SINGLE CELL ORIGIN OF BIGENOTYPIC AND BIPHENOTYPIC B CELL PROLIFERATIONS IN HUMAN FOLLICULAR LYMPHOMAS

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B cell lymphomas are generally believed to result from monoclonal proliferations of neoplastic lymphocytes derived from a single transformed progenitor (1). The monoclonality of most neoplastic lymphoproliferations is supported by the demonstration of light chain–restricted tumor cell surface Ig, detection of clonal Ig gene rearrangements, and the finding that all cells of the neoplastic clone contain a common cytogenetic abnormality (1–3). However, we have described a number of B cell lymphomas consisting of two apparently different tumor clones with distinctive phenotypic and genetic markers (4–6). The most thoroughly investigated among these cases were initially selected for study because subpopulations of tumor cells within single biopsies could be distinguished by their reactivity with antibodies directed against Ig idiotopes on the cell surfaces. The antibodies were used to separate cells for subsequent Southern blot analysis of Ig genes. Differences in the observed patterns of rearrangements between the two populations suggested the presence of two genetically and phenotypically distinct clones of B lymphocytes within each tumor.

To further investigate the possible relatedness of the subpopulations that make up these so-called biclonal lymphomas, we have examined five biclonal follicular lymphomas using DNA probes specific for the t(14;18) chromosomal translocation, which is a characteristic feature of these neoplasms (7, 8). Our results show that four of five phenotypic and genotypic biclonal lymphomas contained comigrating t(14;18) DNA rearrangements on Southern blot analysis, indicating that the two subpopulations were derived from a single progenitor cell. Although no comigration of t(14;18) gene rearrangements was found in the subpopulations that composed a fifth lymphoma, nucleotide sequence analysis of cloned breakpoint DNAs confirmed the relatedness of the subpopulations. Sequence analysis of the productive Ig heavy and light chain genes showed that somatically acquired differences of the Ig genes accounted for the appearance of genotypic and phenotypic biclonality.

Our results indicate that Ig gene rearrangements and idiotope expression may

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not be consistently accurate clonal markers since they are subject to variability as a result of somatic mutation. Although chromosome 18 translocation-specific DNA rearrangements are more reliable, they may fail in some cases since the t(14;18) translocation breakpoint involves the excluded Ig heavy chain gene, which is also subject to somatically acquired alterations in B cell lymphomas. Somatic mutation can be quite extensive in follicular lymphomas, and for one of the lymphomas in our study most mutations accumulated outside of the expressed V regions, particularly in the J-C introns.

Materials and Methods

Lymphoma Tissues. Lymphoma tissues serving as a source of DNA for Southern blot analyses and genomic DNA cloning were obtained from lymph node biopsies of patients with follicular small cleaved cell lymphoma. Patients BH, LK, and BB in this study have been described in earlier studies as patients 2 and 3 (5) and patient 2 (9), respectively.

Molecular Methods. DNA was extracted from lymph node biopsy tissues, digested with restriction enzymes, and size fractionated in agarose gels using previously described procedures (2, 10). Methods for Southern blot transfers and hybridizations have been described earlier (11). DNA probes were radiolabeled by primer extension, with the Klenow fragment of DNA polymerase I (12). The structures of the Ig constant and heavy chain joining region probes have been described (2). The J_κ probe consisted of a 1.2-kb Sac I fragment spanning the entire κ joining region. The chromosome 18 probes pFL-1 and pFL-2 have been described previously (13–15).

Genomic DNA fragments were isolated from follicular lymphoma DS essentially as described previously (11, 13). Briefly, after complete restriction enzyme digestion of lymphoma DNA and preparative agarose gel electrophoresis, regions of the gel containing DNA fragments of interest were excised and DNA was electroeluted from gel slices for further purification. The purified DNA fragments were then ligated into λgt10 or λ590 phage vectors and packaged in vitro; recombinant phage were plated and screened as described (16), using appropriate radiolabeled Ig heavy or light chain DNA probes.

DNA sequences were determined by the method of Sanger et al. (17) on DNA fragments subcloned into either M13mp18 or M13mp19 single-strand phage vectors (18).

Flow Cytometry. Fluoresceinated polyclonal goat antiserum to human κ, λ, and μ Ig chains and goat anti-mouse IgG were obtained from Tago Inc. (Burlingame, CA). mAb to human γ heavy chain was obtained from Coulter Immunology (Hialeah, FL). Monoclonal murine antiidiotype reagents were produced as previously described (19–21). Single cell suspensions of tissue samples from the patients were stained with fluorescent antibodies and analyzed by fluorescence flow cytometry (FACS IV; Becton Dickinson & Co., Mountain View, CA). The fluorescence data were analyzed using a histogram of the log of fluorescence intensity versus cell number.

Results

The results of genomic Southern blot analyses using Ig gene probes are shown in Fig. 1 for four representative follicular lymphomas with biclonal features. For both the WP and BB lymphomas, biopsies removed from two different sites within each patient were compared for potential differences in Ig gene rearrangement patterns. In WP, DNA from the left inguinal lymph node biopsy (lane *1*) contained an additional heavy chain and κ light chain gene rearrangement when compared with the right inguinal lymph node biopsy (lane *2*). In BB, DNA from the left neck node biopsy contained an additional heavy chain and λ light chain gene rearrangement (lane *1*) when compared with the epitrochlear node. For both lymphomas, the observed differences in the heavy and light chain gene

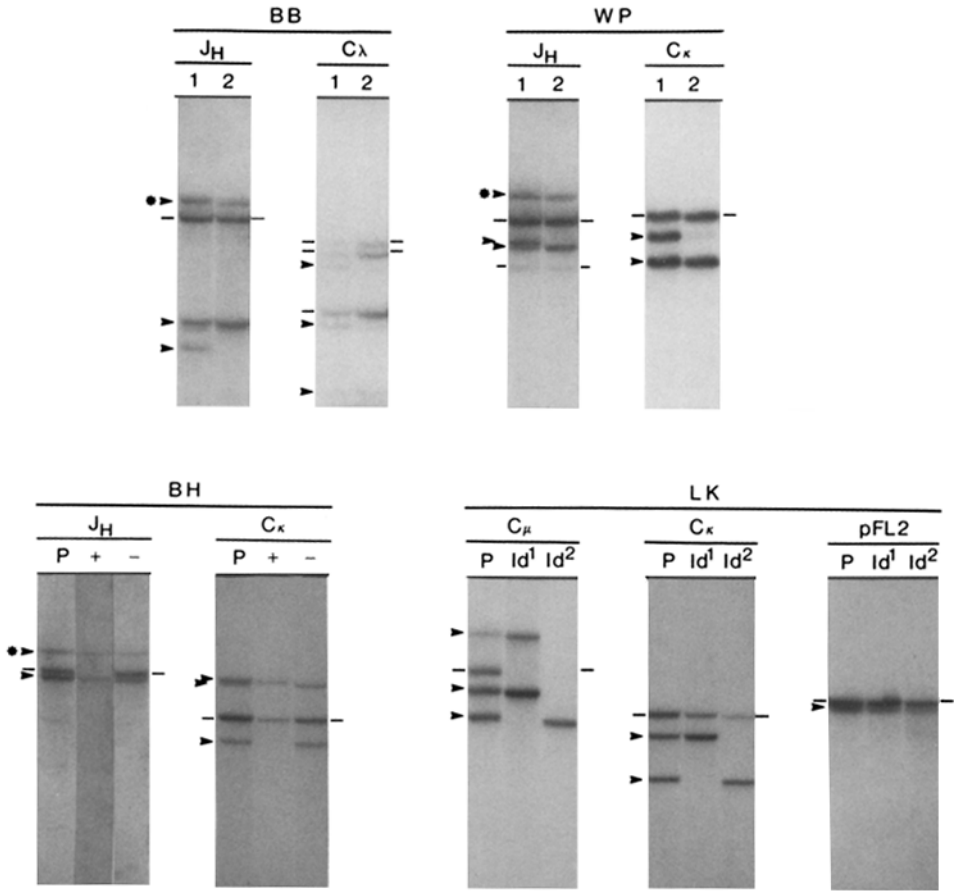

FIGURE 1. Analysis of Ig gene DNA and t(14;18) breakpoints in follicular lymphomas with biclonal features. DNA was extracted from lymph node biopsy samples from patients WP and BB, or from sorted cell populations of lymphoma biopsies from patients BH and LK. The Ig probes used in the hybridization analyses are indicated above the gel lanes. BB DNA was digested with Eco RI; WP, BH and LK DNAs were digested with Bam HI, except for the pFL-2 analysis of LK DNA where the BstE II restriction enzyme was used. Germline configurations for Ig genes are denoted with dashes. All bands marked with arrows correspond to rearranged Ig genes. Bands marked with asterisks also hybridized with pFL-1 (WP, BH) or pFL-2 (LK, BB) chromosome 18 DNA probe and represent t(14;18) breakpoint containing DNA fragments. Samples analyzed were: BB: (lane *1*) left neck node; (lane *2*) epitrochlear node. WP: (lane *1*) left inguinal node; (lane *2*) right inguinal node. BH: (*P*) presorted cell population; (+) idiotope bearing cells; (−) idiotope lacking cells. LK: (*P*) presorted cell population; (Id^1 and Id^2) cells bearing idiotope recognized by first or second antiidiotope antibodies, respectively.

configurations suggested that within each patient two genotypically distinct subpopulations might be simultaneously present in the left inguinal lymph node and the left neck lymph node biopsies respectively.

For the BH lymphoma, it was possible to physically separate two distinct subpopulations from a single biopsy specimen. This was accomplished with a FACS, using an antibody that recognized an idiotypic determinant present on only a fraction of the tumor cell population, as we have described in detail previously (5). As shown in Fig. 1, when DNA was examined from the two

subpopulations, the pattern of heavy chain gene rearrangements was identical but each subpopulation contained its own unique κ light chain gene rearrangements. In this case, the two sorted subpopulations appeared to be related by having identical heavy chain gene rearrangements, suggesting a divergence from a common progenitor after heavy chain gene rearrangement but before light chain gene rearrangement.

A similar separation, using two different antiidiotype antibodies, each specific for one or the other subpopulation, was carried out on the LK lymphoma, with different results. In this case, no common μ heavy chain or κ light chain gene rearrangements were observed for the two B cell populations. Thus, there was no evidence from the Ig gene studies that the two populations were related to one another through a common progenitor.

To further investigate the clonal relationships of the subpopulations within these four follicular lymphomas, t(14;18) translocation breakpoints were examined as potential clonal markers. For these analyses, we used either the pFL-1 or pFL-2 chromosome 18 DNA probes, which we have previously shown are capable of detecting most t(14;18) translocation breakpoints in follicular lymphomas (13, 14). In the WP and BB lymphomas, only one rearranged pFL-1 or pFL-2 chromosome 18 DNA fragment was observed, comigrating at the same position in DNA obtained from the two biopsies in each patient (bands denoted with asterisks in Fig. 1). For the BH lymphoma, identical rearranged pFL-1–containing fragments were observed for each FACS-separated subpopulation. The same result was obtained for the LK FACS-separated subpopulations, using the pFL-2 probe (Fig. 1). Therefore, in spite of the extensive differences observed in the Ig gene rearrangement patterns for each of these lymphoma subpopulations, only a single clonal t(14;18) breakpoint could be detected on genomic Southern blot analyses of lymphoma DNA. The results suggested that, despite genetic and in some cases phenotypic differences in the various B cells comprising each of the follicular lymphomas, all of the tumor cells in each patient's biopsies contained the same t(14;18) translocation and likely arose from a single progenitor.

Extensive Southern blot analyses using Ig and chromosome 18 DNA probes on a fifth lymphoma (DS) suggested that the subpopulations may not be related through a common progenitor cell. As shown in Fig. 2, Southern analysis with the Ig J_H and chromosome 18 DNA probes on Hind III–digested DNA showed no common rearranged bands in the two DS subpopulations. Although each sample contained a similarly sized Eco RI band detected with the J_H probe, analysis with a chromosome 18 DNA probe showed hybridization to the comigrating band in lymph node 1 but not its counterpart in lymph node 2. After Bam HI digestion, a single rearranged band of similar size was observed in both samples but only the band seen in lymph node 2 hybridized to a chromosome 18 DNA probe. Analysis of the λ light chain genes showed no comigrating rearrangements between the two DS lymphoma subpopulations. The two subpopulations in this lymphoma occupied anatomically separate nodes, which were removed from the patient at two different time points, ~2 yr apart. Lymphoma cells from the two biopsy sites were also found to be phenotypically distinct, since an antiidiotype mAb directed against one of the subpopulations did not react with the second subpopulation (Fig. 3), although both were similar in that they

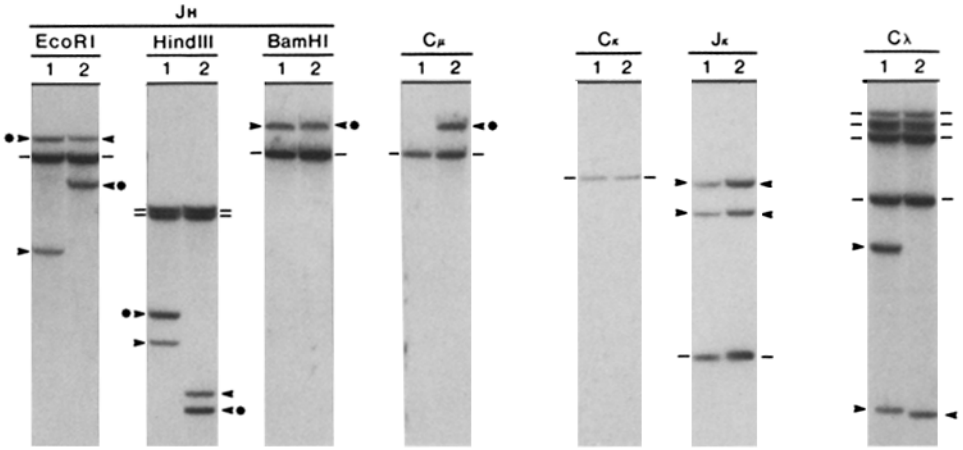

FIGURE 2. Southern blot analysis of Ig gene DNA and t(14;18) breakpoints in the DS lymphoma DNA. DNA was extracted from two separate biopsies (lymph nodes 1 and 2) of the DS follicular lymphoma. Ig probes for the C_μ, J_H, C_K, J_K, and C_λ genes were used in the hybridization analyses as indicated above the respective gel lanes. The J_H analysis was performed after Eco RI, Hind III, and BamHI digestion of lymphoma DNA, respectively. DNAs were digested with BamHI for C_μ and C_K analysis, Sac I and J_κ analysis, and Eco RI for C_λ analysis. Dashes denote germline configurations for Ig genes. Arrows indicate rearranged Ig genes. Asterisks indicate rearranged Ig fragments that also hybridized to chromosome 18 DNA probes and correspond to t(14;18) breakpoint containing fragments.

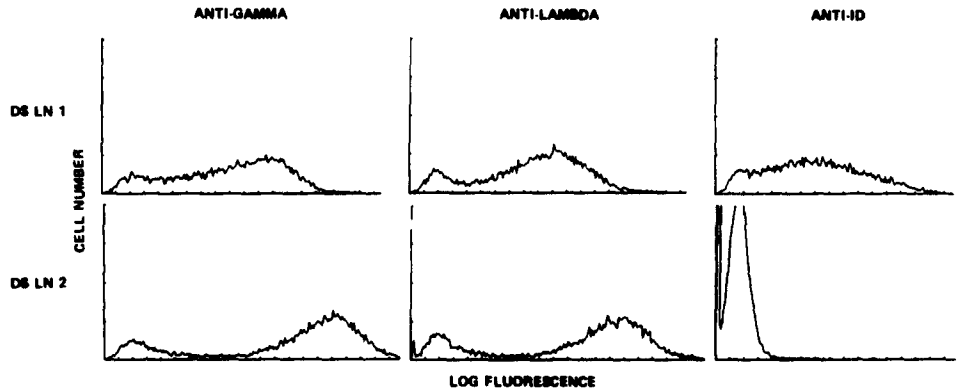

FIGURE 3. FACS analysis of DS lymphoma subpopulations. Tumor cells from the two DS lymphoma biopsies were stained with appropriate antibodies (*top*: anti-λ, *middle*: anti-γ, *bottom*: antiidiotype). Anti-light and -heavy chain antibodies were fluorescein conjugated; binding of antiidiotype antibody was determined with fluorescent anti–mouse IgG. The graphs show the number of cells staining with various intensities of fluorescence.

expressed γ heavy chain and λ light chain surface Ig. The results of the above analyses on two separate biopsies of the DS lymphoma suggested that each subpopulation contained unique genetic and phenotypic markers and that they were not related through a common progenitor.

To further investigate this case, we cloned t(14;18) breakpoint DNA from each subpopulation. The resultant t(14;18) breakpoint DNA clones are shown in Fig. 4; mapping of each fragment for various restriction enzyme cutting sites showed that the two breakpoint DNA fragments were identical except that the

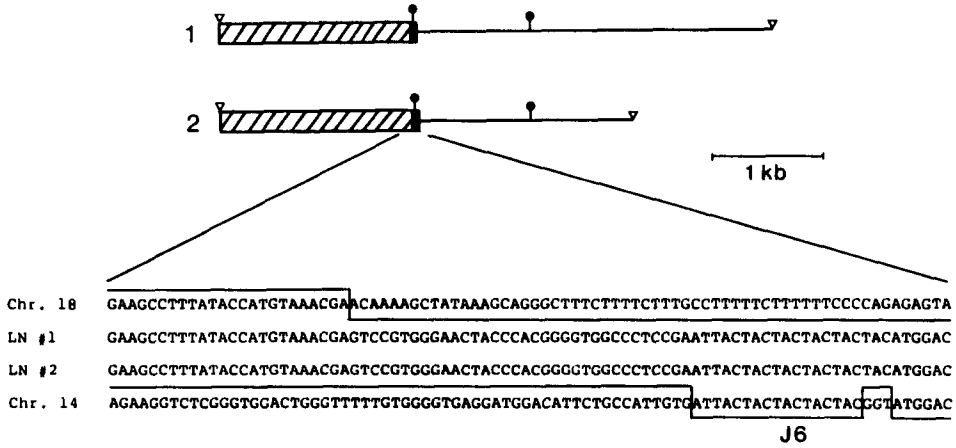

FIGURE 4. Physical maps and nucleotide sequences of the t(14;18) breakpoint DNA fragments isolated from DS lymphoma subpopulations. (*Top*) The rearranged DS lymphoma Hind III fragments containing t(14;18) crossovers are shown. DNA fragments are shown in a 5′ to 3′ orientation with respect to the Ig heavy chain gene. (*Bottom*) The nucleotide sequences for the DS t(14;18) crossovers are displayed along with the germline chromosome 18 or 14 sequences for comparison. The sequences are displayed in a 5′ to 3′ orientation; blocks of homologous nucleotides shared by adjacent sequences are enclosed in boxes. Arrows denote points where the t(14;18) sequences diverge from chromosome 18 or 14 germline sequences. Restriction sites are Hind III (▽) and Bgl II ( ). These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00645.

fragment from lymph node 1 contained an additional 1,500 bp at its 3′ end consistent with its larger size on genomic Southern blots. Nucleotide sequence determinations on the DNAs showed that the t(14;18) recombination sites for these two fragments were identical in sequence (Fig. 4). Comparison of the breakpoint DNA sequences with germline chromosome 18 and 14 sequences showed that both crossovers occurred within J6 and contained identical 34-bp insertions of likely *N*-nucleotide origin (13, 22). Further mapping and hybridization studies indicated that the size differences of the two t(14;18) DNAs resulted from a small deletion within the breakpoint fragment from lymph node 1, which encompassed the Hind III site that normally resides between J_H and $S\mu$. Since this Hind III site is still present in the breakpoint fragment from lymph node 2, it indicates that the small deletion was acquired by the precursor of subpopulation 1 after t(14;18) had occurred and after divergence of the two subpopulations. In addition, the translocated allele from subpopulation 1 (but not 2) had undergone a class switch from a μ to γ heavy chain C region (Fig. 2).

To determine whether divergence of the two subclones may have occurred before completion of Ig gene rearrangement, cloning and sequencing studies were performed on the productive heavy and light chain genes from the two DS lymphoma biopsies. The cloned Hind III DNA fragments containing the productive heavy chain variable region genes from the DS lymphoma subpopulations are shown in Fig. 5. Physical maps of the two fragments showed them to be very similar except that the fragment from lymph node 1 contained an additional 1.2 kb of DNA at its 3′ end. Nucleotide sequence determinations showed that the two V_H genes were highly homologous but contained more than 50 nucleotide

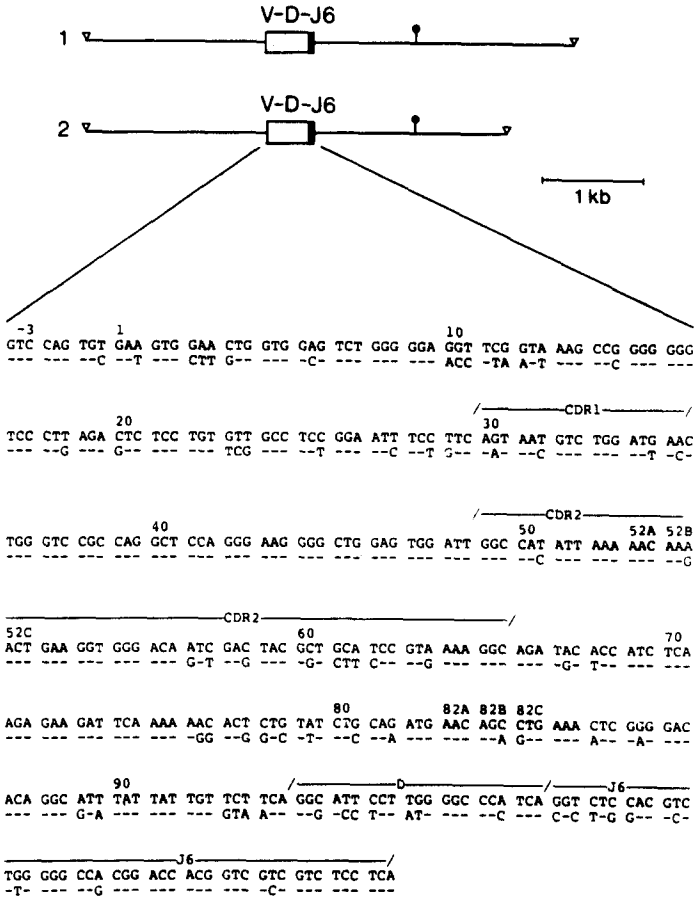

FIGURE 5. Physical maps and nucleotide sequences of productive V-D-J genes isolated from DS lymphoma subpopulations. (*Top*) The productive heavy chain V-D-J genes from the DS lymphoma subpopulations are shown oriented in a 5′ to 3′ orientation. Open boxes denote V segments and solid boxes indicate D and J segments. Restriction sites are Hind III (∇) and Bgl II ( ). (*Bottom*) Nucleotide sequences for the heavy chain V genes from each subpopulation are depicted 5′ to 3′, left to right; the sequence from LN 1 is shown above that for LN 2. The complementarity-determining regions CDR1 and CDR2 are indicated, as are the D and J segments. Dashes indicate identical nucleotides shared by the two sequences. Single letters denote differences. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00645.

differences interspersed with blocks of identical sequence (Fig. 5). In both fragments, D-J joining occurred within joining segment J6, likely at precisely the same nucleotide even though several nucleotide differences were observed in the vicinity of the D-J joint, particularly within the joining segment. Several nucleotide differences at the presumed site of V-D joining also were observed, but the D segments appeared to be of equal length and identical at 16 of 24 nucleotides. The large blocks of homologous sequence shared by the two genes and the lack of insertions or deletions at the V-D or D-J joints are strong evidence that the two fragments encoded the same functional V_H genes before divergence. The observed differences likely were due to somatic mutations of these functional

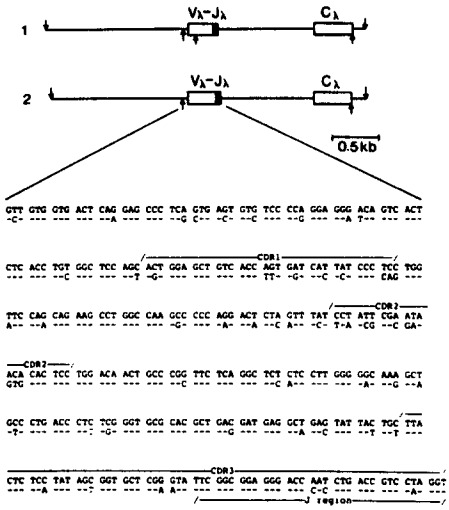

FIGURE 6. Physical maps and nucleotide sequences for the productive λ light chain genes from DS lymphoma subpopulations. (*Top*) The productive λ light chain genes from the DS lymphoma are shown oriented 5′ to 3′, left to right. Open boxes denote V or C segments and solid boxes denote J segments. Restriction enzyme recognition sites: Eco RI (↓), Bam HI (↑). (*Bottom*) Nucleotide sequences for the λ V-J genes are shown 5′ to 3′, left to right. CDR1, 2, and 3 are indicated. Dashes indicate identical nucleotides shared by the two sequences and single letters denote differences. The sequence from LN 1 is shown above that for LN 2. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00645.

genes similar to those we have previously observed in V genes of cells from other follicular lymphomas (11, 23). Somatic mutations in the J-C intron can also account for the loss of the normal 3′ Hind III site in the fragment from lymph node 1, which explains its larger size when compared with the fragment from lymph node 2.

Nucleotide sequences for the productive λ light chain V genes are shown in Fig. 6. The V_L sequences are highly homologous except for numerous point mutations in both the V_λ and J_λ gene segments. However, both fragments contained the same V_λ gene segment identically joined to the same J_λ segment. The data indicated that both fragments resulted from a single V-J joining event and the observed nucleotide differences accumulated independently in each fragment following divergence of the subpopulations. The C_κ gene segments were deleted in both subpopulations, consistent with the findings that the DS lymphoma cells expressed λ light chain–containing Ig, since nonproductive κ gene rearrangement is frequently followed by C_κ deletion (24, 25). Southern blot analysis with a J_κ hybridization probe showed that the nonproductive κ gene rearrangements were identical in the DS lymphoma subpopulations (Fig. 2). Therefore, all recombination events involving the heavy and light chain Ig genes were identical in DNA fragments isolated from the DS lymphoma biopsies, indicating that the common precursor cell for the two subpopulations had progressed completely through the stages of gene rearrangement before divergence.

To investigate the extent of variation for DNA flanking the productive V_λ genes, nucleotide sequences were obtained for the complete λ V-J-C genes from the two DS subpopulations. Approximately 2,400 nucleotides of sequence were obtained which started 300 nucleotides 5′ to the V_λ region, extended through the J_λ-C_λ intron, and terminated at the 3′ end of the C_λ gene segment. The data showed that the 5′ flanking V_λ segments and C_λ genes from the two subpopulations were >99% homologous. By comparison, the V_λ-J_λ segments shown in Fig. 6 were 82% homologous. The greatest sequence divergence, however, was

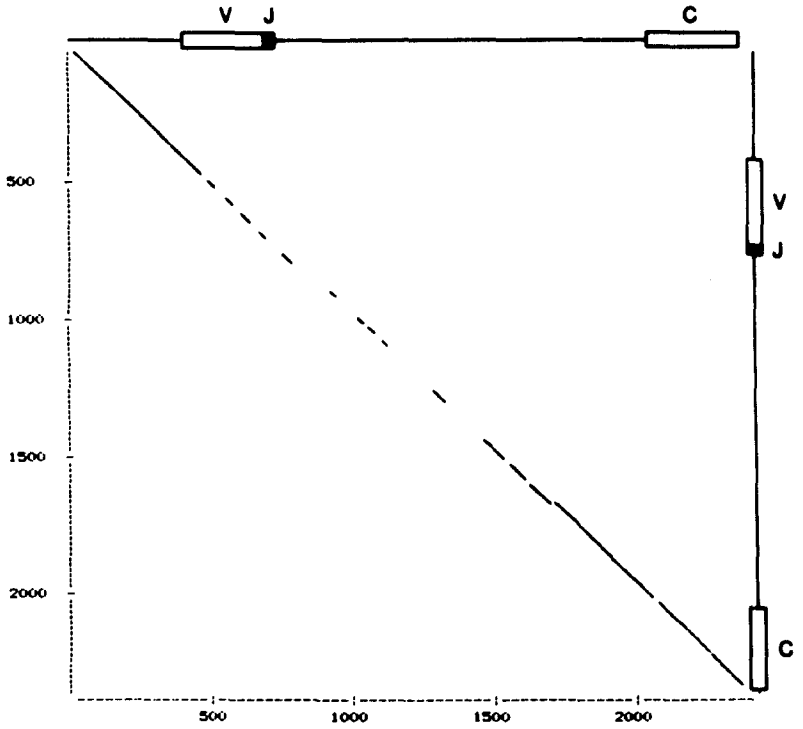

FIGURE 7. Dot-matrix analysis showing the divergence of the two productive DS λ light chain genes. The nucleotide sequences determined for the λ light chain V-J-C genes from the DS lymphoma were analyzed using the matrix comparison function of the microgenie sequence analysis program. Program parameters were set at MINMATCH = 19 and MINPER = 95, which resulted in a mark along the diagonal line for every segment of 20 nucleotides containing at least 19 identical positions between the two sequences. Offset diagonal lines resulted from small deletions/insertions between the two sequences.

observed in the J_λ-C_λ intron. Many nucleotide differences, both single and in small clusters, and small insertions/deletions of 1–20 nucleotides were observed in a large portion of this region. The net loss of ~50 nucleotides in this region of the fragment from subpopulation 2 when compared with the fragment from subpopulation 1 likely accounted for its slightly smaller size on Southern blot analysis (Fig. 2). A dot-matrix analysis comparing the sequences obtained for the entire λ genes from the two DS lymphoma subpopulations is shown in Fig. 7. This analysis graphically portrays the sequence data and shows that mutations accumulated in a large, broad region of the productive λ genes with most of the mutations occurring in the 5′ three-quarters of the J_λ-C_λ intron. Occasional sequence differences were also observed in the productive C_λ genes (data not shown) and also near an unrearranged C_λ gene since cloning, mapping, and hybridization studies showed that the additional C_λ fragment migrating in a nongermline position in DNA from lymph node 1 contained only germline DNA (data not shown). These studies confirmed that somatic mutation can be quite extensive in follicular lymphomas and showed that the specific target for mutation in the DS lymphoma was much larger than the rearranged V_λ region gene.

Discussion

The vast majority of neoplastic processes are assumed to be monoclonal proliferations, implying that all constituent cells arise from a single transformed progenitor cell. However, assessment of clonality based on Ig light chain type has occasionally demonstrated that some human B cell neoplasms are biclonal with respect to this marker. We and others have described lymphomas, leukemias, and myelomas comprising subpopulations characterized by differences in their expressed light chain type (5, 26, 27). More recently, antiidiotype antibodies have been used to define specific subpopulations of B cells, within morphologically monomorphic lymphoid neoplasms that contain different idiotypic determinants associated with their surface Ig molecules (5, 9, 23, 28, 29). Configurations of Ig or TCR genes have also been used as clonal markers in human lymphoid disorders, and several examples have been reported in which patterns of gene rearrangements suggest bi- or multiclonal lymphomas (4–6). In some instances, differences in Ig gene rearrangements were shown to segregate with surface phenotypic markers to distinct tumor subpopulations (5, 6).

This study was undertaken to reassess the clonality of several B cell lymphomas that contained distinct subpopulations with Ig phenotypic and/or genotypic differences. We used, as an independent measure of clonality, the configuration of chromosome 18 DNA at or near the t(14;18) breakpoint, as assessed by the mobility of DNA fragments on genomic Southern blots or actual DNA sequences of t(14;18) crossover points. The crossover points for this chromosomal translocation have been shown to cluster at two different sites on chromosome 18 (13, 14). On genomic Southern blot analysis, the configuration of rearranged chromosome 18 DNA, as detected by one or the other t(14;18) breakpoint cluster region probe, represents a tumor-specific marker since the actual crossover point at the DNA level is unique to each patient's lymphoma. This has been confirmed by nucleotide sequence analysis of t(14;18) breakpoints from a number of follicular lymphomas and lymphoma cell lines (13–15, 30, 31).

When we tested the subpopulations within each patient's tumor for this clonal marker, they were found to share the same configuration for t(14;18) DNA. These findings clearly indicated that the two tumor subpopulations in each patient were related by virtue of having the same t(14;18) breakpoint and thus necessarily derived from a common progenitor cell. If the two tumor subpopulations were completely unrelated, i.e., of separate origins, we expected to observe different configuations for t(14;18) breakpoints in each of the subpopulations. The latter represents a minimal criterion for what we would term true biclonality, i.e., two tumors of identical morphologic type arising *de novo* within the same patient and having separate and distinct origins.

The t(14;18) chromosomal translocation likely occurs very early in B lymphocyte differentiation, probably during heavy chain gene rearrangements, due to an error in D-J joining as shown by several previous studies (13, 30, 31). The sequence features of the DS lymphoma breakpoint are consistent with this proposal since the crossover occurred directly 5′ to heavy chain joining segment J6. The early occurrence of t(14;18) in B cell ontogeny raises the possibility that in follicular lymphoma patients, various tumor subclones may derive from a progenitor cell carrying a t(14;18) on one heavy chain allele and a germline

configuration or D-J rearrangement on the other. Tumor subclones may potentially arise at a number of different time points following t(14;18), including a stage before D-J or V-D joining on the productive heavy chain allele and/or before κ or λ light chain gene rearrangement. To more accurately determine the point of divergence in the DS lymphoma subpopulations, we used the precise configurations of V-D, D-J, and V-J joints for the productive heavy and light chain Ig genes as another measure of clonality. Our analyses indicated that the subpopulations likely contained identical joints in their productive Ig genes, confirming that the common precursor cell for the two subpopulations had completely progressed through all stages of heavy and light chain gene rearrangement before divergence of the subpopulations.

None of the tumors analyzed in this report proved to be truly biclonal. Rather they represent examples of what might be termed apparent biclonality; that is, tumors comprising subpopulations that are evolutionarily related but distinct due to differences acquired during tumor progression. These differences may result from different V, D, J, or C segment utilization by the members of each subpopulation. Segment utilization differences may result from different *de novo* gene rearrangements in individual cells derived from a common primitive precursor that has not yet completely rearranged its Ig genes. Clonal Abelson virus–transformed pre-B cell lines that retain the capacity for *de novo* Ig gene rearrangements represent in vitro analogs of this phenomenon, i.e., they are related by having identical D-J rearrangements, but subclones can arise that have independently undergone V-D rearrangements (32). Alternatively, segment utilization differences may result from continuing V-V-D-J or V-V-J rearrangement within an otherwise monoclonal B cell population. Recent examples of this phenomenon have been described in murine lymphoma and Abelson pre-B cell lines (33, 34). Thus, in apparent biclonal proliferations, several mechanisms may account for Ig gene restriction fragment length differences, but all involve changes superimposed on a single progenitor resulting in progeny that differ by virtue of secondarily acquired, independent mutational events.

An additional mechanism to explain apparent biclonality in tumors derived from a single parent cell is somatic mutation in rearranged Ig genes, as we have determined for the lymphoma DS. After divergence from a common progenitor containing a t(14;18) cytogenetic abnormality and completely rearranged Ig genes, subpopulations separately accumulated extensive point mutations and deletions of their Ig genes and flanking sequences. This process resulted in alteration of restriction enzyme recognition sites and acquisition or loss of tumor-specific idiotypic determinants. It is clear from our results that these alterations do not define clonally separate tumors within the host, but rather reflect the separate maturational histories of two subpopulations derived from a common progenitor. Therefore, expressed idiotope and Ig gene fragment lengths cannot be regarded as consistently stable clonal markers, at least in follicular lymphomas. Further studies are required to determine their stability in other morphologic subtypes of lymphoma and leukemia.

Nucleotide sequence analysis of the DS lymphoma showed that numerous point mutations had accumulated in the expressed heavy and light chain V region genes. Although we have previously described somatic mutation of expressed V

TABLE I

Nucleotide Changes in Framework and Complementarity-determining Regions

Region	Base change/base pair*							Replacement substitution	
	Replacement		Silent		Total		R/S	Expected‡	Observed§
	n	%	*n*	%	*n*	%		%	%
Heavy chain									
FR (228)	27	11.84	16	7.02	43	18.86	1.69	75.44	62.8
CDR1 (18)	3	16.67	1	5.56	4	22.22	3	87	75
CDR2 (60)	6	10	4	6.67	10	16.67	1.50	76.40	60
CDR3 (69)	11	15.94	6	8.70	17	24.64	1.83	70.53	64.7
Total (375)	47		27		74				
Light chain									
FR (213)	15	7.04	18	8.45	33	15.49	0.83	74.4	45.45
CDR1 (33)	7	21.20	2	6.06	9	27.27	3.50	75.76	77.78
CDR2 (21)	7	33.33	3	14.29	10	47.62	2.30	73	70
CDR3 (60)	3	5	4	6.67	7	11.67	0.75	71	42.86
Total (327)	32		27		59				

* Percent base change/base pair was derived by summing the replacement or silent nucleotide differences in the sequences for each region and dividing by the total number of base pairs in each region (37).

‡ Expected replacement substitutions was derived by summing all possible replacement substitutions for each codon within the respective region and dividing by the total number of potential substitutions (replacement or silent) for all codons within the region.

§ Percent observed replacement substitutions was calculated by summing the observed replacement substitutions for each region (Figs. 5 and 6) and dividing by the total number of replacement and silent substitutions for each respective region.

region genes in human follicular lymphomas, the DS data are notable for the extensive number of mutations observed. As shown in Table I, 74 nucleotide differences were observed between the two DS heavy chain V region isolates, and 59 were observed for the λ light chain V genes. This is 5–10 times more extensive than we previously observed for several independent isolates of expressed V_H and V_L genes obtained from serial biopsies of other patients with follicular lymphoma. Since patient DS had never been treated with antineoplastic therapy, the observed mutations reflect the unperturbed biology of the tumor.

The extent of sequence heterogeneity within each DS subpopulation is not known, since only single copies of each V region were isolated and sequenced. Within the limits of our analyses, it appeared as if all cells at each site in DS were homogeneous with respect to idiotope expression and Ig gene fragment lengths. However, our previous studies (11, 23) on other patients with follicular lymphoma have shown that V region sequence and/or idiotope heterogeneity is frequently, if not invariably present within a given biopsy site. The combination of findings from patients studied here and elsewhere suggests that somatic mutation of Ig genes is an active, ongoing process in follicular lymphomas. Our data also suggest that there may a spectrum for the degree of somatic variation that can occur among follicular lymphomas, possibly indicating different inherent rates of mutation or potentially different levels of selection for favorable or

unfavorable mutations in the lymphoma cells by endogenous antiidiotype antibody or antigen.

We had previously noted a significant bias against mutations affecting amino acid sequences in the framework regions and clustering of such replacement mutations in one complementarity-determining region (CDR)[1] of Ig genes from a patient with follicular lymphoma (11). Clustering of replacement mutations during a normal immune response may occur in heavy or light chain CDRs or both, supporting the notion that the respective CDRs are important for binding of antibody to antigen (35). A bias for mutations in CDRs was seen in the DS light chain data (Table I), where we observed clustering of the expected levels of replacement substitutions in CDRs 1 and 2, but much lower levels elsewhere in the gene. However, no significant bias was observed in the heavy chain gene, since the expected proportions of replacement substitutions were found throughout the coding region. As we have suggested previously, one potential explanation for the nonrandom distribution of somatic mutations is that endogenous selective forces were acting on the tumor cells and favored the retention of cells with functional surface Ig molecules. In spite of the more uniform distribution of mutations in the heavy chain V genes, the DS data may be consistent with this hypothesis, in light of the observed suppression of replacement mutations in the framework regions and CDR-3 of the λ V region genes. In murine systems, clustering has been observed in CDRs only in the light chain genes and not the heavy chain gene in B cells responding to influenza hemagglutinin antigen (36). For the DS lymphoma, the light chain CDRs may have been more important than the heavy chain CDRs for the interaction of host factors with surface Ig.

Our sequence data from the DS lymphoma also showed that the target for somatic mutation was much larger than the productive V region gene and extended well into the J-C intron. Although mutations have been observed in the J-C intron of V genes isolated from murine B cells responding to defined antigens (37), many more mutations were observed in the DS lymphoma cells, probably reflecting the accumulation of somatic mutations with continued cell division of the tumor clone. The numerous mutations in the DS λ light chain genes define a broad DNA region susceptible to the mutational process. At least for the λ light chain genes, the target appears to be defined by a high density of mutations centered on the J-C intron with a lower density of mutations extending into the productive V region gene. However, we cannot rule out the possibility that the actual target for high frequency mutation is much larger and that the observed gradient of mutations resulted from positive or negative selection on the cells harboring them.

Summary

To investigate the possible relatedness of the subpopulations that make up so-called biclonal lymphomas, we examined five bigenotypic and biphenotypic follicular lymphomas using DNA probes specific for the t(14;18) chromosomal translocation, which is a characteristic feature of these neoplasms. On Southern blot analysis, both subpopulations from four of five lymphomas contained comigrating t(14;18) DNA rearrangements, confirming the single cell origins for

[1] *Abbreviation used in this paper:* CDR, complementarity-determining region.

these neoplasms. No comigrating t(14;18) DNA rearrangements were observed in the fifth lymphoma, but nucleotide sequence analysis of cloned, breakpoint DNA showed identical t(14;18) crossovers in the two subpopulations. The migration differences of both the Ig and chromosome 18 DNA rearrangements were shown to result from somatically acquired mutations of the Ig genes from the fifth lymphoma. These studies indicate that Ig gene rearrangements and idiotope expression are not consistently stable clonal markers since they are subject to variability as a result of somatic mutation. Although translocated chromosome 18 DNA rearrangements are more reliable, they may also vary among cells of some tumors since somatic mutation can affect, as well, DNA of translocated alleles in follicular lymphomas.

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