous Southern blot analyses revealed that the κ locus of all LAM cell lines presented 1 deleted and 1 re-arranged allele each (Roncella *et al.,* 1993). Indeed, RT-PCR disclosed that a κ chain transcript was present in all clones studied. Sequence analysis of PCR products from 4 clones (BM-D4, BM-H4, PB-D8 and PB-B2.1) showed that the Vκ gene used by all of them was the B1 gene, which belongs to the VKVII family. This gene was originally described as being non-functional because of a nucleotide change in the ATG starting codon (ATG→ATA) (Lorenz *et al.,* 1988); this nucleotide change was also present in LAM cells (not shown). However, the gene differed from the B1 germline counterpart by the presence of 12 somatic mutations (3.7%, Fig. 2). In addition to the mutation in the starting codon, the Vκ-Jκ rearrangement in LAM cells generated an out-of-frame sequence that completely excluded the possibility that the κ gene could encode a functional K chain.

Translocated c-myc sequence

Taking advantage of preliminary Southern blot studies (Saglio *et al.,* 1993), we designed a PCR approach to amplify and sequence the region encompassing the *c-myc* translocation (Fig. 3). A J_H primer was used in conjunction with a primer mapping to the second exon of *c-myc.* A PCR product of about 1,700 bp was amplified from BM-C4 and PB-D8 malignant clones, while no product was obtained using the LAM-K lymphoblastoid B-cell line (not shown). Sequence analysis disclosed that the *c-myc* breakpoint was located at nucleotide 3170 juxtaposed to the J_H6 segment (Fig. 4). There was no evidence for N-segment additions. As shown in Figure 3, myc intron factors 1 and 2 were lost whereas myc intron factor 3 was partially preserved; this finding is similar to a previous report (Yu *et al.,* 1993).

The translocated *c-myc* sequence had 7 nucleotide substitutions in the first intron and 3 mutations in the portion of the second exon analyzed (Fig. 4). These substitutions were present in both of the clones analyzed and absent in LAM-K cells, thus indicating that they were somatic mutations rather then allelic variants. In the second exon, replacement mutations were present at codon 9 (AAc→AAg, Asn→Lys), codon 11 (AaC→AgC, Asn→Ser) and codon 58 (AcC→AaC, Thr→Asn).

p53 analysis

The *p53* functional assay (Flaman *et al.,* 1995) was employed to determine whether 2 representative clones, each from 1 group with different EBV-fused termini, had *p53* mutations capable of impairing its function. *p53* cDNAs, derived from different cells (LAM-K, BM-C4 and PB-D8 cell clones), were PCR-amplified and analyzed by gap repair in yeast. In detail, unpurified PCR products were co-transfected with HindIII-StuI–linearized pRDI-22 plasmid. HindIII-StuI–linearized pRDI has 2 regions homologous to the terminal regions of the PCR products. After co-transfection followed by homologous recombination *in vivo* (gap repair), a p53 expression vector having the wild-type promoter region (derived from pRDI-22) and the core region of the p53 open reading frame (derived from the PCR product) was reconstituted. If the PCR product contained a mutation, the reconstituted expression vector expressed a mutant p53 protein, unable to transactivate the *ADE2* reporter gene. In this assay, yeast colonies expressing such a mutant p53 originate small red colonies,

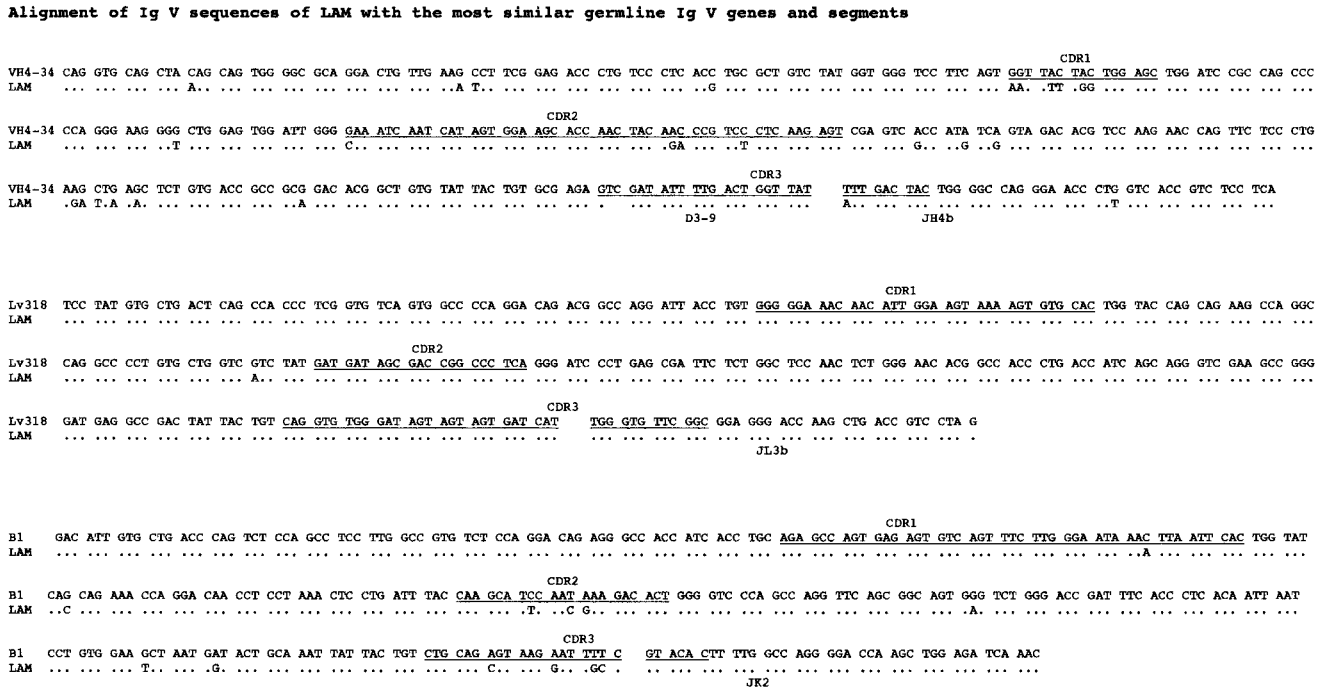

FIGURE 2 – Nucleotide sequences of re-arranged LAM Ig V genes. Nucleotide sequence of the LAM Ig V genes is shown and compared to the corresponding germline Ig V sequence. CDRs are underlined.

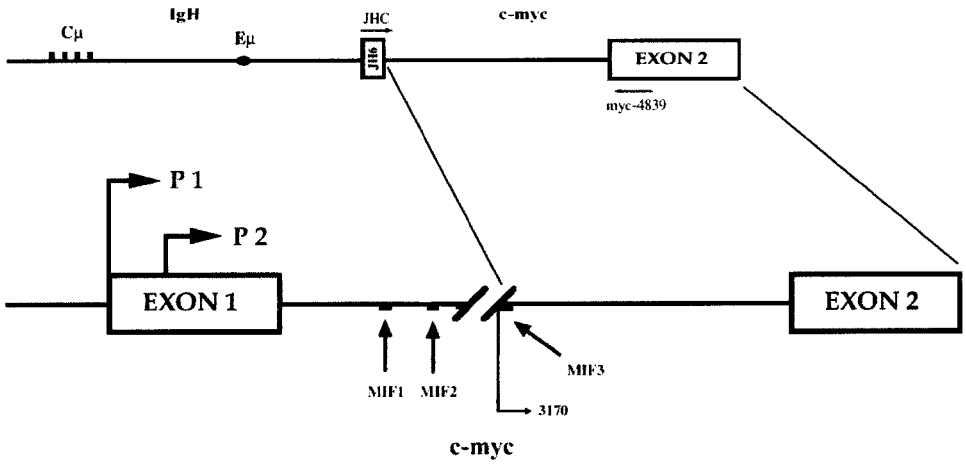

FIGURE 3 – Schematic representation of PCR strategy used to amplify re-arranged *c-myc* in LAM cells, with the breakpoint on the *c-myc* gene anf some relevant features indicated. Upper figure represents the *c-myc* gene juxtaposed to the Ig heavy-chain region as deduced by nucleotide sequence of the region encompassing the re-arrangement. JHC and *c-myc* 4839 were the primers used for PCR amplification. In the lower figure, the *c-myc* breakpoint is shown in relationship to myc intron factor (MIF) regions. P1 and P2 indicate promoters present in the first intron. 3170, nucleotide number of the breakpoint (refers to submitted *c-myc* sequence X00364).

while colonies expressing wild-type p53 originate normally sized white colonies. Both neoplastic cell clones (BM-C4 and PB-D8) expressed completely inactive p53 alleles (near 100% red colonies) (Table I), while the non-neoplastic LAM-K cell line expressed wild-type *p53* alleles. This result is compatible with either the presence in BM-C4 and PB-D8 cell clones of 2 alleles with inactivating mutations or the presence of a single mutated allele accompanied by loss of the other allele.

To determine whether BM-C4 and PB-D8 cells shared the same *p53* mutations, plasmids containing mutated *p53* cDNA from 11 independent red yeast colonies (6 from BM-C4, 5 from PB-D8) were rescued and sequenced (Table II). A C→T transition at codon 273 resulting in an arginine to cysteine amino acid substitution was present in 5 of 6 BM-C4 yeast colonies and in 3 of 5 PB-D8 yeast colonies. Three yeast colonies (BM-C4-8, PB-D8-4 and PB-D8-5) exclusively showed an insertion of 9 bp, resulting in the insertion of 3 amino acids. One yeast colony derived from each of the cell clones (BM-C4-2 and PB-D8-8) showed the co-existence of both mutations (C→T and +9 bp), most likely derived from PCR cross-over. Therefore, at this level of analysis, the BM-C4 and PB-D8 neoplastic cell clones appeared to have the same 2 mutated alleles.

DISCUSSION

All of the cytogenetic changes observed in LAM cells took place prior to EBV infection. Moreover, we have disclosed a number of stepwise changes that potentially occurred during lymphomagenesis. Figure 5 schematically summarizes a hypothetical cascade of events that contribute to the genesis of BL.

The V_H gene used by LAM cells was 4–34, which contained somatic mutations (Fig. 2) that failed to alter the FR regions of the Ig molecule, suggesting antigen stimulation. This observation is similar to that described previously in 2 other BLs that used the same V_H gene (Riboldi *et al.*, 1994). Moreover, as in the cases reported by Riboldi *et al.* (1994), the Ig produced by LAM cells had cold agglutinin (CA) activity with specificity for the i antigen present on the fetal red blood cells and not for the I antigen expressed by erythrocytes from adults (data not shown). In antibodies encoded by the V_H4–34 gene, the i/I binding capacities are determined primarily by the FR1 structure, while sequences of HCDR3 dictate the fine specificity for either i or I (Li *et al.*, 1996). Although with CA, antigen binding is determined mainly by the H chain, the L chain is also important since the presence of incompatible light chains abrogates antigen binding. Based on the preservation of FR regions, in particular FR1, and the choice of a compatible λ chain (see below), it appears likely that stimulation by a carbohydrate structure related to the i antigen, which is not expressed exclusively by red blood cells (Bhat *et al.*, 1993; Grillot-Courvalin *et al.*, 1992), may play a role at a certain point during lymphomagenesis. Whether this stimulation occurred concomitantly with that of the specific antigen that also caused accumulation of point mutations in the V_H chain genes is unclear.

The sequence of the re-arranged Vλ gene showed that it belonged to V_λ3 family. In contrast with the re-arranged V_H gene, this gene was minimally mutated. Moreover, N additions were not obvious at the Vλ–Jλ junction (Fig. 2). The virtual absence of somatic mutations on the Vλ gene suggests that this re-arrangement was a relatively late event that took place in a κ-expressing cell after it had reached the GC and accumulated V_H gene mutations. Expression of RAG1 and RAG2 has been shown in human GCs (Meffre *et al.*, 1998; Giachino *et al.*, 1998). This finding, although somewhat debated based on observations of experimental animals (Monroe *et al.*, 1999; Yu *et al.*, 1999), has been used to suggest that this mechanism contributes to "antibody diversification" and/or rescue of B cells with deleterious mutations on Ig V genes (Rajewsky, 1998).

Although the clones expressed a monotypic λ light chain, we were able to amplify a re-arranged κ gene in all of the LAM clones. Sequencing the PCR product revealed that the re-arrangement was non-productive due to frameshift mutations in the CDR3 region. Moreover, the Vκ gene re-arranged was the B1 gene, the unique member of the VKVII family which is non- functional because of a mutation in the ATG starting codon (Lorenz *et al.*, 1988). The timing of the κ chain re-arrangement could not be determined precisely, though the presence of a significant number of somatic mutations suggests that it took place before the Vλ gene re-arrangement. The other κ allele was deleted (Roncella *et al.*, 1993), a finding that makes analyses of molecular events in LAM cells more complicated. One possible explanation, based on the non-functional re-arrangement of the remaining κ allele and its mutation pattern, is that the deleted κ gene was indeed the one used by the clone to code for the Ig molecules, potentially up to their transit in the GC. There, both productive and non-productive Vκ genes were likely to accumulate point mutations before deletion of the productive κ allele and the switch to the λ light chain. A similar example of the Ig V light-chain switch has been reported in a

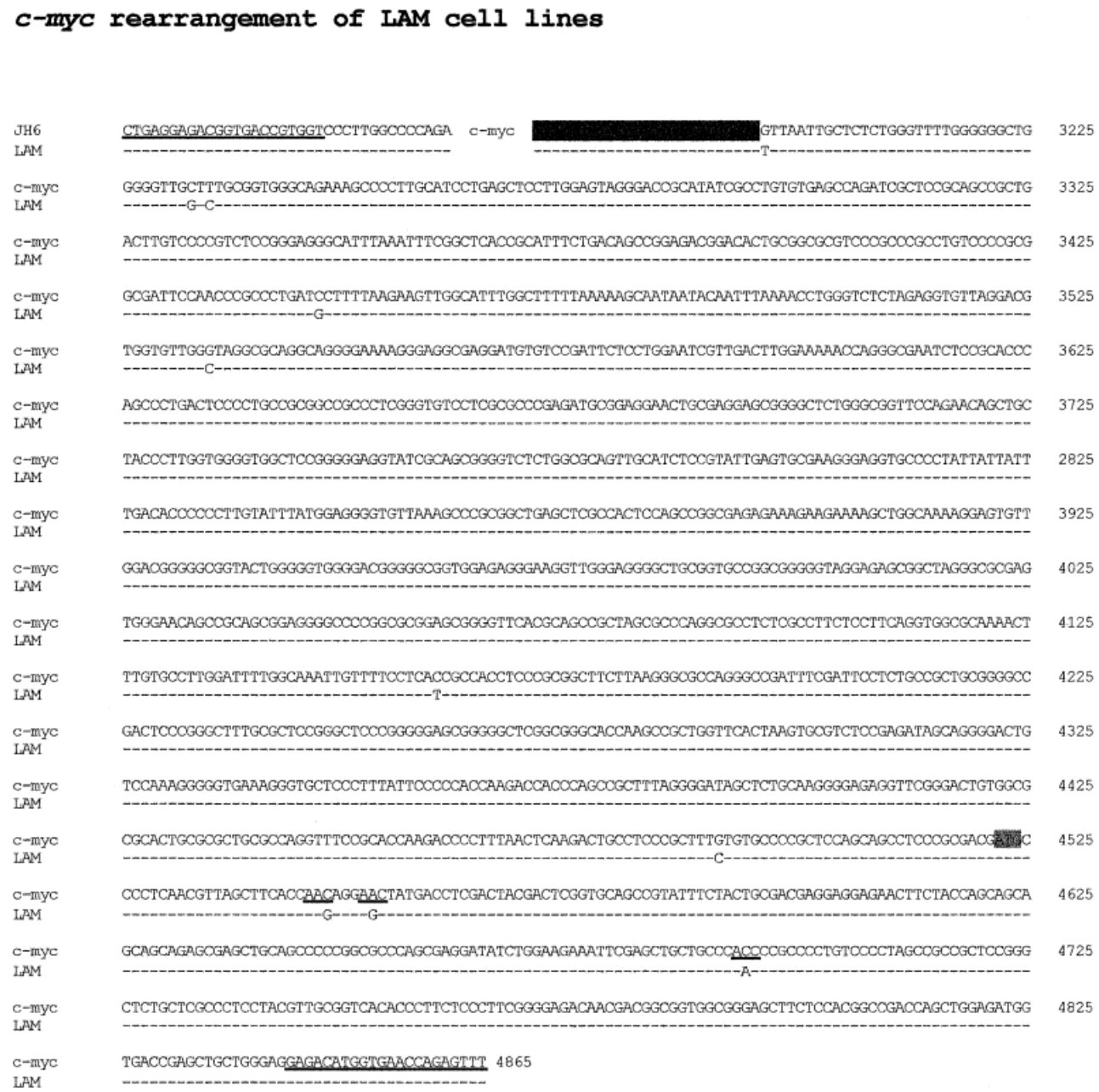

FIGURE 4 – Nucleotide sequence of region encompassing the *c-myc* re-arrangement on Ig heavy-chain locus on chromosome 14 in LAM cells. Underlined sequences at top and bottom represent primer areas. Preserved portion of myc intron factor 3 is highlighted. The ATG starting codon (at position 4522) is highlighted. The 3 mutated codons in the *c-myc* coding region are underlined.

normal B cell line derived from peripheral blood (de Wildt *et al.*, 1999).

The re-arranged *c-myc* gene was amplified and sequenced in 2 representative clones, from PB and BM (PB-D8 and BM-H4), with different EBV-fused termini. The sequences did not differ between the 2 clones. Ten nucleotide substitutions were observed in the amplified segment. Interestingly, replacement mutations in the second exon were present at codons 9, 11 and 58. This area encodes the N-terminal transcription activation domain (Henriksson and Luscher, 1996). In particular, Thr^{58} and other amino acids in its proximity have been described as often being targeted by somatic mutations in the re-arranged *c-myc* allele in BL (Bhatia *et al.*, 1994). Moreover, phosphorylation of Thr^{58} is necessary for the *c-myc*–suppressing activity of p107, a protein related to the retinoblastoma protein. The lack of Thr^{58} phosphorylation results in increased transforming activity *in vitro* (Hoang *et al.*, 1995), likely due to decreased proteasome-mediated turnover (Bahram *et al.*, 2000). Therefore, these changes may have contributed to further acquisition of malignant properties by LAM cells. The translocated *c-myc* oncogene was likely to have mutated during cell passage in the GC because of the proximity of the Ig genes. Whether translocation of *c-myc* also took place at the same site could not be determined, though the occurrence of recombination events, including isotype switching and possibly secondary Ig gene re-arrangements, makes this hypothesis particularly appealing (Kuppers *et al.*, 1999).

Sequence and functional analyses showed that both *p53* alleles from malignant LAM cells carried mutations resulting in protein inactivation. These mutations were somatically acquired and

present in both groups of clones with different EBV-fused termini. The timing of these mutations could not be ascertained. However, the involvement of *p53* in inducing apoptosis (Liebermann *et al.*, 1995) and the abundant evidence demonstrating that *c-myc* overexpression predisposes cells to apoptosis (Cutrona *et al.*, 1995; Polack *et al.*, 1996). Hence, it is possible that *p53* mutations favored the expansion of cells that already had a translocated *c-myc*.

The presence of multiple EBV-fused termini is generally taken as evidence for multiple infection events (Raab-Traub and Flynn, 1986), though alternative explanations have been offered, including recombination among EBV episomes. These may be used to explain the heterogeneity of EBV-fused termini in certain, otherwise monoclonal, tumor samples taken *ex vivo* (Delecluse *et al.*, 1993) since EBV recombination may occur in some cellular components of a given malignant clone. Following this event, because of the presence of a multiplicity of EBV copies, the malignant cells would display a combination of original as well as recombinant EBV termini. In LAM cells, however, cellular clones could be isolated with single, albeit different, EBV-fused termini. Subsequent infectious events, rather than recombination of episomes, perhaps best explain this finding.

TABLE I – ANALYSIS OF *p53* Status in the Different Cell Lines by Yeast Functional Assay

Colonies	Colonies		Red total (%)
	Red	White	
Control	149	540	21.6
LAM-K	246	854	22.4
BM-C4	2,965	92	97.0[1]
PB-D8	1,578	37	97.7[1]

As control, pLS76 expression plasmid containing wild-type *p53* cDNA was PCR-amplified using primers P3 and P4 and *Vent* DNA polymerase.–[1]$p < 0.0001$ (χ^2 test, compared to control).

TABLE II – SEQUENCING ANALYSIS ON INDEPENDENT *p53* Mutant Clones

	Arg^{273}>Cys	9 bp Insertion[1]
BM-C4-1	+	–
BM-C4-2	+	+
BM-C4-5	+	–
BM-C4-6	+	–
BM-C4-7	+	–
BM-C4-8	–	+
PB-D8-2	+	–
PB-D8-4	–	+
PB-D8-5	–	+
PB-D8-7	+	–
PB-D8-8	+	+

[1]The 96p, in-frame insertion (sequence GGG TGT AAC) coding for Gly-Cys-Asn occurred between codon 239 and codon 240.

Collectively, the above data reiterate the notion that in AIDS-related BL EBV infection represents a late event in cells that have already accumulated a number of cytogenetic changes (Roncella *et al.*, 1993; Gutierrez *et al.*, 1997). This observation raises a number of questions related to both the promoting factors that facilitate the clonal expansion of malignant B lymphocytes as well as the role of EBV in the pathogenesis of lymphoma. Although these questions remain unresolved, it is tempting to speculate that stimulation by specific antigens or even by a superantigen represents an important promoting effect on transforming B cells, at least until their entrance into the GC, where *c-myc* translocation may occur. As for the pathogenetic role of EBV, doubts may be raised primarily by the observation that in AIDS-related BL, as well as in other BL cases, type I latency of the virus occurs (Rowe *et al.*, 1986). With this type of latency, which was also observed in LAM cells (Roncella *et al.*, 1993), EBNA2 or EBNA3 antigens are not expressed and, hence, cannot promote cell proliferation. Likewise LMP1, which prevents apoptosis (Henderson *et al.*, 1991; Wang *et al.*, 1987, 1990), is absent. However, EBNA1, which is consistently expressed by these BL cells, can have a promoting role in lymphomagenesis (Wilson *et al.*, 1996). This concept is also supported by our previous observation that all LAM cells invariably expressed EBV (Roncella *et al.*, 1993), indicating that the presence of the virus provided a growth advantage.

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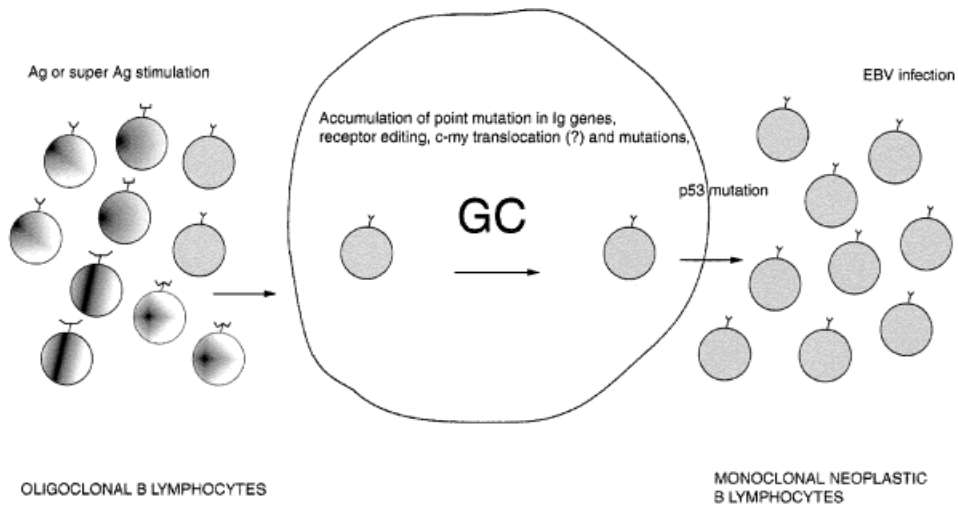

FIGURE 5 – Schematic representation of factors possibly involved in AIDS-related BL pathogenesis. Antigen (or superantigen-like molecules, such as i antigen) drives initial expansion of a certain number of B-cell clones (oligoclonal expansion). One of these clones accumulates a number of cytogenetic changes possibly related to the other molecular events occurring in the GC (somatic hypermutation, Ig isotype switch and receptor editing). *p53*-inactivating mutations appear to be necessary for survival of these cells. Whether mutations of *p53* occur during the transit of GC or outside the GC is not known. Heterogeneity of EBV-fused termini in neoplastic cells suggests that EBV infection is a late event and might contribute to malignancy in immunocompromised subjects.

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