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Clonal evolution of a follicular lymphoma: Evidence for antigen selection

(somatic mutation/immunoglobulin gene)

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ABSTRACT **The potential role antigens play in growth stimulation or in clonal selection of follicular lymphomas is unknown. To study this issue, we sequenced the immunoglobulin heavy chain variable region genes expressed by a follicular lymphoma from multiple biopsy specimens and also cloned and sequenced the corresponding germ-line variable gene from this patient. Comparison to the germ-line gene revealed numerous nucleotide substitutions in all of the lymphoma variable gene sequences. Some of the substitutions may have occurred in the nonmalignant precursor B cell that gave rise to this lymphoma because they were shared among all of the variable genes, but many of the mutations accumulated as the malignant clone expanded. The mutations were distributed in such a way that strongly suggested the majority of tumor cells had been positively selected through their antigen receptor. This was especially evident for the mutations that developed late in the clonal evolution of this lymphoma. These findings indicate that antigen stimulation may be involved in the growth of follicular lymphoma tumors.**

Follicular lymphomas (FLs) are thought to be malignancies of germinal center B cells (1–3). The process of somatic hypermutation of immunoglobulin genes, which occurs during the generation of memory B cells in germinal centers (4, 5), appears to be a general feature of FLs and can result in considerable intraclonal heterogeneity (6). Tumor cells without surface immunoglobulin are presumably generated in most FLs by mutations that result in stop codons and/or dysfunctional residues. It is therefore significant that the vast majority of FLs are surface immunoglobulin positive even after treatment of patients with tumor-specific anti-idiotypic antibodies (7, 8). Long-term retention of an idiotypic determinant has also been demonstrated in one case of FL that mutated its immunoglobulin genes (9). These observations suggest there may be selection for surface immunoglobulin expression in FLs and are consistent with the notion that antigen stimulation may play a role in the growth of these tumors.

The role of antigen selection during the clonal evolution of a FL can be studied by analyzing immunoglobulin gene mutations (10, 11). Mutations appear to mostly localize to the immunoglobulin variable regions and occur randomly in the framework regions (FWRs) and complementarity-determining regions (CDRs) (12–14). Antigen contact is thought to occur primarily through amino acid residues in the CDRs (15). As a result, these mutations can be positively selected and variable genes from antigen-selected clones often have large accumulations of replacement (R) mutations in their CDRs (10, 16, 17). Moreover, clustering of R mutations in CDRs can be used as an indicator of antigenic selection (10, 12). Past studies of immunoglobulin genes in FLs were potentially limited with regard to mutation analyses because the corresponding germ-line variable genes were never identified (9, 18, 19).

In the present study we have determined the nucleotide sequences for the heavy chain variable region (V_H) genes of a FL that had been sampled on multiple occasions over a 2-year time period.* The V_H segment expressed by this case was found to be a mutated version of an already reported germ-line gene termed V_H4-21 (20). Analysis of the V_H gene mutations suggests that an antigen may have been acting to stimulate the growth of the tumor and to select cells for growth that retained their immunoglobulin receptor but mutated the binding site.

MATERIAL AND METHODS

Tumor Tissue and DNA Isolation. Spleen and lymph node biopsies of a patient with FL, mixed small and large cell type, were used as sources of tumor DNA. Genomic DNA was isolated using previously described procedures (21).

PCR Amplification of V_H Genes. Rearranged V_H genes were amplified from 1 μg of biopsy DNA using six 5′ V_H family-specific leader primers (V_HL1–V_HL6) in combination with a 3′ heavy chain joining region (J_H) primer as described (22). Germ-line V_H4 genes were amplified under similar conditions using the V_H4L primer with the 3′ V_H4SS primer (5′-GGCTCACACTCACCTCCCCT), which annealed to the spacer between the 3′ heptamer nonamer recombination signal sequences (23).

Cloning and Sequencing of PCR Products. For cloning, gel-purified PCR products were treated with T4 polynucleotide kinase (New England Biolabs) and blunt end ligated into phosphatase-treated *Sma* I-cut M13mp19 using T4 DNA ligase (Boehringer Mannheim). Following purification, the ligated DNA was used to electrotransform *Escherichia coli* XL1-Blue (Stratagene) using a Gene Pulser (Bio-Rad). Recombinant plaques, identified by their colorless appearance in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside and isopropyl β-D-thiogalactoside, were grown in 2 ml of 2× YT medium for 6–8 hr and processed for isolation of DNA following PEG precipitation as described (24). The generation of single-stranded DNA through asymmetric amplification of gel-purified PCR products was carried out as described (22). Dideoxy sequencing was carried out with Sequenase (United States Biochemical) following the manufacturer's protocol. Sequences were analyzed using the University of Wisconsin Genetics Computer Group (Madison) programs (25).

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Abbreviations: V_H, heavy chain variable; J_H, heavy chain joining; CDR, complementarity-determining region; FWR, framework region; FL, follicular lymphoma; R, replacement (mutation); S, silent (mutation).

*The sequences reported in this paper have been deposited in the GenBank data base (accession no. M94768).

Screening for V_H4-21-Related Germ-Line Genes. Following amplification and cloning of germ-line V_H4 genes from the patient's spleen specimen, randomly selected recombinant M13 plaques were grown in 2× YT medium for 8–10 hr and spotted onto nylon membrane (Optiblot, IBI). After crosslinking with UV light and prehybridization at 42°C for 1 hr (hybridization buffers used were those recommended by the membrane supplier), the DNA spots were hybridized at 42°C for 6 hr with the V_H4-21-FW1 oligonucleotide (5-CTACAGCAGTGGGGCGCA) that had been end labeled with ^{32}P. V_H4-21-FW1 anneals to FWR1 of the V_H4-21 and closely related V58 germ-line genes (see Fig. 2) but is mismatched at four internal positions with all other known V_H4 family genes. The membranes were then washed non-stringently with 6× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate)/0.1% SDS at 23°C for 10 min and then at 42°C for 10 min. These conditions were confirmed to give strong signals even with DNA that contained G → A and/or C → T mismatches at positions 6 and 1, respectively, of V_H4-21-FW1 (the expressed gene is mismatched at position 6).

Somatic Mutation Analysis. For n random mutations, the number of R mutations should = $0.75n$ and the number of expected silent (S) mutations = $0.25n$ (26). Without selection, the R and S mutations should distribute among the various V_H regions according to their respective sizes—i.e., R_{CDRs} = 0.23 R, R_{FWRs} = 0.77 R, S_{CDRs} = 0.23 S, and S_{FWRs} = 0.77 S. A binomial mutation model developed by Schlomchik *et al.* (12) was used to calculate the probability (P) that kR mutations would occur in the CDRs given n total mutations, $P = [n!/k!(n - k)!]q^k(1 - q)^{n-k}$, where q = the probability a R mutation will locate to the CDRs = (0.23)(0.75) = 0.17. If one assumes that immunoglobulin function is maintained, then the number of R_{FWRs} should be doubled—i.e., let $n = n$ observed + R_{FWR} in the above formula—to obtain a more accurate P value.

RESULTS

Nucleotide Sequence Analysis of FL V_H Genes. Four different FL biopsy specimens were analyzed (Table 1) that have been previously described relative to this patient's clinical course (7). The 1982 spleen sample, obtained shortly after the initiation of chemotherapy, provided FL cells that were used in the production of a tumor-specific anti-idiotypic monoclonal antibody. Most of the lymphoma cells from all four samples displayed high levels of surface immunoglobulin by flow cytometric analysis. The anti-idiotypic antibody, however, failed to stain the majority of FL cells from the 1984 biopsy. DNA isolated from each biopsy was amplified with a 3′ J_H primer and one of the six 5′ V_H family-specific leader region primers. Following electrophoresis and staining with ethidium bromide, products of the correct size were observed only with the V_H4L and J_H primer combination (Fig. 1). For further analysis, gel-purified PCR products generated with the V_H4L and J_H primers were either cloned into M13 or used as templates in subsequent "asymmetric" PCR reactions.

Table 1. Patient specimens analyzed in this study

Specimen no.	Date	Tissue	Clinical	Phenotype*
1	5/7/82	LN	During Chemo	Ig^+, Id^+
2	5/7/82	SP	During Chemo	Ig^+, Id^+
3	11/1/83	LN	During mAb Tx	Ig^+, Id^+
4	5/30/84	LN	After mAb Tx	Ig^+, Id^-

LN, lymph node; SP, spleen; Chemo, chemotherapy; mAb Tx, monoclonal antibody treatment; Id, lymphoma idiotype. For additional information, see ref. 7.
*Determined by surface staining and flow cytometry.

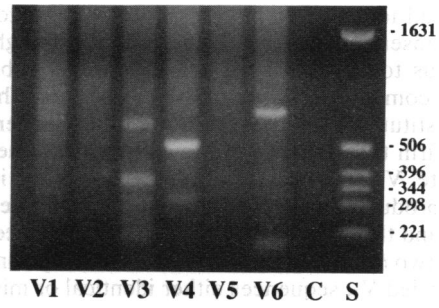

FIG. 1. PCR amplification of rearranged V_H genes. Shown are PCR products following electrophoresis in 1.5% agarose and staining with ethidium bromide. The products were generated by amplification of DNA prepared from a tumor specimen for 30 cycles with each one of the six 5′ V_H family-specific leader region primers V_H1L–V_H6L (lanes V1–V6, respectively) and the 3′ J_H primer. Products from a reaction containing the V_H4L and J_H primer combination but where no DNA was added are in lane C. Molecular weight standards (*Hin*fI-digested pBR322) are in lane S. Virtually identical results were obtained from all four of the FL specimens.

Asymmetric amplification with the V_H4L and J_H primers was utilized to generate single-stranded DNA that was directly sequenced to determine a V_H gene consensus sequence for each tumor specimen. In Fig. 2, the V_H consensus sequences associated with each biopsy are shown relative to the V_H4-21 germ-line gene. Among the 10 reported functional germ-line genes from the V_H4 family [V79, V12G-1, V11, V2-1, V58, V71-4, V71-2 (23); V_H4-22, V_H4-21 (20); V_H4-MC9 (28)], V_H4-21 shows the greatest homology to the lymphoma sequences and is the likely germ-line origin (see below). The V_H consensus sequences for FL specimens 1, 2,

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          1                          ←FW1→
# 1       --------a- ----a----- ---------- t--------- ---------- ----------
# 2       --------a- ----a----- ---------- t--------- ---------- ----------
# 3       --------a- ----a----- ---------- t--------- ---------- ----------
# 4       ---------- ----a----- ---------- ---------- ---------- ----------
VH4-21    CAGGTGCAGC TACAGCAGTG GGGCGCAGGA CTGTTGAAGC CTTCGGAGAC CCTGTCCCTC
                     *   *   *    *

          61                                  CDR1
# 1       ---------- ---------- ---t-----A AA----A--- ---Ct----- ----------
# 2       ---------- ---------- ---t-----A AA----A--- ---Ct----- ----------
# 3       ---------- ---------- ---t-----A AA----A--- ---Ct----- ----------
# 4       ---------- ---------- ---t----AA AA----A--- ---Ct----- ----------
VH4-21    ACCTGCGCTG TCTATGGTGG GTCCTTCAGT GGTTACTACT GGAGCTGGAT CCGCCAGCCC

          121       ←FW2→                        CDR2
# 1       --c------- ---------- --------G- G--------- C----C---- ----------
# 2       --c------- ---------- --------G- G--------- C----C---- ----------
# 3       --c------- ---------- --------G- G--------- C----C---- ----------
# 4       --c------- ---------- ---------- ---------- -------t-- ----------
VH4-21    CCAGGGAAGG GGCTGGAGTG GATTGGGGAA ATCAATCATA GTGGAAGCAC CAACTACAAC

                                                     ←FW3→
# 1       ---------- G-G------- ---------- --t------- ---------- ----------
# 2       ---------- G-G------- ---------- --t------- ---------- ----------
# 3       ---------- G-G------- ---------- --t------- ---------- ----------
# 4       ---------- ---------- ---------- A-t------- ---------- ----------
VH4-21    CCGTCCCTCA AGAGTCGAGT CACCATATCA GTAGACACGT CCAAGAACCA GTTCTCCCTG

          241
# 1       -CaA---C-- ---------- ---------- ---C-a---- ---------- -
# 2       -CaA---C-- ---------- ---------- ---C-a---- ---------- -
# 3       -CaA---C-- ---------- ---------- ---C-a---- ---------- -
# 4       -CaA---C-- ---------- ---------- ---C-a---- ---------- -
VH4-21    AAGCTGAGCT CTGTGACCGC CGCGGACACG GCTGTGTATT ACTGTGCGAG A
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FIG. 2. V_H gene consensus sequences for different biopsies. The consensus V_H gene sequences for each of the four different lymphoma specimens indicated on the left were determined by asymmetric PCR and by analysis of cloned PCR products. Nucleotide differences from the V_H4-21 germ-line gene that can result in amino acid replacements (R) are indicated in bold uppercase letters, whereas silent substitutions (S) are shown in lowercase letters. Identity with the V_H4-21 gene is indicated by a dash (–). The functional immunoglobulin domains defined by Kabat *et al.* (27) are indicated above the sequences as FWRs or CDRs and the appropriate number. The V_H4-21-FW1 oligonucleotide used for screening cloned V_H4 germ-line genes from this patient is underlined. Nucleotides marked with an asterisk indicate the positions V_H4-21-FW1 is mismatched with all of the reported V_H4 genes except V_H4-21 and V58.

and 3 are all identical and are 92% homologous to V_H4-21. The V_H consensus sequence from sample 4 is slightly more homologous to V_H4-21 at 94%. It lacks eight substitutions that were common to samples 1–3 but contains three additional substitutions not present in the other sequences.

To confirm the accuracy of the consensus sequences and to evaluate V_H sequence heterogeneity within individual biopsies, products from separate PCR reactions were cloned into M13 and then sequenced. Nucleotide sequence analysis of at least two randomly selected clones from specimens 2, 3, and 4 revealed V_H sequences either identical or mismatched at only one nucleotide with the consensus sequences obtained by asymmetric PCR (not shown). For specimen 1, V_H genes from eight randomly selected clones were sequenced and are shown in Fig. 3. Observe that three of the clones (LN82-4, -7, and -8) are identical to the consensus sequence while four others (LN82-2, -3, -5, and -6) have one additional substitution each. One clone (LN82-1), however, lacks five substitutions present in most of the other sequences but is otherwise identical. This gene and the V_H consensus sequence from specimen 4 are both equally homologous to V_H4-21 (94%) and contain the least number of total substitutions. Nucleotide sequence information was also obtained for all of the CDR3 regions but no differences were found among any of the consensus or cloned V_H sequences, which all showed a single productive variable–diversity-joining rearrangement (not shown).

Cloning of Germ-Line V_H4 Genes. To confirm that V_H4-21 was the germ-line origin of the lymphoma V_H sequences, unrearranged V_H4 family genes were amplified from the patient's spleen specimen. The PCR products were cloned into M13, and recombinant clones were screened under low stringency with an oligonucleotide (V_H4-21-FW1) that was designed to identify all potential "V_H4-21-like" germ-line genes. After screening 44 clones, 5 were identified that hybridized with the V_H4-21-FW1 oligonucleotide. Sequence analysis showed that 4 of these clones were identical to the published V_H4-21 sequence that has been isolated now from several different individuals (20, 28, 29). The other identified clone was a composite of the V_H4-21 and V_H4MC-9 germ-line genes and therefore appears to be a PCR-generated artifact

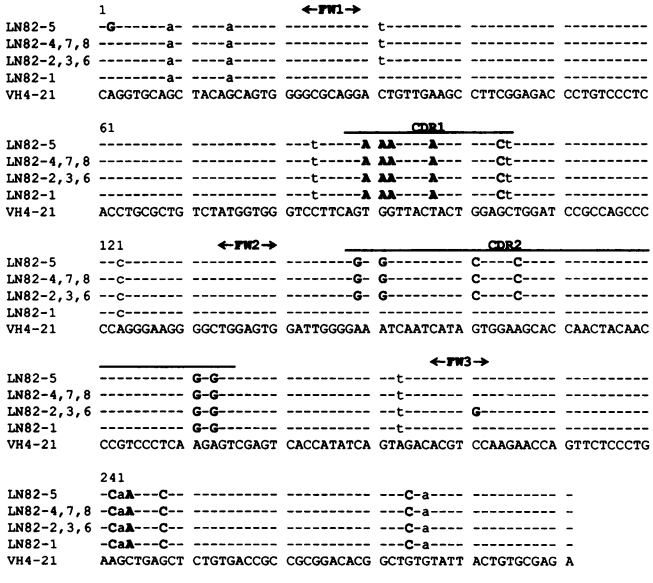

FIG. 3. Variation of V_H gene segments within a biopsy. Nucleotide sequences were determined for eight randomly selected rearranged V_H4 genes cloned following PCR amplification from specimen 1, an early lymph node biopsy obtained in 1982. The various clones were labeled numerically following an LN82 prefix and are indicated on the left. The sequence format is the same as in Fig. 2.

(30). These data clearly establish that the germ-line repertoire of this patient contains an unaltered V_H4-21 gene. Moreover, additional germ-line genes that may be closely related to V_H4–21 or the V_H gene expressed by this lymphoma are not present or else failed to amplify with our primers. Although this later possibility cannot be totally ruled out, it is nevertheless unlikely because of the high degree of homology among all of the known V_H4 family genes especially around the amplification primer sequences (23). These data, therefore, strongly argue that all of the nucleotide differences in this lymphoma V_H segment from V_H4-21 are indeed acquired mutations.

Distribution of Mutations. As shown in Fig. 2, the consensus V_H sequences from specimens 1, 2, and 3 all differ from germ line at the same 23 nucleotide positions. In Table 2, the type (R or S) and location (FWR or CDR) of the mutations associated with this dominant lymphoma V_H sequence are summarized. Observe that the CDRs contain over twice the number of R mutations expected by chance or observed in the FWRs, even though the CDRs comprise only 23% of this V_H gene. Using a binomial model, the probability of obtaining by chance 11 R mutations in the CDRs given 23 total mutations is only 0.0005. Even if it is assumed that FL cells must express functional immunoglobulin, which can be taken into consideration by doubling the number of R mutations observed in the FWRs, the probability for observing 11 R mutations in the CDRs is still only 0.002. This analysis indicates, therefore, that at least some of the CDR R mutations have almost certainly been selected.

DISCUSSION

We analyzed mutations in the immunoglobulin V_H genes expressed by a FL from multiple tumor biopsy specimens. Besides serving as clonal markers, mutations in V genes can provide important information regarding the role of antigen during clonal selection. To accurately identify the locations and types of mutations that may have been present, the germ-line counterpart for the expressed V_H gene was cloned and sequenced from this patient. Because germ-line genes were not identified in past studies, only nucleotide differences among clonally related FL V_H genes could be analyzed (9, 18, 19). The large number of closely related V_H segments in humans can make identification of an appropriate germ-line counterpart quite difficult (31). In this study, however, we were able to take advantage of the fact that the lymphoma V_H segment belonged to a relatively small family (V_H4) for which nucleotide sequence information is known for most of the genes (20, 23, 28).

Prior to treatment with an anti-idiotype antibody, this patient's lymphoma sampled at three different sites over a 16-month period appeared to be remarkably homogeneous and was dominated by a single clone. Clonal dominance was evident from the analysis of multiple V_H4 genes cloned from several independent PCR amplifications. Moreover, because of clonal homogeneity the V_H gene consensus sequences, which were identical for each pretreatment biopsy, could be easily determined by directly sequencing templates gener-

Table 2. Distribution of mutations in the dominant V_H sequence

Location	No. observed R	No. observed S	No. expected R	No. expected S
FWR	4	7	13	5
CDR	11	1	4	1

Expected values were calculated as described in the text assuming that the 23 mutations would have a random distribution and R/S composition.

ated by asymmetric PCR amplification. Relative to germ line, the dominate clone contained 23 total substitutions in its V_H gene. For subsequent analysis, each V_H substitution was assumed to have occurred independently and was classified as being R or S and having a CDR or FWR location. Expected numbers of R or S mutations in the CDRs or FWRs were determined by further assuming that all nucleotides in this V_H segment had an equal probability of undergoing mutation. Although these assumptions are typically made when analyzing mutations in V genes, some may not be strictly true—e.g., see ref. 32. Nevertheless, they represent reasonable approximations given the current state of understanding and also facilitate quantitative analysis of V gene mutation distributions (12). Examination of the V_H sequence associated with the dominate clone revealed that it contained 11 R mutations in the CDRs, a number significantly greater than expected if the 23 mutations had a totally random distribution. From this analysis it appears, therefore, that many of the CDR R mutations must have been selected. Moreover, the clonal dominance present in this tumor could readily be explained by antigen-mediated selection.

Positive selection is especially evident when those mutations that occurred during the later stages of the dominant clone's evolution are analyzed separately (see Fig. 4). Since 15 mutations were common to all of the V_H sequences, some or all of these could have developed in the nonmalignant

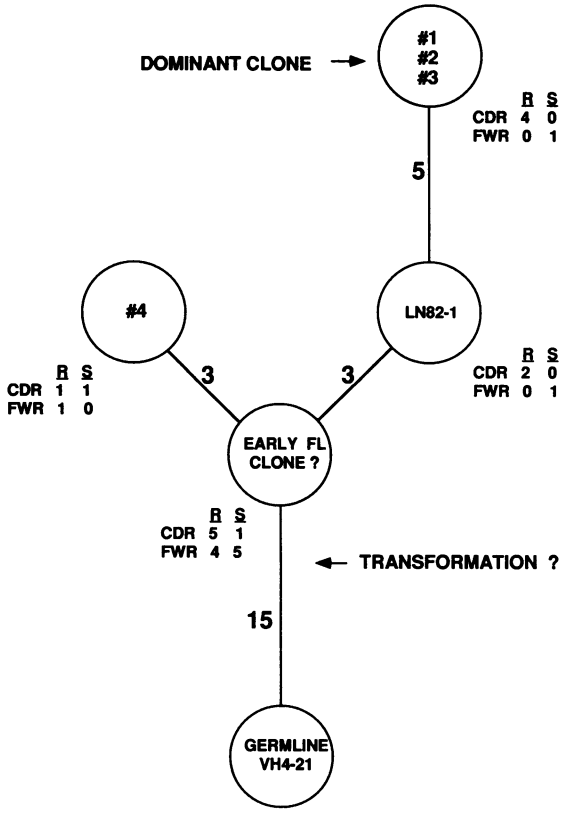

FIG. 4. Relationship of lymphoma V_H genes. The V_H gene consensus sequences for specimens 1, 2, and 3 are depicted along with an intermediate gene (LN82-1) cloned from specimen 1 in a genealogic tree based on shared and unshared mutations. The early FL clone sequence is a hypothetical branch point defined by the 15 mutations common to all of the lymphoma V_H sequences. The number of mutations acquired between different sequences is indicated along the connecting lines and the type (R or S) and location (FWR or CDR) of the acquired mutations have been tabulated. The clonotype present at transformation is not known but could be any point between the germ-line gene, V_H4-21, and the hypothetical early FL clone.

precursor B cell from which this FL originated. As a result, their distribution could reflect the action of selective forces different from those acting on the lymphoma cells (33). Of the 23 total mutations present in the dominant clone, however, it is clear that at least 8 must have occurred after transformation because they were not present in all of the V_H consensus sequences. Moreover, finding an intermediate clone in specimen 1 that contained only 3 of the 8 mutations further establishes their lymphoma cell origin and indicates a sequential pattern of development. Examination of these 8 mutations revealed that 6 are R mutations in CDR2 and 2 are S mutations in FWR1. Extreme clustering of R mutations in the CDRs such as this most likely occurred because of positive selection. The probability, from the binomial model described above, that this distribution of 8 mutations occurred by chance is very low ($P = 0.0005$).

Antigen stimulation may also have played an important role early on in the evolution of this FL, although better knowledge of the original V_H clonotype is required to properly address this point. In theory, the earliest FL clonotype could have been any point between the hypothetical intermediate represented by the 15 substitutions that were common to all of the V_H sequences and the germ-line gene. If some of the V_H mutations were present before lymphomagenesis, as is likely, this would further suggest that antigen stimulation occurred prior to transformation (34) and could therefore also be involved in oncogenesis.

In contrast to the CDRs, fewer FWR R mutations were observed than expected by chance in all of the lymphoma V_H sequences. To maintain function, it appears that FWRs can accommodate only about half of the possible number of R mutations (12). Selection for functional immunoglobulin could, therefore, account for the paucity of FWR R mutations we observed. Also relevant to the issue of immunoglobulin selection is that immunoglobulin loss variants were never detected by flow cytometry in this case, even after treatment with an anti-idiotypic antibody (7). In addition, none of the V_H clones possessed any mutations, deletions, or insertions that would prevent or limit translation. These and similar findings from other studies as outlined above lend support to the notion that maintenance of immunoglobulin expression may be a general feature of FLs. It is tempting to speculate, therefore, that the maintenance of immunoglobulin expression and immunoglobulin-mediated clonal selection have a similar basis.

Besides FLs, antigen receptor-mediated selection has been suspected of playing an important role in the growth of other types of lymphoid malignancies. Friedman *et al.* (11) recently provided evidence for immunoglobulin-mediated clonal selection studying V_H mutations in a human B-cell lymphoma that initially was classified as low grade and expressed a well-defined autoantibody. Unlike this study, however, it was suggested that antigen was providing negative selective pressure by selecting against CDR R mutations to maintain autoantigen binding affinity. Biased use of V_H genes in chronic lymphocytic leukemia (35), a low-grade malignancy of $CD5^+$ B cells, as well as in low-grade $CD5^+$ mouse lymphomas (36) could result from antigenic selection as has been suggested. However, differentiating between the selection of normal B cells or lymphoma cells can be difficult (37), and because these $CD5^+$ B-cell tumors do not mutate their immunoglobulin genes, individual cases cannot be evaluated for evidence of antigen-driven proliferation or clonal evolution as was done here. Antigen receptor stimulation has also been implicated in the development of viral-associated lymphoid neoplasms in mice (38, 39). In this model of leukemogenesis, virus envelope proteins provide a chronic mitogenic signal through antigen receptor binding, which is required for tumor cell proliferation. Along this line, human chronic

lymphocitic leukemias have been identified that appear to be proliferations of cells responding to retroviral infections (40).

The nature of the selective force that appears to be functioning through the immunoglobulin receptor expressed by this FL is not known. However, self antigens should be considered as potential candidates, especially in light of recent findings that autoantibody reactivity of FL immunoglobulin is not uncommon (41). In this regard, it is particularly interesting that the V_H4-21 gene, which is the germ-line counterpart for the lymphoma V_H sequences, may be frequently used by autoantibodies. In particular, cold agglutinins, rheumatoid factors, and anti-DNA antibodies have been identified that use somatically mutated versions of V_H4-21 (29, 42).

Most of the FL cells from the specimen that had been collected after treatment of the patient with a monoclonal anti-idiotypic antibody failed to stain with the treatment antibody. Loss of anti-idiotypic reactivity has been previously observed following this type of therapy and has been attributed to the outgrowth of tumor variants that arise through mutation of immunoglobulin genes (6). Examination of the V_H sequence from the idiotype-negative specimen revealed it lacked six R mutations in CDR2 that were present in the V_H sequence from the FL cells that were used to produce the anti-idiotype antibody. In this case, therefore, lack of anti-idiotype staining may be due to the outgrowth of an early more germ-line-like FL clone rather than one that has acquired additional V gene mutations.

From analysis of this as well as an additional case (A. D. Zenenetz, T. H. L. Chen, and R.L., unpublished data) it appears that the selection of FL cells may be analogous to the selection that occurs in nonmalignant germinal centers for B-cell clones that express high-affinity antibodies (43, 44). Although antigen-mediated selection of germinal center cells is not well understood, crosslinking of surface immunoglobulin *in vitro* has been reported to prevent programed apoptotic death of these cells by up-regulation of Bcl-2 protein (34, 45). Since almost all FLs constitutively express high levels of Bcl-2 protein due to a t(14;18) chromosome translocation (46), our data suggest that the immunoglobulin receptor complex provides an additional signal(s) required for selection.

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