V_H and V_L Gene Analysis in Sporadic Burkitt's Lymphoma Shows Somatic Hypermutation, Intraclonal Heterogeneity, and a Role for Antigen Selection

By Caroline J. Chapman, Jo X. Zhou, Chris Gregory, Alan B. Rickinson, and Freda K. Stevenson

Tumor cell lines and one tumor biopsy from seven cases of Epstein-Barr virus (EBV) genome-negative sporadic Burkitt's lymphoma (BL) have been investigated for usage and mutational pattern of Ig variable region genes. The V_H genes were derived from the V_H 3 (one) and V_H4 (six) families and both the IgM-positive (six) and the IgA-positive (one) were all mutated from their germline counterparts. The V_L genes were derived from Vκ1 (one), Vκ3 (one), Vλ1 (four), and Vλ2 (one) families and were also somatically hypermutated. Biopsy material from one of the IgM-positive cases showed V_H and V_L sequences that matched the derived cell line, with additional intraclonal sequence heterogeneity, indicating that the tumor cells had undergone posttranformation somatic mutation. Mutational patterns in V_H genes did not show a conventional role for antigen in selecting tumor cell sequences. In contrast, patterns in V_L sequences were consistent with a role for antigen in five of seven cases. The pattern of extensive scattered somatic hypermutation and intraclonal variation is similar to that in V_H sequences of EBV genome-positive endemic BL, although the degree of mutational activity is less. These common features indicate that B cells involved in the two variants of BL may share a common clonal history.

BURKITT'S LYMPHOMA (BL) is a high-grade B-cell lymphoma that presents in two distinct geographic areas. The high incidence or endemic form is restricted to certain parts of equatorial Africa and New Guinea, and the low incidence or sporadic form is seen worldwide.[1] In recent years, the incidence of sporadic BL has risen due to an increase in immunosuppressed patients, particularly those with human immunodeficiency virus (HIV) infection.[1-3] Both types of BL carry a characteristic reciprocal chromosomal translocation involving chromosome 8 at the site of the c-*myc* protooncogene and one of the three Ig gene loci on chromosome 14, 2, or 22.[4,5] This translocation can lead to constitutive active transcription of the c-myc gene, particularly when accompanied by mutations in the regulatory or protein-coding sequences.[6-9] The translocation event appears, therefore, to contribute to subsequent malignant transformation, although other influences may be required.[1] For endemic BL, Epstein-Barr virus (EBV) may provide an additional influence, with cases almost always associated with the presence of the EBV genome. However, sporadic BL has a more uneven association with EBV, ranging from 10% to 85% positivity in different areas.[10,11]

A further distinction between the two forms is in organ distribution, with endemic BL having a high frequency of jaw involvement.[1] Both forms tend to give rise to abdominal tumors, but each involves different regions, and bone marrow disease is more common in sporadic BL.[1,12-14] Morphology and cell surface marker analysis have suggested that the cell of origin in BL derives from the germinal center,[15] although the site of growth of endemic BL is often in tissues that do not normally contain germinal centers, such as jaw or ovary.[16]

Information relevant to the clonal history of neoplastic B cells is being provided by analysis of the immunoglobulin variable region genes, V_H and V_L. The first point is that usage of V-genes from the available repertoire can be asymmetric, as found dramatically for B-cell tumors secreting cold agglutinins, which are largely encoded by a single V_H gene.[17,18] There is also evidence for asymmetry of V-gene usage in B-cell lymphomas[19,20] and in chronic lymphocytic leukemia.[21,22] In addition, sequence analysis can show if the cell of origin has traversed the germinal center and been exposed to the somatic hypermutation mechanism operative in that site.[23] Accumulation of mutations that lead to amino acid substitutions in the complementarity-determining regions (CDRs) may indicate a role for antigen in stimulating growth and survival of the expressing B cell.[24] Somatic mutation and antigen selection may occur before or after neoplastic transformation. In cases of follicular lymphoma, there is evidence for continued exposure to somatic hypermutation after transformation,[23,25] whereas in myeloma, the malignant plasma cell appears to have undergone somatic mutation before the final neoplastic event, but to accumulate no further mutations.[26]

In the case of endemic BL, analysis of V_H genes showed extensive somatic hypermutation in six of six cases, with evidence for ongoing mutation as in follicular lymphoma.[27] However, the pattern of mutations did not indicate a role for antigen selection in the expressed sequences.[27] The V_H genes of five cases of sporadic BL occurring in association with acquired immune deficiency syndrome (AIDS) have also been investigated, and somatic mutation is again a clear feature.[28-30] In sporadic BL, evidence for antigen selection from V_H gene sequences has been uneven, but generally unconvincing,[29,30] although a single case that demonstrated intraclonal class switching and somatic mutation showed accumulating replacement mutations in the CDRs.[28] The present study has analyzed both V_H and V_L genes from seven cases of EBV-negative sporadic BL in HIV-negative children to compare the clonal histories of the two types of BL that occur in this age group.

From the Molecular Immunology Group, Tenovus Laboratory, Southampton University Hospitals, Southampton; Department of Immunology, University of Birmingham Medical School, Birmingham; and CRC Laboratories, Department of Cancer Studies, University of Birmingham Medical School, Birmingham, United Kingdom.

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Address reprint requests to Caroline J. Chapman, PhD, Molecular Immunology Group, Tenovus Laboratory, Southampton University Hospitals, Tremona Rd, Southampton SO16 6YD, United Kingdom.

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MATERIALS AND METHODS

Cell lines. The BL cell lines were established from tumor biopsies from HIV-negative children or young adults with a histological diagnosis of BL. The L3055 patient was a 17-year-old male of Arabic origin with an initial diagnosis of Burkitt type acute lymphoblastic leukemia (L3 morphology). All the other cell lines were isolated from children of European origin and have been described elsewhere.[31-35] All the lines were negative for the EBV genome. Six of seven had the most common t(8;14)(q24;q32) chromosomal translocation, while BL2 had the t(8;22) (q24;p11) translocation. Six of seven had been previously described as reacting with V_H4 specific antibodies 9G4 and LC1.[36] Biopsy material from which the L3055 cell line was derived was also available, and an EBV-transformed normal B-lymphocyte cell line (LCL) from the patient DH, was also available for germ line analysis.

Analysis of V genes. Total RNA was isolated from the cells using RNAzol B (Biogenesis, Bournemouth, UK) and cDNA produced using Moloney murine leukemia virus reverse transcriptase and an oligo dT primer (Promega, Madison, WI). 1/20 of the cDNA was then amplified by polymerase chain reaction (PCR) using either a mixture of oligonucleotide primers specific for each of the V_H leader sequences (V_HL1-6)[37] together with an appropriate $C\mu$ or $C\alpha$ constant region primer[38] to amplify the heavy chain genes or a mixture of oligonucleotide primers specific for each of the $V\kappa$[17,39,40] or $V\lambda$ leader sequences[41] together with an appropriate $C\kappa$[39] or $C\lambda$[29] constant region primer to amplify the light chain genes. PCR conditions were as previously described.[39]

Amplified products were gel-purified, cloned by ligation into pGEM-T vector, and transfected into JM109 competent bacteria (Promega). Nucleotide sequence analysis was by the dideoxy chain termination method, with alignment being made to EMBL/GenBank and V-BASE sequence directory (Tomlinson et al, MRC Centre for Protein Engineering, Cambridge, UK) using MacVector 4.0 sequence analysis software (IBI, New Haven, CT). At least two independent PCR amplifications were performed on each sample, and a minimum of five sequences analyzed.

Analysis of germline genes. DNA was isolated from DH-LCL using a Blood-AMP DNA isolation kit (Quiagen Ltd, Dorking, UK). The $V\lambda$ genes were amplified by PCR using primers specific for leader ($V\lambda1$ Leader) and λ nonamer sequences ($V\lambda1$non)[41] which specifically amplify the germline repertoire. The amplified products were purified and analyzed as before.

Analysis of mutational patterns in V genes. The number of expected replacement (R) mutations in the CDR was calculated according to the methods of Chang and Casali.[42] The binomial distribution model was used to calculate whether the excess or scarcity of R mutations in CDRs and FWRs resulted by chance.[42]

RESULTS

V_H gene usage. The V genes used by the seven sBL lines and the percentage homology to their closest germ line counterpart are shown in Table 1, and the deduced amino acid sequences of these lines, as compared with their germ line counterpart, are shown in Fig 1. The full nucleotide sequences have been deposited (EMBL accession numbers Z74663, Z74665, Z74668, Z74671, Z74672, Z74674, Z74693, Z74695). Six of seven of the BL lines are derived from the V_H4 family, with two of these using the V_H4-18 gene and two using the V_H4-21 ($V_{4\text{-}34}$) gene. The sequences have been aligned to the V_H4-21/DP63 gene rather than the $V_{4\text{-}34}$ gene since there is one nucleotide difference, position 15 in FWR1, between the two. This V_H4 usage is suggestive of a bias in V_H4 gene rearrangement in sBL, also reported by Moazzeni et al,[36] although the numbers of sequences analyzed here are too small to be statistically significant. In the other tumor cell line, DG75, a V_H3 gene is used, although an additional full-length V_H4 transcript (DP71) was identified as a repeated sequence from cDNA. On sequencing, a frameshift at the D-J join was found, indicative of a transcribed, but nonfunctional, V_H gene (data not shown, the nucleotide sequence has been deposited, EMBL accession no. Z74667). All the sequences showed evidence for somatic hypermutation with percentages ranging from 1.0 to 7.5 (Table 1) and the IgA sequence (DH) showed the same level of mutation as the IgM sequences (3.0%).

No J_H gene restriction was seen and in three of seven of the tumors (BL2, LOUCKES, DH). There was evidence for somatic mutation in J_H, with only one of the mutations giving rise to a replacement amino acid (LOUCKES). D regions were assigned to the closest known D_H genes, but no restriction in the D_H gene usage could be seen, and the D segment was often complex with low homology to known D segment genes (Fig 2).

V_L gene usage. The deduced amino acid sequences of the seven cell lines, compared with their germline counterpart, are shown in Fig 3, with the V_L-CDR3-J_L join region shown at the nucleotide level in Fig 4. The full nucleotide sequences have been deposited (EMBL accession numbers Z74662, Z74664, Z74667, Z74670, Z74673, Z74691, Z74692, Z74694). There was no evidence for preferential light chain use in the BL lines with two of seven using $V\kappa$ and five of seven using $V\lambda$ genes (Table 1).

V_κ gene usage. $V\kappa1$ and $V\kappa3$ genes were used by LOUCKES and DG75 sBL tumor cell lines, respectively, and were mutated from their germline counterpart (Fig 3). $J\kappa2$ and $J\kappa4$ genes were also used and mutations in the $J\kappa$ regions were seen in one of the lines (LOUCKES). Both the sequences were somatically mutated with percentages of 2.1% and 2.4% (Table 1). As was seen in the heavy chain, DG75 produced a full-length nonfunctional $V\kappa$ transcript ($V\kappa1$(L1)-$J\kappa3$) in addition to the functional $V\kappa3$ transcript shown. This transcript contained a large nucleotide deletion at the 3′ end of the CDR3 and the 5′ end of the $J\kappa$ region that caused a frameshift at the V-J join (data not shown, the full nucleotide sequence has been deposited, EMBL accession no. Z74666). This type of frameshift has also been reported in another BL line Ly91.[43]

V_λ gene usage. $V\lambda1$ and $V\lambda3$ genes were used by five of seven of the BL tumor cell lines and are shown in Fig 3. The $V\lambda$ genes were also all mutated from germ line (Table 1) with percentages ranging from 1.1% to 4.7%. As with the heavy chain, DH-BL (IgA) was no more mutated than the IgM sequences (2.6%). The $J\lambda2/3$ germ line V_L gene was rearranged in all cases. In the case of RAMOS, another full-length nonfunctional V_L gene was also transcribed.

Intraclonal heterogeneity. For the BL tumor cell line L3055, 20 V_H sequences and 10 V_L sequences, from at least two independent PCRs, were analyzed. In total only three nucleotide differences, between sequences, were observed giving a mutation frequency of 0.03% (consistent with our Taq polymerase error rate of 1 in 5,000-bp). Biopsy material from this patient was also available and was analyzed for

Table 1. V-Genes of Tumor-Derived BL Lines

		V_H			V_L		
BL Cell Line	Ig Class	V_H/J_H	GL Donor	% Mutation*	V_L/J_L	GL Donor	% Mutation*
BL30	Mλ	V_H4/J_H4b	V_H4-21	1.0	$V_\lambda1/J_\lambda2/3$	DPL2	1.1
RAMOS	Mλ	V_H4/J_H6b	V_H4-21	2.1	$V_\lambda2/J_\lambda2/3$	DPL11	4.7
BL2	Mλ	V_H4/J_H5a	V4-18	7.5	$V_\lambda1/J_\lambda2/3$	DPL5	2.6
LOUCKES	Mκ	V_H4/J_H3b	V4-18	6.1	$V_\kappa1/J_\kappa2$	02	2.1
DH-BL	Aλ	V_H4/J_H4b	V4-33	3.0	$V_\lambda1/J_\lambda2/3$	DPL3	2.6
L3055	Mλ	V_H4/J_H3b	V4-11	3.1	$V_\lambda1/J_\lambda2/3$	DPL5	1.1
DG75	Mκ	V_H3/J_H4b	V3-23	5.8	$V_\kappa3/J_\kappa4$	A27	2.4

* Percentage mutation of the V_H and V_L genes used by the sBL tumor cell lines from their germ line counterpart.

the presence of sequence heterogeneity within the sample (Fig 5). The majority of the biopsy sequences were identical to the L3055 BL line, but 25 additional nucleotide changes were seen in 16 of 42 sequences analyzed, often with more than one mutation per sequence and with one of these mutations giving rise to a stop codon. This gives a percentage mutation frequency of 0.2%, which is considerably higher than our Taq polymerase error rate seen in the cell line using identical conditions. These extra somatic mutations were seen in both the heavy and light chain genes and are indicative of intraclonal variation in the biopsy material. The full nucleotide sequences have been deposited (EMBL accession numbers Z74672-Z74691).

Mutational analysis of the V_H genes. V_H gene analysis has been restricted to the less polymorphic V_H4 gene family used by six of seven BL cell lines. Two of these use the relatively nonpolymorphic V_H4-21 gene, while three of four of the other V_H genes align to the V_H4-11 and V_H4-18 germline genes that have been shown to display a high inherent replacement mutation frequency in their CDR regions.[42]

The number of R mutations in the CDR and FWR regions are shown in Table 2. In some of the sequences, the number of R mutations seen in the CDRs was larger than that expected by chance, but these values were not statistically significant. In two of the cell lines, BL2 and LOUCKES, there are a significantly fewer number of R amino acids in the FWR regions suggesting a conservation of antibody structure.

Mutational analysis of the V_L genes. The distribution of somatic mutations in the V_L sequences was also assessed. The number of expected replacement mutations in the CDR and FWR regions are shown in Table 2. In all cases, the number of R mutations seen in the CDR regions was larger than that expected by chance, and these mutations cannot be accounted for simply by V-J joining or N region additions. In five of seven of the tumors, the values were statistically significant implying that antigen may play a role in selection of these cells. In fact, even when the *P* value did not reach significance, the few mutations present in the CDR3 were all R mutations. The FWR sequences of two of these lines (DG75 and LOUCKES) were also more conserved than expected by chance.

DISCUSSION

Burkitt's lymphoma is a term originally applied to tumors involving jaw tissue of African children.[44] This endemic form was subsequently found to have a Western sporadic counterpart, involving cells with similar morphological and

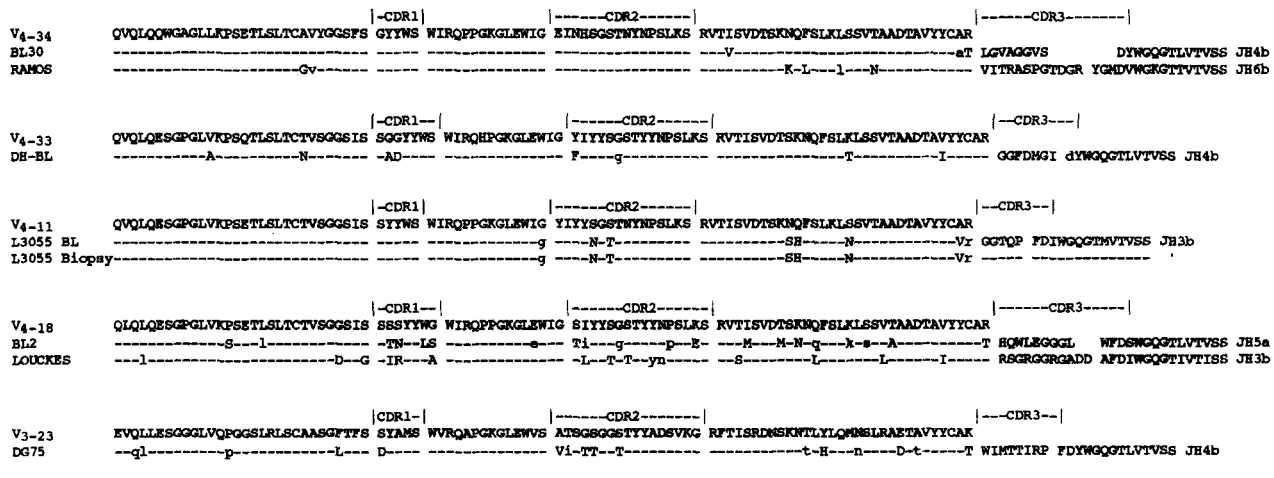

Fig 1. Deduced amino acid sequences of the V_H regions of the BL lines. Comparisons are made with the closest germ line genes. Tumor line and biopsy material from L3055 are shown. Upper case, replacement mutations; lower case, silent mutations.

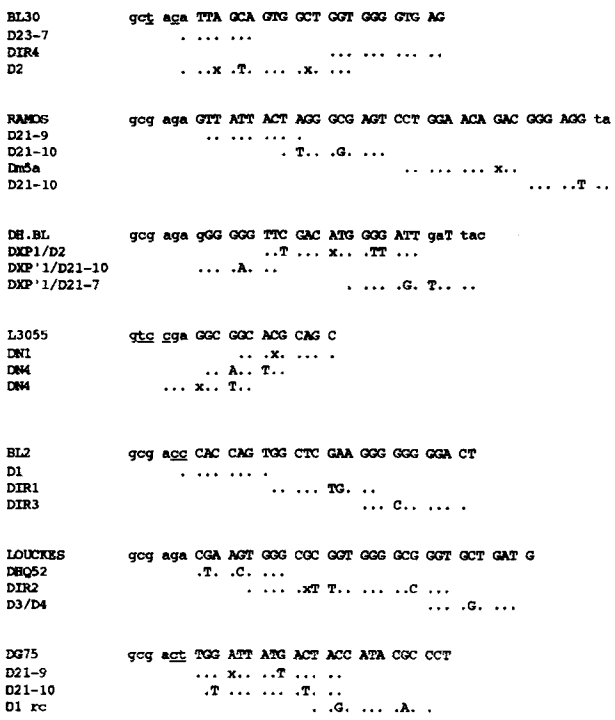

Fig 2. Nucleotide sequence alignment of the D regions to the closest known germline D segment gene. Lower case, V_H and J_H regions; underlined, mutations from germline; x, missing nucleotide; rc, reverse configuration.

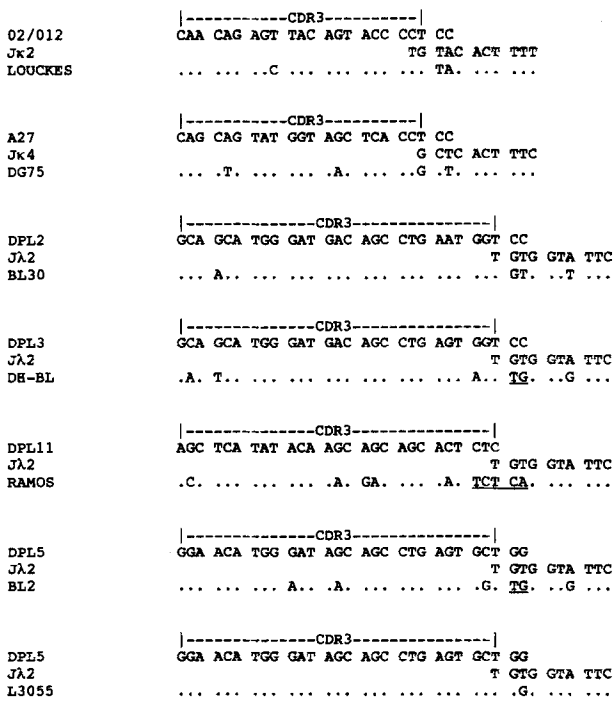

Fig 4. Nucleotide sequence alignment of the V_L-CDR3-J_L regions to indicate derivation of V-J amino acids and mutations. Areas of N region addition are underlined.

phenotypic properties.[16] The relationship between the two forms was strengthened by the finding of a shared chromosomal abnormality, consisting of a reciprocal translocation between chromosome 8 and one of the Ig gene loci, usually chromosome 14.[1,4] However, it has been reported that there may be differences in the site of cleavage of chromosome 14 in the two forms of BL, with endemic BL having breaks in the joining (J) region, and sporadic BL in the switch region.[1,45] Since translocation could occur during the process

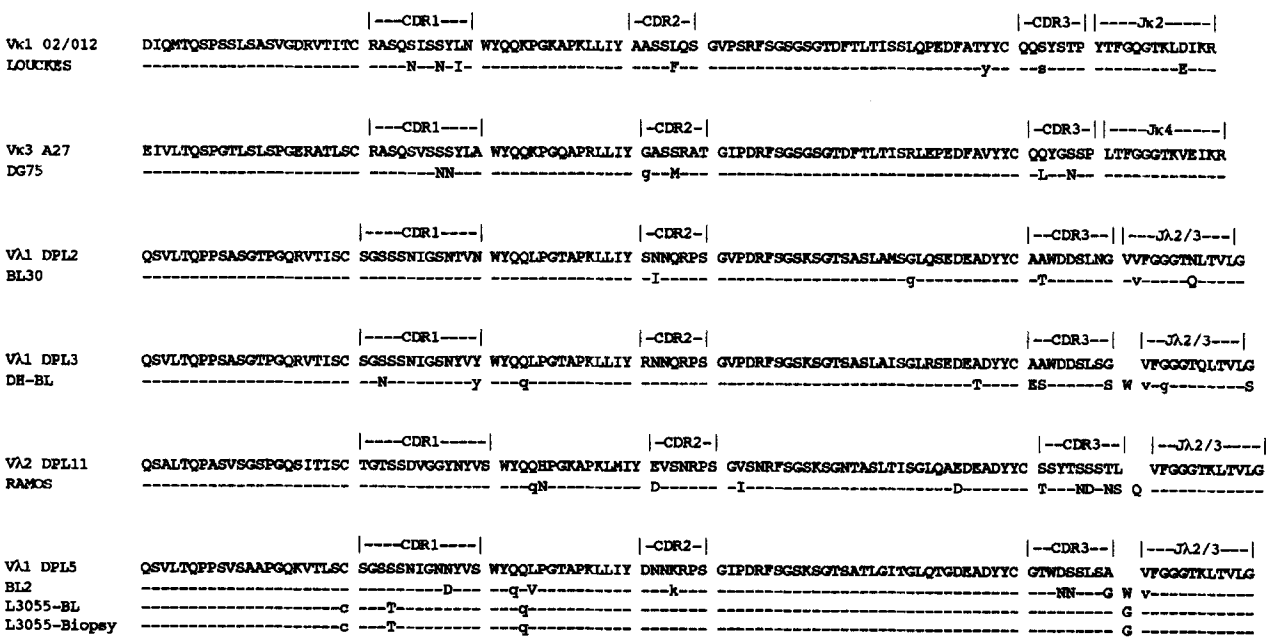

Fig 3. Deduced amino acid sequences of the V_L regions of the BL lines. Comparisons are made with the closest germ line genes. Tumor line and biopsy material from L3055 are shown. Upper case, replacement mutations; lower case, silent mutations.

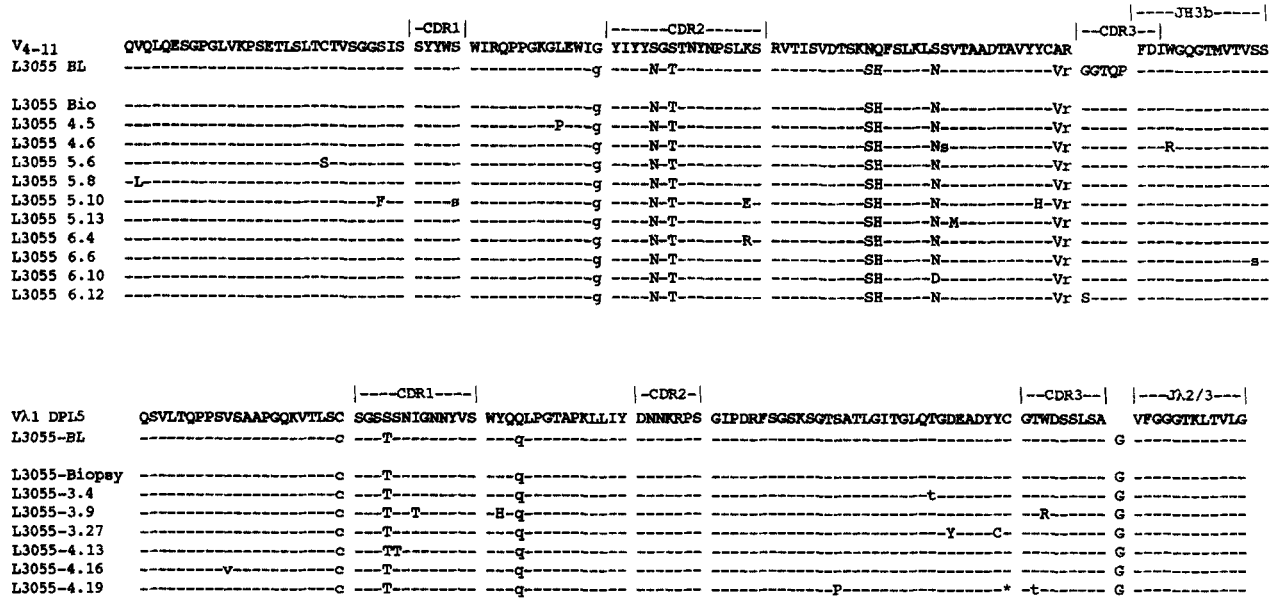

Fig 5. Clonal heterogeneity of the V_H and V_L regions of the BL biopsy L3055. Prefix 3.n-6.n denote separate PCR reactions. Upper case, replacement mutations; lower case, silent mutations; * stop codon.

of Ig gene recombination, this might suggest that endemic BL arises from a precursor cell that acquires a translocation during V_H-D_H-J_H combination in the bone marrow. In contrast, sporadic BL could arise by translocation in a mature B cell undergoing Ig class switching in the germinal center.[23,46]

However, analysis of V_H gene sequences in cases of endemic BL has shown extensive somatic hypermutation,[27,47] indicating that even if translocation occurs at an early stage of B-cell development, the translocated cell can move to the germinal center of the lymph node and be exposed to the somatic hypermutation mechanism operative at that site.[23,24] The finding of intraclonal heterogeneity in the endemic BL tumor cell population was consistent with continuing accumulation of somatic mutations in the clone, with a pattern similar to that of follicular lymphoma.[48]

Events in sporadic BL appear to be quite similar to those in endemic BL. A recent report has also indicated quite high levels of somatic mutation in V_H sequences from cell lines from sporadic BL, although the fact that five of eight were derived from the polymorphic V_H3 family prevented confi-

Table 2. Analysis of Mutations in sBL Sequences

	V_H			V_L		
BL Cell Line	$R:S_{CDR}$ Obs*[a] $R:S_{FWR}$ Obs	R_{CDR} Exp† R_{FWR} Exp	*P*(CDR)‡	$R:S_{CDR}$ Obs* $R:S_{FWR}$ Obs	R_{CDR} Exp† $R_{FWR\ Exp}$	*P*(CDR)‡
BL30	0:0	0.5	0.5497	2:0	0.7	0.1218
Mλ	2:1	1.7	0.4215	0:1	1.5	0.1156
RAMOS	0:0	1.1	0.3022	7:1	2.7	0.0064
Mλ	4:2	3.4	0.2930	3:1	6.3	0.0400
BL2	7:4	4.0	0.0540	4:1	1.6	0.0438
Mλ	5:6	12.4	0.0011	1:1	3.6	0.0484
LOUCKES	6:3	3.3	0.0591	4:1	1.3	0.0183
Mκ	6:3	10.1	0.0280	0:1	3.3	0.0078
DH-BL	3:1	1.7	0.2934	4:1	1.5	0.0379
Mλ	4:1	5.0	0.2051	1:1	3.6	0.0444
L3055	2:0	1.8	0.2998	1:0	0.7	0.4080
Mλ	4:3	5.0	0.2113	0:2	1.5	0.1163
DG75	7:1	ND	ND	5:1	1.3	0.0019
Mκ	3:6			0:0	3.3	0.0086

Abbreviation: ND, not done.

* $R:S_{CDR/FWR}$ Obs is the observed number of replacement (R) and silent (S) mutations seen in the CDRs and FWRs of sBL sequences.

† $R_{CDR/FWR}$ Exp is the total expected number of replacement mutations in CDRs and FWRs.

‡ $P_{CDR/FWR}$ is the probability that an excess of R mutations, in the CDRs, resulted by chance. Significant *P* values are underlined.

dent assignment of nucleotide changes to somatic mutation.[47] For the less polymorphic V_H4 genes in our study, a more confident assignment could be made, and the mean level of mutation of 3.8% was less than our previous finding of 5.6% in endemic BL.[27] Assignment is more certain for V_L genes, and the mean level of mutation of 1.8% found by Klein et al[47] compares well with our mean level of 2.4% for sporadic BL, and is considerably less than the accumulated mean of 6.0% for the endemic form.[47] Our data therefore confirm the suggestion that there is a higher degree of somatic mutation in endemic BL.[47]

In addition, we were able to show continuing somatic mutation occurring in biopsy material from a case of sporadic lymphoma, L3055, which indicates that as in endemic disease, the translocated cell can still be under the influence of the hypermutation mechanism. The somatic mutations identified, 25 nucleotide changes in 42 sequences (0.2%), can be contrasted with the detection of three nucleotide changes in 30 sequences (0.03%) derived from the in vitro line, the latter being consistent with Taq polymerase error. These results differ from those published by Tamuru et al,[49] who failed to detect intraclonal heterogeneity in five cases of sBL. However, this may be due to restrictions in material recoverable from paraffin sections. Further work on more patients is needed to clarify the point.

A further parallel with endemic BL was shown in the mutational pattern of V_H sequences, with scattering throughout the sequence providing no evidence for antigen selection.[27] Comparable results were found in two cases of BL, both of which involved the V_H4-21 gene, which arose in patients with AIDS.[30] However, an analysis of intraclonal somatic mutations in a similar clinical setting indicated that ongoing replacement mutations could accumulate in CDRs.[28] With regard to V_L, we were surprised to find a pattern in five of seven cases with clear clustering of replacement mutations in the CDRs, in a manner that fulfilled the current criteria for a role for antigen. There is less information available on V_L sequences in BL, but in the accumulated sequences of 10 cases of endemic and sporadic BL, there was evidence for a concentration of replacement mutations in CDR2.[47] We are currently analyzing the V_L sequences of a group of endemic BL cases, and they appear to have a distribution of mutations similar to the sporadic cases (unpublished observations, February 1996).

Immunoglobulin variable region gene analysis has shown close similarities between endemic and sporadic forms of BL, with perhaps the only differential feature being a higher degree of somatic hypermutation in the endemic form. Clearly, both tumors have been exposed to the mutator and continue to undergo somatic mutation. This aspect of clonal history is, therefore, not influenced by the presence of EBV. Antigen selection may have occurred, but malignant behavior is unlikely to rely on this mechanism in cells that are clearly independent of normal restraint.

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