The Role of Clonal Selection in the Pathogenesis of an Autoreactive Human B Cell Lymphoma

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Summary

To study the association of autoimmunity and human B cell neoplasia, we have established a model of a B cell lymphoma which expresses a pathogenic autoantibody of defined specificity. The Ig V_H gene expressed in this neoplasm was analyzed longitudinally using clinical specimens taken from the splenic lymphoma (S) at diagnosis and from lymph node relapses 3 and 4 yr later (N3 and N4). Southern analysis and oligonucleotide hybridization experiments demonstrated that clonally related predominant and minor tumor cell populations were present in S at diagnosis, and that the minor population became the predominant population in the relapse specimens, N3 and N4. Although the Ig specificity and idiotype were the same at diagnosis and at both relapses, analysis of the expressed V_H gene sequences showed 14 base changes between S and N3, and 2 further changes at N4. Little sequence heterogeneity was observed at each sampling time, indicating that the ongoing mutation frequency was low. The relevant germline precursor V_H gene was determined from autologous germline DNA and compared to the expressed genes. Based on the pattern of shared and unshared mutations, we were able to establish the genealogic relationship of the germline V_H gene and the expressed clonotypes of S, N3 and N4. Taken together, the findings from Southern blotting, oligonucleotide hybridization, and sequence analysis permit us to describe a molecular aspect of tumor progression, "clonotypic shift", wherein subpopulations of the malignant clone, marked by different V gene clonotypes, emerge and predominate at different time points in the evolution of the lymphoma. Furthermore, the sequential and nonrandom pattern of the V_H mutations, correlated with the observed conservation of autospecificity and idiotype, implies that clonal selection may have influenced the pathogenesis of the lymphoma.

Autoimmune phenomena can occur in association with several human clonal B cell disorders, such as idiopathic cold agglutinin disease (1), chronic lymphocytic leukemia (2), nonHodgkin's and Hodgkin's lymphomas (3, 4), and the clonal B cell expansions seen in HIV infection (5–7). The reasons for the association of autoimmunity and B cell neoplasia are poorly understood, and it is unlikely that a single mechanism will explain every example. In the normal humoral immune response, antigen plays a central role in provoking clonal expansion of B cells; theoretically, antigen could play an analogous role in some B cell clonal diseases. Disease states in which autoantibodies are found in association with B cell clonal disorders offer the opportunity to investigate the role of antigen, specifically autoantigen, in the pathogenesis of the clonal disease.

The Ig gene can serve as a marker in clonal analysis because unique combinations of V and J or V, D, and J segments are formed in the preimmune repertoire and are generally conserved within a clone thereafter. The nucleotide sequence of the CDR3, with its N sequences, is generated by these rearrangements and is unique to a particular clone. These features of the Ig gene have been exploited as analytic tools in studies of the clonality of murine and human immune responses and lymphoid neoplasms (8–12).

The Ig gene can also serve as a genealogic record of the processes of genetic diversification and clonal selection in a defined B cell clone. A nonrandom distribution of sequentially occurring mutations in the variable region gene can be interpreted as evidence of antigen driven clonal selection (13). This approach has been applied to studies of normal primary and secondary immune responses in murine models (14). Analysis of the pattern of somatic mutations has also been applied to murine autoimmune responses, and has demonstrated parallels between normal and autoimmune responses in the mouse (15). In human studies of normal immune responses, lymphoid malignancy, and autoimmune disease, how-

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ever, the Ig gene has been used principally as a clonal marker (10, 16–18); its potential to reveal information about clonal selection by a specific antigen in human disease has been exploited to only a limited degree (19–21).

Our laboratory has been interested in studying the association of autoimmunity and B cell neoplasia at a molecular level, and we have developed a model system in which the mechanism of that association is well defined. The model consists of a B cell lymphoma from a patient with autoimmune hemolytic anemia mediated by a monoclonal IgM cold agglutinin with specificity for a red cell carbohydrate antigen, Pr_2 (22). It is known from studies of EBV transformed cell lines derived from the lymphoma (23), that the neoplasm secretes the pathogenic autoantibody.

From these studies we hypothesized that this lymphoma arose from a pre-existing, expanded, autoreactive B cell clone, and that a member of this clone sustained one or more transforming events, resulting in the neoplasm. In following the patient's disease, we observed that the specificity and idiotype of this lymphoma were conserved over a 4-yr period. This observation suggested the additional hypothesis that clonal selection mediated by idiotype or by autoantigen (perhaps in an altered form), might have played a role in the evolution of the neoplastic B cell clone. Based on these two hypotheses, we predicted that if the neoplastic clone in this model system demonstrated diversity of nucleotide sequence at the Ig locus, then the pattern of that sequence divergence would reflect clonal expansion and the influence of antigen mediated clonal selection.

To test this prediction, we have undertaken a longitudinal molecular analysis of the Ig gene in this model system, using EBV cell lines and several clinical specimens from this patient. Interestingly, we found genetic diversity manifest as shifts in the predominant V_H clonotype during the evolution of the malignant clone. Furthermore, the pattern of nucleotide changes in the V_H gene fits well with a model of antigen mediated clonal selection favoring preservation of Ig idiotypy and specificity.

Materials and Methods

Patient Material. This model system has been described previously (22, 23). Briefly, a patient with IgM cold agglutinin mediated autoimmune hemolytic anemia was found to have a splenic lymphoma. EBV transformed B cell lines were established from splenic lymphocytes by limiting dilution. The karyotype of the cell lines had the same abnormalities as the tumor, and the antigen specificity, anti-idiotype binding, and isoelectric focussing patterns of the IgM secreted by the cell lines were the same as in the patient's serum (23). Lymph node biopsies were obtained at relapses of the lymphoma 3 yr after the first chemotherapy induced remission, and again 15 mo after a second remission. Sections of each clinical specimen were stained for light microscopy, and were snap-frozen or cryopreserved at −80°C. Granulocytes were harvested from peripheral blood by collecting the white cell layer which overlies the red cell layer after density gradient centrifugation of buffy coats through Sepracell (Sepratech, Oklahoma City, UK). Differential counts of these preparations revealed >95% granulocytes. The specificity of the anti-erythrocyte autoantibody was determined by hemagglutination using group O human erythrocytes with and without ficin treatment (24). Idiotype analysis in serum was performed by an ELISA assay as described by Jefferies et al. (25).

Cell Surface Staining and Flow Cytometry. Cell surface staining was performed by incubating cell suspensions of spleen or lymph node with a combination of fluorescein-anti-CD20 (Coulter B1; Coulter Electronics, Hialeah, FL) and allophycocyanin-anti-Id (25) or allophycocyanin-anti-IgM (Fisher Biotech, Orangeburg, NY) in staining medium on ice for 15 min and then washing 3× with staining medium. Stained cells ($0.5–1.0 \times 10^6$) were then applied to a dual laser/dye laser flow cytometer (FACSTAR^{PLUS}; Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with appropriate filters for multicolor immunofluorescence (26). Samples included propidium iodide (1 μgm/ml) in the medium which allowed dead cell exclusion. Labeling of antibody with allophycocyanin has been described previously (27).

Southern Hybridization. Genomic DNA was isolated by standard phenol/chloroform extraction (28). 5–10 μgm of DNA was digested with Eco RI, Hind III, Bam H1, or Xba 1, electrophoresed through 0.5% agarose, and transferred to Duralon nylon membrane (Stratagene, La Jolla, CA). The probes used were J_H, a 3.3-kb genomic fragment containing four of the six germline heavy chain joining segments, (29, 30), J_κ, a 1.8-kb genomic fragment (31), and C_μ, a 1.2-kb fragment containing the first two exons of the C_μ region (32). Probes were labeled with P^{32} (Amersham, Arlington Heights, IL) by random priming using the Prime-It kit (Stratagene). Hybridization was carried out in 7% SDS/0.5 M $NaHPO_4$, pH 7.2, and membranes were washed with decreasing SSC concentrations, with a final wash in 0.1% SDS/0.1 × SSC at 65°C. Autoradiographs were exposed on XAR film (Kodak, Rochester, NY) at −70°C for 1–3 d.

cDNA-PCR. Total RNA was extracted from homogenized lymph node specimens with a guanidinium isothiocynate method (33). First strand cDNA synthesis from 30 μgm of total RNA used AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) and a C_μ specific oligonucleotide primer (34) (see below). One tenth of this product was subjected to PCR amplification using synthetic oligonucleotide primers Leader and DJ or C_μ at final concentrations of 0.2 μM, and the buffer recommended by the manufacturer for its Amplitaq recombinant thermostable Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), except that the $MgCl_2$ concentration was 0.75 mM. Thirty cycles of amplification were carried out in a programmable thermocycler (Coy Laboratory Products, Ann Arbor, MI), with soak temperatures of 94°, 55°, and 72°C for 1 min each followed by 9 min at 72°C.

Cloning. PCR products were treated with Klenow fragment (Boehringer-Mannheim GmbH, Germany) (28), gel purified, recovered by electroelution, and treated with T4 polynucleotide kinase (Stratagene) in the presence of 10 μM ATP. Blunt end ligation into SMA-1 cut, phosphatase treated PBS M13− (Stratagene) was carried out with T4 DNA ligase (International Biotechnologies, Inc., New Haven, CT) at room temperature overnight. The entire ligation reaction was cloned in the XL-1 Blue strain of *E. coli* (Stratagene) by heat pulse of competent bacteria or by electroporation (BioRad Laboratories, Rockville, NY). Bacterial colonies were screened by blue/white selection and the presence of insert confirmed by restriction analysis of alkaline lysis plasmid DNA preparations (28).

Sequencing. Double stranded plasmid DNA was sequenced in both directions by the dideoxynucleotide termination method (35) using the Sequenase II kit (United States Biochemical Corp., Cleveland, OH). Sequences were analyzed using the DNA Inspector II (Textco, West Lebanon, NH) and the Wisconsin Analysis package (36).

Table 1. *Patient Material and Nucleotide Sequences for Longitudinal Analysis*

Timepoint	Specimen	Serology	Idiotype	Isolates	Sequencing technique
Diagnosis	Serum	IgM anti-Pr_2	+		
(S)	Spleen tissue	IgM anti-Pr_2	+	3	gDNA,PCR-LC,Leader-DJ
(EBV)	EBV Cell line	IgM anti-Pr_2	+	7	cDNA,cloning, $C\mu$
				1	gDNA,PCR-LC,Leader-DJ
1st remission	Granulocytes			4	gDNA,PCR-LC,Leader-Fr3
Year 3	Serum	IgM anti-Pr_2	+		
(N3)	Lymph node	IgM anti-Pr_2	+	9	cDNA,PCR-LC,Leader-DJ
				1	gDNA,PCR-LC,Leader-DJ
				1	cDNA,PCR-seq,Leader-DJ
Year 4	Serum	IgM anti-Pr_2	+		
(N4)	Lymph node	IgM anti-Pr_2	ND	8	cDNA,PCR-LC,Leader-$C\mu$
				1	gDNA,PCR-LC,Leader-DJ
3rd remission	Granulocytes			1	gDNA,PCR-LC,Leader-Fr-3

The serum and tissue specimens from each time point used in the longitudinal analysis of the lymphoma are shown, along with antibody specificity and the presence of the private idiotype. Granulocytes were prepared from peripheral blood obtained during disease remissions. The template sources and primers used to determine the V_H gene sequence at each time point are indicated, as are the number of independent bacterial isolates sequenced from each amplification-ligation-cloning procedure. The sequences from 7 independent EBV cell lines at the time of diagnosis were obtained from unamplified cDNA and were originally reported by Silberstein, et al. (23). *Abbreviations*: gDNA - genomic DNA; cDNA - complementary DNA; ND - not done; PCR-LC - PCR amplification-Ligation-Cloning procedure; PCR-seq - Direct sequencing of PCR product; cloning - cloning of unamplified $C\mu$ primer extended cDNA.

Oligonucleotide Hybridization. Four pairs of 17 mer oligonucleotide probes were chosen as described in the text. 10 pM of each probe was end-labeled with P^{32}-ATP and hybridized at 37°C overnight. Two pairs of PCR amplification primers were used to amplify V_H genes from each template. The Leader-DJ primer pair was shown by ethidium stained gels to amplify specifically the expressed V_H gene from the neoplastic clone, so amplification products of this primer pair served as controls for the specificity of hybridization conditions. The Leader-Fr3 primer pair amplified bands of the appropriate size by ethidium stained gel from all templates, so these amplification products served to include related germline genes in the material to be probed with the 8 oligonucleotide probes. Genomic DNA from each lymphoma specimen, from the patient's granulocyte DNA diluted 1:10 or 1:30, and from normal, unrelated lymphocyte DNA, was subjected to 30 cycles of PCR amplification. These dilutions of the patient's granulocyte DNA showed no Leader-DJ amplified band on ethidium stained gel, while undiluted granulocyte DNA showed a faint band, indicating a small amount of contamination with DNA from the lymphoma. The amplification products were denatured in 0.3 M NaOH for 5 min, neutralized with 1.2 M Tris pH 7.0, and the equivalent of 10% of the original product was dotted under vacuum onto 8 replicate Duralon strips. The strips were probed in parallel with the 8 oligonucleotide probes in 6 × SSC, 40 mM NaH_2PO_4, 1% SDS, and 100 μgm/ml salmon sperm DNA (Sigma Chemical Co., St. Louis, MO) and washed in 6 × SSC/0.1% SDS at increasing temperature for increasing stringency. Autoradiographic exposures after each wash ranged from 15 min to 2 d. For each probe individually, the final washing temperature and duration of autoradiographic exposure were chosen to optimize the distinction between control positives and control negatives.

Oligonucleotides. Oligonucleotides were synthesized by automated phosphotriester chemistry (Applied Bioscience Inc., Foster City, CA), diluted to stock concentrations and used without further purification. The sequences were as follows: *Leader*: 5′ ATGGACTGGACCTGGAGCATC 3′; *DJ*: 5′ GCCCAGTAGTCATCTCCTCT 3′; *Fr3*: 5′ CGGCCGTGTCGTCAGATCTTA 3′; C_μ: 5′CAGGAGACGAGGGGGAA 3′; *A-germline*: 5′ CCCACTCCCAGGTTC 3′; *A-mutant*: 5′ CCCACTCTCAGGTTC 3′; *B-germline*: 5′ AGAGAAACCAGGCCA 3′; *B-mutant*: 5′ AGAGAAAGCAGGCCA 3′; *C-germline*: 5′ GTGAGACTTCACCCA 3′; *C-mutant*: 5′ GTGAGACCTCACCCA 3′; *D-germline*: 5′ TCCTGTGTCCTCTCC 3′; *D-mutant*: 5′ TCCTGTGCCCTCTCC 3′.

Statistics. The probability model for detection of positive or negative selection from patterns of replacement and silent mutations within variable region genes is described by Shlomchik et al. (37). The binomial distribution is used to model random mutation, and to calculate the probability (p value) that an observed distribution of mutations occurred by chance. The assumptions underlying this model have been validated experimentally (37).

Results

Serial Samples: S, N3, N4

The materials available for comparative studies in this patient are described in Table 1. The lymphoma sample obtained from the spleen at the time of diagnosis will be referred to as 'S', the sample from the first lymph node relapse 3 yr after diagnosis will be referred to as 'N3', and the sample from

the second lymph node relapse 4 yr after diagnosis will be referred to as 'N4'.

Histology of Serial Samples

The histologic appearance of the lymphoma changed from S to N3. At diagnosis, the lymphoma was composed mostly of small, uniform cells, and by Rappaport classification (38) was a diffuse, well differentiated lymphoma, described as plasmacytoid. In contrast, at N3 the histologic classification was diffuse lymphoma, of mixed cell type, with small cells and large cleaved cells, many of which resemble histiocytes. The N4 histology was diffuse, poorly differentiated lymphoma. The histologic features of N3 and N4 are generally correlated with more aggressive malignant behavior and a worse prognosis (38, 39). Some studies have suggested that finding progressive histologic changes in a lymphoid neoplasm, as in Richter's Syndrome (17, 40, 41), may indicate the emergence of new, distinct neoplastic clones, although the molecular evidence on which that proposal was based did not include analysis of clonality by nucleotide sequencing. On the other hand, one study of histologic change from follicular lymphoma to diffuse lymphoma, in which one tumor was examined by sequence analysis, concluded that the diffuse lymphoma was clonally related to the follicular lymphoma (18).

Conserved Specificity and Idiotype in Serial Samples

Serology. At the times of S, N3, and N4, the patient suffered exacerbations of autoimmune hemolytic anemia, with a fall in hemoglobin, and elevation of the reticulocyte count. As shown in Table 1, the serum at each time point demonstrated a monoclonal IgM cold agglutinin with anti-Pr_2 specificity. During the remissions between time points S, N3, and N4, the serum cold agglutinin titer was several fold lower than at diagnosis or relapse, and the hemoglobin level and reticulocyte counts returned to normal.

Anti-Idiotype Analysis. A murine monoclonal antiidiotype antibody identifies, by an ELISA assay, a private idiotype on this patient's cold agglutinin. The antiidiotype antibody does not crossreact with other human paraproteins including those with similar anti-Pr_2 specificity (25). As shown in Table 1, sera obtained at the times of N3 and N4 remain reactive with this antiidiotype antibody. Similarly, the presence of the idiotype on the S and N3 cell surfaces is demonstrated by two color flow cytometry (Fig. 1) using allophycocyanin labelled antiidiotype antibody and suspensions of lymphoma cells. Two-color analysis using antiidiotype versus anti-human IgM (not shown) revealed a direct linear correlation at both time points between idiotype and IgM expression on the cell surface. These observations indicate that both the antigen specificity and the unique idiotype have been conserved through the evolution of the lymphoma.

Clonal Relatedness of Serial Samples

Genomic DNA extracted from S, the EBV transformed cell line, N3 and N4 was digested with Eco R1, Hind III, Bam H1, or Xba 1, electrophoresed, and transferred to nylon membranes. The blots were hybridized with probes for the joining region of the heavy chain, J_H, or the joining region of the light chain, J_κ, or the IgM constant region, C_μ. Both heavy chain alleles in this tumor are rearranged. In the Hind III (Fig. 2 *b*) and Xba 1 (not shown) digests, the J_H hybridizing restriction fragments are of the same size in all samples. The light chain rearrangement pattern with the J_κ probe is also the same at all time points (not shown). However, in the Eco R1 (Fig. 2 *a*) and the Bam H1 (not shown) digests, one of the bands shifts to a smaller restriction fragment in N3, and the other band apparently shifts to a smaller fragment in N4. Finally, there is a less intense, third J_H hybridizing band in the Eco R1 digested DNA of the S sample, running at the same size as the 7.2-kb band in the N3 DNA. (Fig. 2 *a*, lane *3*).

Because it demonstrates that the three lymphoma specimens share rearranged bands, the Southern analysis provides strong evidence that the specimens are clonally related. The Hind III and Xba 1 digests show the same pattern at all time points, and in the Eco R1 digest, S and N3 share the 9.7-kb band, and N3 and N4 share the 7.2-kb band. Sequence analysis of the expressed D regions (see Fig. 3 and results below) confirmed, on the basis of shared unique sequences, that S, N3 and N4 are clonally related. To explain the different restriction patterns in the Eco R1 and Bam H1 digests, we hypothesized that the neoplastic clone had undergone genetic changes which gave rise to distinguishable subpopulations within the lymphoma, as has been reported in some follicular lymphomas (42). A different subpopulation was predominant at each time point, and the N3 subpopulation was de-

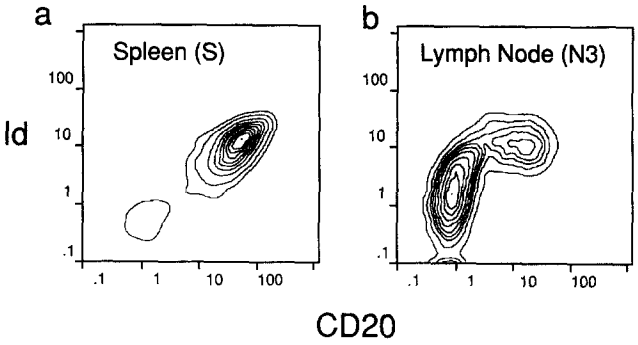

Figure 1. Continued cell surface expression of a private idiotype. Surface expression of a private idiotype (Id) on the spleen (S) and year 3 node (N3) tissues was demonstrated by two color flow cytometry. The log fluorescence intensity of antiidiotype antibody coupled to allophycocyanin is plotted on the vertical axis, and of monoclonal anti-human CD20 (Coulter B1, a pan B cell marker) coupled to fluorescein on the horizontal axis. Contour lines represent 5% increments in cell number. Fig. 1 *a* shows a single predominant cell population which is Id^+ and $CD20^+$, indicating that the spleen specimen is composed almost entirely of idiotype bearing B cells. Fig. 1 *b* shows two populations of cells. One population is Id- and CD20-, and probably represents background T cells in the lymph node. The second population is Id^+ and $CD20^+$, and represents recurrent lymphoma in the node.

[1] *Abbreviations used in this paper:* N3, N4, lymph node relapses 3 (yr) and 4 (yr), respectively; S, splenic lymphoma.

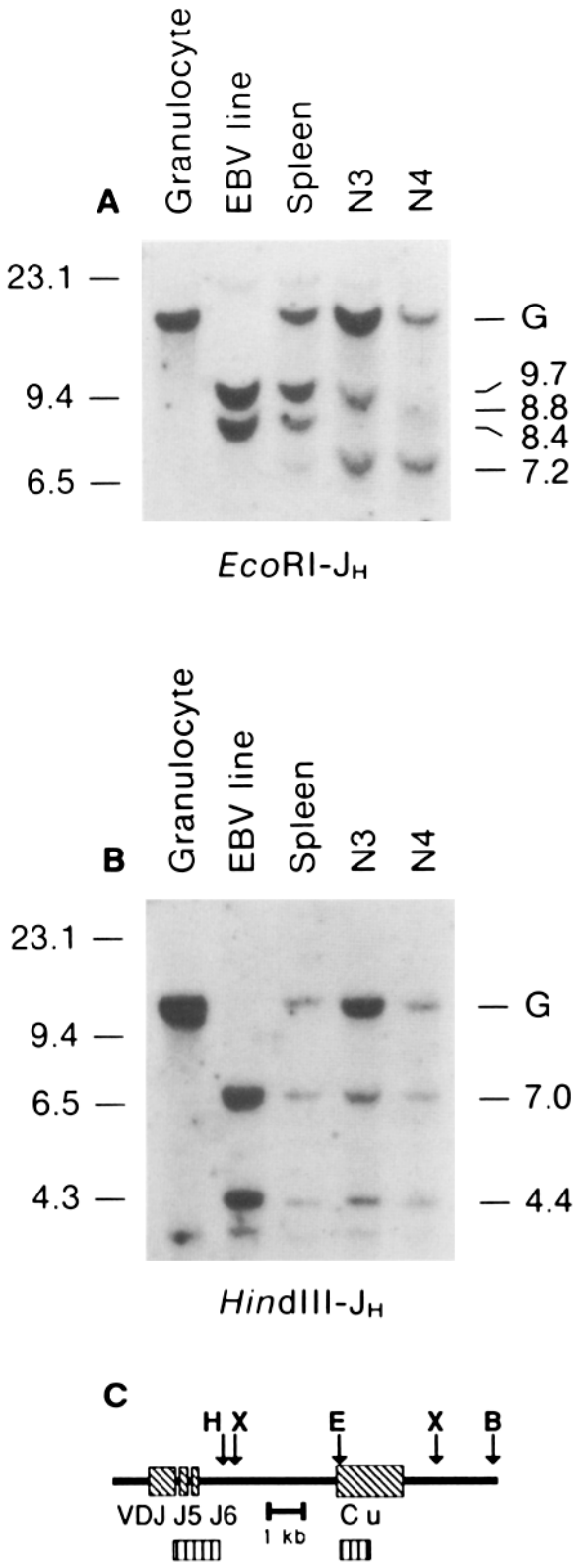

Figure 2. Immunoglobulin gene rearrangements in serial lymphoma samples. Hybridization of the J_H probe with restricted DNA from the serial lymphoma samples is shown, using Eco R1 in Fig. 2 *A*, and Hind III in 2 *B*. From left to right, the lanes contain autologous granulocyte DNA, EBV cell line DNA, spleen DNA, N3 DNA, and N4 DNA. Size markers are indicated on the left, and the size of each rearranged band is shown on the right. The position of the germline band for each enzyme is indicated as G. Both the Eco R1 (2 A) and Hind III (2 *B*) digests demonstrate two rearranged heavy chain alleles. The bands representing the rearranged Ig genes shift to smaller sizes over time in the Eco R1 digest, but remain at the same size in all specimens in the Hind III digest. The faint bands present in all lanes are crossing reacting bands. The 8.8-kb band in Eco R1 digested N4 DNA was diffuse in numerous repetitions of the experiment. Fig. 2 *C* is a map of a rearranged human heavy chain locus showing the positions of the restriction sites which would define the 3′ end of the J_H hybridizing fragment. Boxes with diagonal hatching represent coding regions, and boxes with vertical hatching represent the probes. H - Hind III; B - Bam H1; X - Xba 1; E - Eco R1.

tectable by Southern analysis at diagnosis as the faint third band visible in the Eco R1 digest of S DNA. The J_H hybridization pattern observed in the Eco R1 digest in N4 may represent another subpopulation derived from the population most prevalent in N3.

V_H Gene Sequence Diversity in Serial Samples

Consensus V_H Sequences. The materials, primers, and techniques used to determine the nucleotide sequence of the expressed V_H gene at different stages in the evolution of the lymphoma are summarized in Table 1, and the sequences are listed in Fig. 3. By consensus sequence, we mean the sequence sharing the most homology with all the sequences determined from one time point, for example, the consensus among the 9 cDNA and 1 genomic DNA sequences determined at N3. Each consensus sequence was obtained from at least two separate PCR amplifications, and from 7 or more independent bacterial isolates (see Table 1) with little variation among those isolates (see Fig. 4). The two major different clonotypes, S and N3/N4, have each been sequenced independently from two separate biologic sources-the S clonotype from the EBV lines and directly from splenic tissue, and the nodal clonotype from the two lymph nodes biopsied a year apart. Finally, both major clonotypes were obtained once by a method which did not rely on the clone-specific DJ primer. Therefore, the consensus sequences presented in Fig. 3 represent the predominant V_H clonotypes present at each time point.

The V_H gene sequences from the S sample and from one of the EBV cell lines were confirmed by the PCR based method used for all the sequences reported here, and were identical to the previously published sequence from the EBV lines except at the third base in codon 12, which represents a typographical error in the published sequences (23) (p. 1635). S and N3 differ by 1 nucleotide in the leader (codon −1), 3 within the leader-VH intron, and 10 within the V_H gene. N4 differs from S by the same 14 nucleotides, and N4 has 2 additional nucleotide changes in the V_H gene. When the sequence at each time point is compared with the germline sequence (VH1GRR, discussed below), 6 nucleotide changes are shared in all expressed sequences, 3 are found only in S and the EBV lines, 11 are found in N3 and N4, and two additional changes are found in N4 only. The nucleotide changes which would result in amino acid changes are indicated on Fig. 3; there are six codons with amino acid changes, and codon 6 has two nucleotide changes each of which would result in an amino acid change.

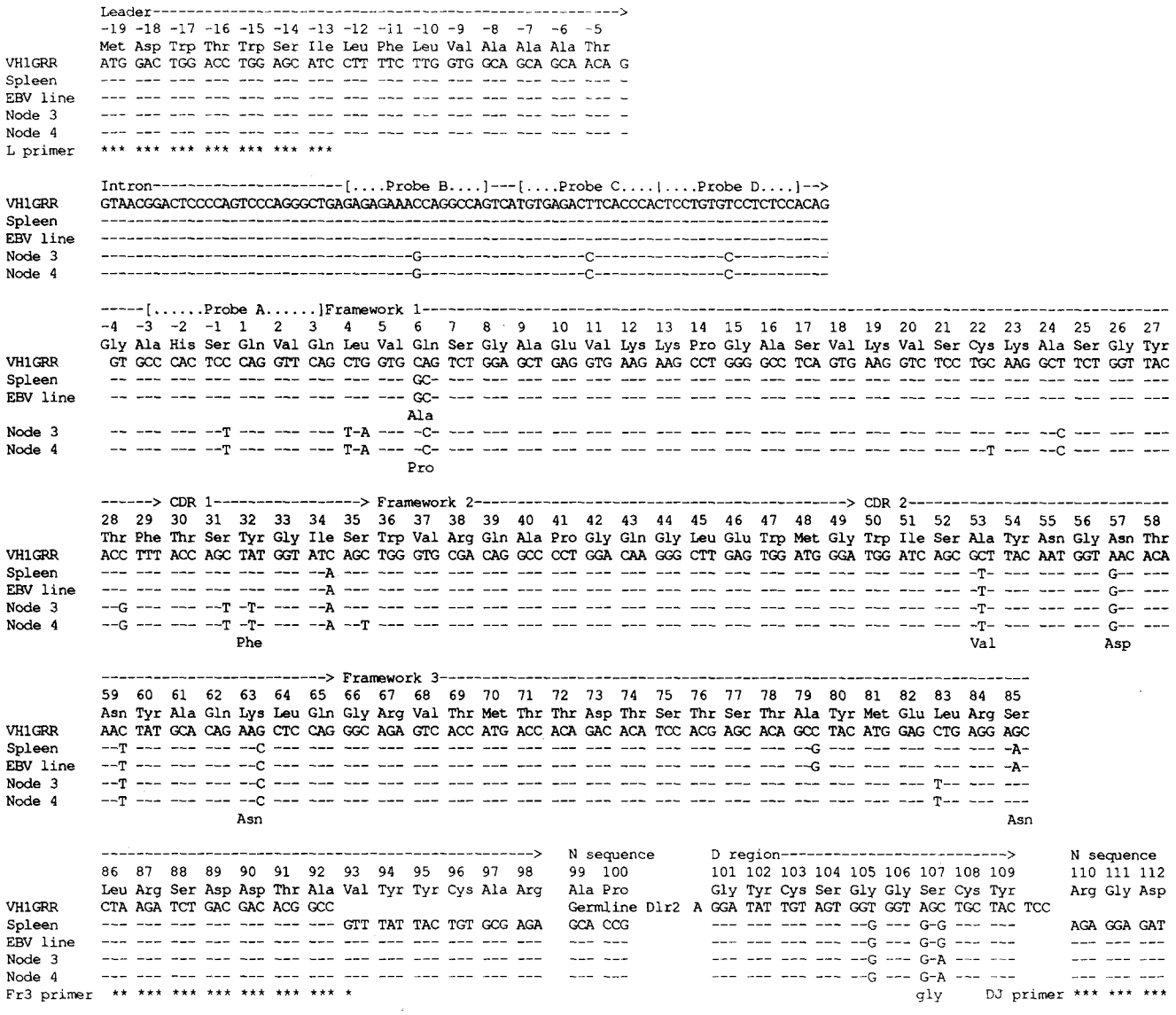

Figure 3. Consensus V_H region sequences from serial samples. The nucleotide and predicted amino acid sequences for the putative germline V_H gene, VH1GRR, the lymphoma at diagnosis (S), the EBV transformed line, and the two lymph nodes 3 and 4 yr after diagnosis (N3 and N4) are shown. Each sequence represents the consensus of all isolates obtained from that time point. Dashes indicate identity with the germline nucleotide sequence, and differences from germline are indicated. The predicted amino acid sequence of the germline gene is shown, and substituted amino acids are shown below each altered codon; silent nucleotide changes have no amino acid indicated. The positions of the Leader (L), Framework 3 (Fr3), and a portion of the DJ amplification primers are indicated with stars. The positions of the 17-mer oligonucleotide probes A,B,C, and D used in hybridization experiments are indicated above the sequence. On the bottom line, the germline Dlr2 sequence is shown as the possible germline counterpart of the expressed D regions. Nucleotides lacking templates in VH1GRR, Dlr2, or J4 are shown as N sequences, and differences between the expressed D regions and Dlr2 are indicated. One nucleotide from the 5′ end and three from the 3′ end of the Dlr2 gene have no apparent counterpart in the expressed genes. The codon numbering corresponds to the previously published sequences (23); the framework 1 region begins with codon 1. These sequence data are available from EMBL/GenBank/DDBJ under the accession numbers X60503, X60504, and X60505.

D Region. The D region from this lymphoma appears most homologous to the Dlr2 sequence from Ichihara et al. (43) as shown in Fig. 3. There are two nucleotide differences from the germline Dlr2 sequence which are common to all the expressed D regions. One of these shared differences results in a serine to glycine change in codon 107, and the other, in codon 105, is silent. The serial samples differ at only one nucleotide in the D region; the S sample and the EBV lines have guanine as the third base of codon 107, while N3 and N4 have adenine. The Dlr2 sequence has cytosine at this position, but codon 107 encodes glycine in all expressed samples.

Sequence Heterogeneity at Each Time Point. To assess the microheterogeneity of nucleotide sequence within the neoplastic clone at each time point, we analyzed several bacterial isolates from each amplification-ligation-cloning procedure. The degree of sequence variation among the sampled isolates reflects the ongoing frequency of nucleotide changes within the tumor population (19). An analogous approach was taken

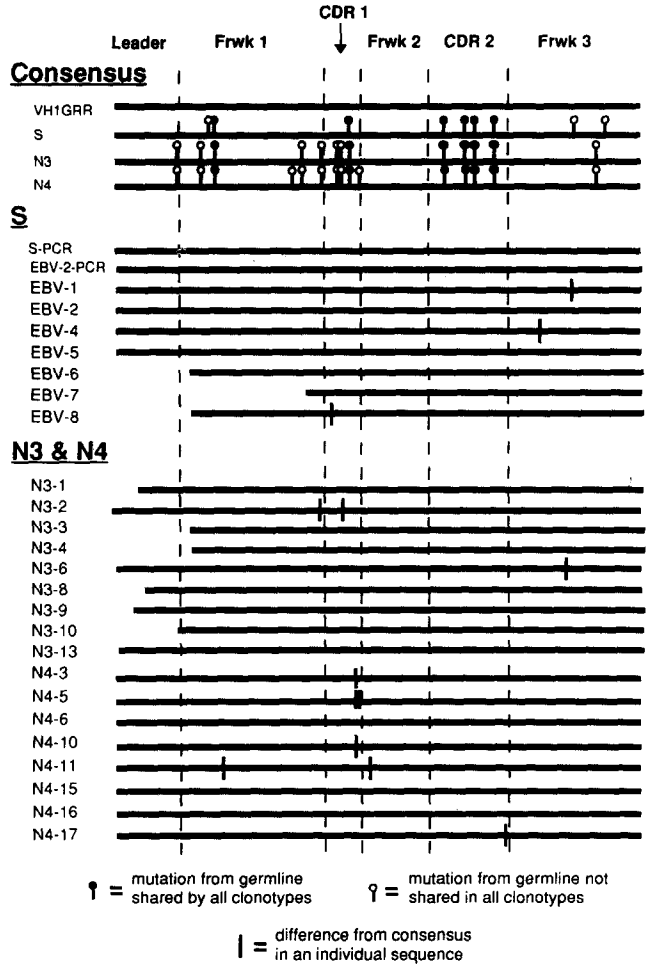

Figure 4. V region diversification and sequence microheterogeneity in S, N3, and N samples. Each sequence is represented as a horizontal line. The top section of the figure shows a comparison of the germline sequence (VH1GRR) with the consensus sequences from each time point (S, N3, and N4). Closed lollipop symbols represent differences from the germline which are shared in all sequences. Open lollipop symbols represent differences from germline which are found only in one clonotype. Below, individual sequences from independent isolates are represented as horizontal lines grouped by the two major clonotypes, S and N3-N4. Within each group, only nucleotide differences from the consensus sequence at that time are shown as vertical bars. The 7 EBV line sequences were previously reported (23), and the S-PCR and EBV2-PCR sequences represent repetitions of those sequences by the PCR based method.

in analyzing sequence heterogeneity at diagnosis (23), by comparing seven independent EBV transformed lines established from the spleen sample.

Fig. 4 shows a schematic representation of the consensus sequences (top 4 lines), of the seven individual heavy chain sequences determined at diagnosis (23), and the additional individual sequences at S, N3 and N4 reported here. A small degree of sequence microheterogeneity is apparent at each time point: 3 bases in S, 3 bases in N3, and 5 bases in N4. Some of this sequence variation may be attributable to infidelity of Taq polymerase, which has been reported to result in an error rate of 1/5000 (44) to 1/9000 (45) errors per base polymerized. Under the assumption that the error rate from Taq polymerase infidelity is as high as 1 error/5000 bases, the expected number of errors that would occur during 30 cycles of amplification of a 450 base sequence is 2.7 errors per amplification procedure. The error rate observed in our laboratory may be lower; we have seen no errors in at least 5,000 bases amplified and sequenced from the expressed V_H genes of EBV transformed clones from this and other patients. Finally, sequence variation was observed at diagnosis (Fig. 4, EBV lines) by a method not dependent on Taq polymerase. Thus, we conclude that a small degree of sequence variation is present within each sample, and that Taq infidelity may account for a few base differences, but does not account for all the observed heterogeneity.

Intermediate Sequences. In addition to the small degree of heterogeneity illustrated in Fig. 4, we found clonotypic sequences which appear to represent intermediate steps between the germline and S, and between the N3 and N4 consensus sequences; we will refer to these as intermediate sequences. First, two sequences from genomic DNA from S (not depicted) were identical to the S and EBV consensus sequences except in codons 79 and 85, which were, respectively, GCC and AGC, the same as the germline gene VH1GRR, instead of GCG and AAC as in the S and EBV consensus sequences. These two sequences may therefore represent an intermediate step between the germline and the consensus S sequences. Second, three of the eight N4 sequences (N4-3, N4-5, and N4-10) have cytosine as the third base in codon 35 while the remaining five N4 sequences have thymine. Since cytosine is found at this position in N3, these three N4 sequences may represent an intermediate step between N3 and N4. The finding of intermediate sequences suggests that the nucleotide changes occurred sequentially rather than as one event.

Determination of the Putative Germline Gene Sequence

The nucleotide sequence of the putative germline gene from which the expressed V_H gene in this tumor is derived is shown as the top line of Fig. 3. The gene was not represented in the EMBL/Genbank databases, and we will refer to this sequence as VH1GRR.

Approach. Previous approaches to the isolation of germline precursor V_H genes have involved screening genomic libraries with a cDNA probe derived from the rearranged gene to obtain a set of candidate germline genes, which were then sequenced (46). In our PCR based method, an analogous set of candidate V_H genes was obtained by amplifying germline genomic DNA with the Leader-Fr3 primer pair (indicated on Fig. 3), which was chosen from the sequences of the rearranged genes. This set was then sampled by sequencing several bacterial isolates. For both approaches, the choice of the most likely germline precursor of the rearranged V_H gene is based on two criteria: the degree of homology between the germline and the expressed genes, and the repeated occurrence of the germline gene in the set of candidates. The potential advantage of the PCR based method lies in the ability to define a more limited set of candidate genes by careful choice of amplification primers. While the assignment of a precursor germline gene can never be made with complete certainty,

several independent lines of argument strongly support the assignment of VH1GRR as the germline precursor in this system.

Choice of Primers. The Leader primer was chosen because it is specific for the V_{H1} family (47) and because it may have a polymorphism near its 3′ end which might further limit its scope of amplification (our unpublished observation). The Fr3 primer was chosen from a portion of the V_H framework 3 common to all of the expressed clonotypes, but as different as possible from other published V_{H1} germline genes (47–49). We anticipated that this primer pair would amplify a limited set of V_{H1} genes which would be closely related to the expressed genes; an appropriate size product from unrelated genomic DNA as well as from autologous granulocyte DNA was observed on ethidium stained gel (not shown).

Repeated Isolation. We performed separate amplification-ligation-cloning procedures on two specimens of the patient's granulocyte DNA obtained 2 yr apart and sequenced a total of 5 isolates from the two transformations. Repeating the amplification-ligation-cloning procedure and forming a consensus sequence among several isolates minimizes the chance that sequence errors introduced during the amplification with Taq polymerase or during the cloning procedure would be interpreted as actual genomic sequence. Of the 5 isolates from the two amplifications, three were identical and two had single bases differences from the others, so that at those two positions, 4/5 isolates were identical and one discordant. These two bases may represent Taq infidelity or, alternatively, there may be two or more nearly identical copies of this germline gene in the genome.

Homology. An unrestricted computerized search of the GenBank/EMBL databases using the FASTA algorithm of the Wisconsin Sequence Analysis package (36) demonstrated that VH1GRR is more homologous to the rearranged gene sequence than any other published gene. The homology between VH1GRR and the rearranged genes from S and N3 is 97% and 94% respectively, counting only the coding regions (including the Leader-V intron would increase the percent homology). The closest match in the databases was a rearranged V_{H1} gene (50) with 95% homology, and the closest V_{H1} germline gene (51) was less than 90% homologous.

Because VH1GRR was isolated from the candidate set 5/5 times, and because VH1GRR has the highest homology of any known V_{H1} gene to the rearranged genes of this clone, we are confident in assigning it as the germline precursor of the expressed rearranged genes.

Oligonucleotide Hybridization

We designed a hybridization experiment with clonotype-specific oligonucleotide probes to seek independent support for two of the important findings described above: first, that the N3 clonotype was present in the spleen sample, and second that VH1GRR is a germline gene. Genomic DNA from S, the EBV line, N3, the patient's granulocytes, and unrelated germline DNA was amplified with either the Leader-DJ or the Leader-Fr3 primer pairs and dotted onto nylon membranes in replicate strips. The strips were hybridized with 17-mer oligonucleotide probes which corresponded to regions of the V_H gene where the germline and N3 sequences differed by one base. The probes were synthesized in pairs, one germline probe identical to VH1GRR (and to S) and one mutant probe identical to the same region of N3 and differing from the germline probe only at the central nucleotide. The positions of the four oligonucleotide probe pairs, A through D, are indicated on Fig. 3.

The hybridization results are shown in Fig. 5. The presence of mutant N3 sequences in the spleen DNA was demonstrated by probes B_{mutant} and D_{mutant} which show faint hybridization to the amplified S DNA under conditions where the EBV line DNA is negative. These findings corroborate the interpretation of the Southern analysis, where the J_H restriction pattern of Eco R1 digested S DNA (Fig. 2 *a*) suggested that the N3 population was present as a minor population in the spleen.

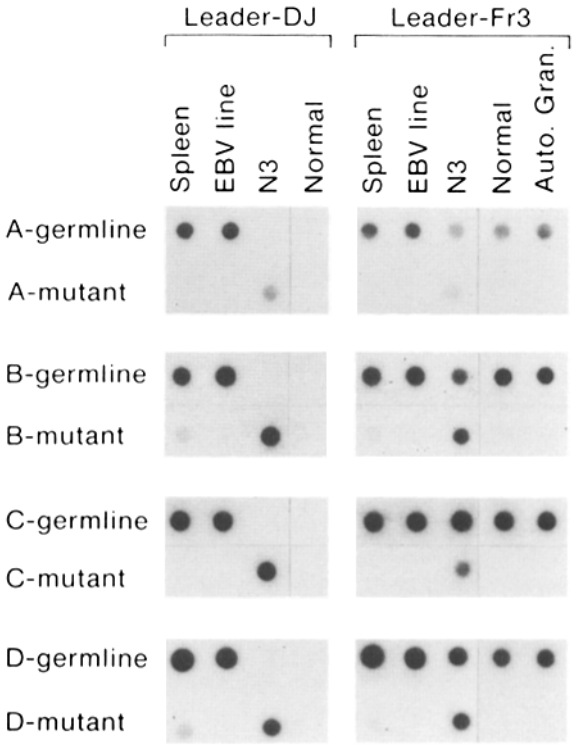

Figure 5. Oligonucleotide hybridization experiments. The Leader-DJ or Leader-Fr3 primer pairs were used to amplify V_H genes from genomic DNA templates from the sources shown, and replicate strips dotted with equal amounts of each amplification product were hybridized with the germline or mutant version of each oligonucleotide probe. For each of 4 probe pairs, the germline and mutant versions differed only at the central nucleotide. The EBV line DNA, N3, and normal DNA amplified with Leader-DJ served as the controls by which the optimal washing temperatures and exposure durations to demonstrate the single base differences were chosen. All germline probes hybridized with amplification products which contain either germline V_H genes or the expressed S V_H gene. All mutant probes hybridized only with amplification products which contain the expressed N3 V_H gene. Probes B and D demonstrate the presence of mutant N3 sequences in the spleen sample amplified with both primer pairs, while probes A and C do not.

The hybridization experiment also demonstrates that all 4 germline probes and none of the mutant probes hybridize strongly with amplification products of germline DNA from autologous granulocytes and from unrelated lymphocytes. This finding confirms that at least the 67 bases of VH1GRR spanned by the 4 probes are present in germline DNA.

Mechanism of V_H Gene Change Between the Serial Samples

Two molecular changes at the Ig locus have been observed between the serial samples: restriction fragment size reductions, and nucleotide changes. To investigate the possibility of DNA deletions that might explain the size reduction in J_H hybridizing Eco R1 and Bam H1 restriction fragments, the Xba 1 digested DNAs were probed with the C_μ probe (see map, Fig. 2 *C*). The same size C_μ hybridizing fragment was observed in all specimens (not shown), eliminating the possibility of deletions 3′ of the J region. Deletion of DNA 5′ of the VDJ between the 5′ Eco R1 site and the 5′ Hind III site remains a likely mechanism to explain the changes on Southern blot because such a deletion could alter the restriction pattern in Eco R1 and Bam H1 digests but not in Hind III or Xba 1 digests, as observed. Alternatively, the restriction fragment length changes could be due to point mutations creating new Eco R1 and Bam H1 sites.

The mechanism of the nucleotide changes between the germline and expressed V_H genes is most likely point mutation. Sequential accumulation of nucleotide changes is characteristic of somatic point mutation, and several observations indicate that the nucleotide changes from the germline to S and N3 indeed occurred sequentially. First, the existence of intermediates between the germline and S, and between N3 and N4 suggests a sequential process. Second, in the oligonucleotide hybridization experiment, there is discordance in the hybridization of mutant probes B and D versus probes A and C to S DNA amplified with either Leader-DJ or Leader-Fr3 under conditions where the EBV cell line DNA is negative with all four probes (Fig. 5). A hybridization signal is detectable with B_{mutant} and D_{mutant}, consistent with the presence of the N3 population as a minor constituent of the spleen specimen, as discussed earlier. No such signal is seen in the corresponding dots for A_{mutant} or C_{mutant}. This discordance suggests that the B and D mutations may have occurred before the splenectomy and the A and C mutations after the splenectomy. The independent and sequential nature of the changes observed here is most consistent with the mechanism of point mutation, although the theoretic possibility of multiple, overlapping gene conversion events (52, 53) cannot entirely be ruled out.

Discussion

We have made serial observations of histology, specificity, idiotype, V_H gene rearrangement, and V_H gene nucleotide sequence in a human B cell lymphoma associated with autoimmune hemolytic anemia. In addition, we have determined the sequence of the putative germline precursor of the V_H gene expressed by this neoplasm. Comparison of the results over time demonstrates conservation of Ig structure at the serologic level, but considerable diversity among clonally related neoplastic subpopulations at the nucleotide level. Based on these findings, we propose a model for the genesis and evolution of this lymphoma, expressed schematically in Fig. 6 and 7. Fig. 6 shows the early clonal expansion and intraclonal divergence in relation to clinical events such as diagnosis, relapse, and treatment. Fig. 7 shows the genealogic pattern of nucleotide sequence divergence within the neoplastic clone. This model incorporates two key observations from the analysis of this case. First, genetic diversity was generated early in the evolution of the neoplasm, and second, that the pattern of nucleotide changes is consistent with antigen mediated selection.

Divergent Populations Within a Single Clone

The Southern blot and sequence data demonstrate that this lymphoma is a monoclonal B cell expansion. Within the neoplastic clone, we have detected two major divergent subpopulations or clonotypes, S and N3, one predominant in the spleen specimen, and one predominant in the lymph nodes but detectable in the spleen. The N4 clonotype has further diverged from N3. The presence of two intermediate clonotypes, between germline and S, and between N3 and N4, and the presence of sequence heterogeneity at each time point suggests that other minor clonotypes may exist within the clone as well. Fig. 6 shows our concept of how divergent clonotypes emerged. A single ancestor gave rise to an expanded B cell population that underwent genetic divergence, which we observe as the base changes shared by all clonotypes. One member of this population was transformed and subsequently continued to expand and diverge, giving rise to the genetic differences between the clonotypes. Alternatively, it is possible that the transforming event occurred before the observed genetic divergence, and that selection acting on the neoplastic clone is responsible for the presence of shared mutations. Ultimately, subpopulations with distinct clonotypes appeared as clinically detectable lymphoma, first in the spleen and later in the lymph nodes. This model of clonal divergence invokes a single step transforming event; however, a multistep transformation process involving more than one subclone cannot be ruled out.

In Fig. 6, the line indicating the splenectomy extends through the top portion of the N3 clonotype, representing the fact that N3 was detectable in the spleen specimen by Southern analysis and oligonucleotide hybridization. This finding along with the sequence data shows that the N3 clonotype was not derived from the S clonotype by accumulation of additional changes, but rather, the two clonotypes share a common ancestor. From this, we draw the conclusion that the observed genetic diversity arose early in the evolution of this clone.

In contrast, the minimal degree of sequence heterogeneity (Fig. 4) within each sample (S, N3, N4) suggests that the frequency of ongoing point mutation at each time point is low. The degree of heterogeneity is less than that observed

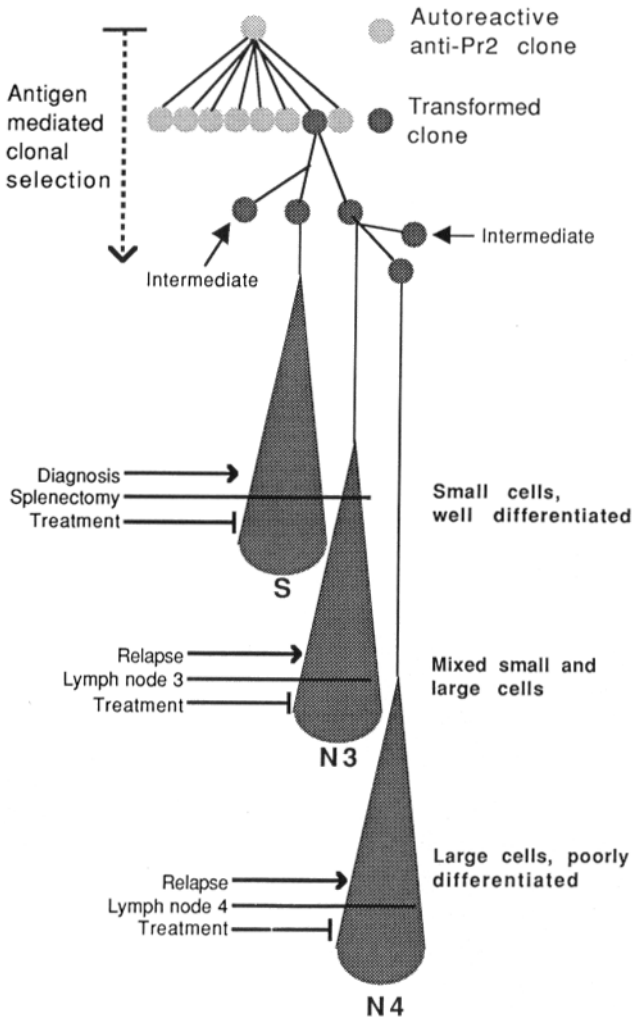

Figure 6. Emergence of subpopulations in relation to diagnosis, sampling and histology. The proposed model for the emergence of divergent subpopulations in an autospecific B cell lymphoma begins with an expended autoreactive B cell clone, represented as lightly stippled circles. Malignant transformation, represented as heavy stippling, occurs in one cell in this clone. The width of the shaded areas represents the relative mass of a subpopulation, and horizontal lines represent specific events, such as clinical detection at diagnosis, and sampling at splenectomy. Diversification by somatic mutation occurs in the V_H region before transformation and continues after transformation, and concurrently, antigen may mediate positive and negative selection. The sequence divergence of the major clonotypes, S and N3, occurs temporally well before any clinical manifestation of lymphoma or cold agglutinin disease. The relative mass of cells with the predominant clonotype is represented as the heavily stippled region, which enlarges until the tumor becomes clinically apparent, and becomes smaller after treatment. The histologic description of each sample is indicated. N3 becomes apparent as a relapse three years after the presentation of S at diagnosis, but N3 does not arise directly from S. The N4 clonotype arises from N3 because it shares all the mutations of N3, but the divergence of N4 from N3 cannot be placed in a precise time frame based on the data available. Finally, the two intermediate clonotypes detected by sequencing are shown as darkly stippled circles.

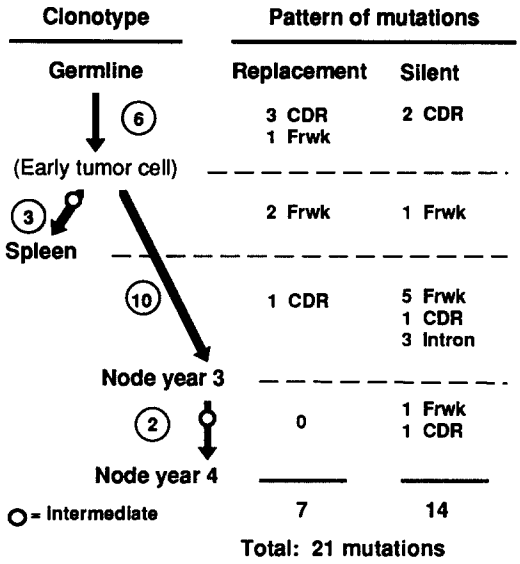

Figure 7. Divergent clonotypes: the pattern of V_H gene mutations. The V_H clonotypes detected in longitudinal analysis are arranged in a geneologic tree based upon shared and unshared mutations, beginning with the VH1GRR sequence as the germline. On the right, the distribution of mutations between CDR and Framework, and between Replacement and Silent, are shown. The numbers in circles are the total number of mutations which occurred between each clonotype. Six mutations are shared in all the clonotypes, and 4/6 result in amino acid replacements of which 3 occurred within CDR regions. Three mutations occur in S only, which is therefore the closest to germline of the expressed V_H genes. In contrast, 12 mutations different from those in S are present in the N3 and N4. Of these 12, 8 are silent, 3 occur in the Leader-V_H intron, and 1 results in a conservative tyrosine to phenylalanine change in codon 32 of CDR1. Comparison of the expressed D regions with Dlr2 demonstrated a similar pattern; of the shared D region mutations, one results in an amino acid replacement and one is silent, while the mutations unique to S or N3 are both silent.

by Cleary (19) in follicular lymphomas, and less than that observed in secondary immune responses in mice (12) where the rate was estimated to be 10^{-3} mutations per basepair per division. This high rate has been termed "somatic hypermutation". The mutation frequency ongoing in each sample of this malignant clone is not typical of somatic hypermutation, and therefore would not account for the observed genetic diversity.

In indolent B cell lymphomas such as this, remission is easily induced by mild, nonablative chemotherapy, but the tumor generally recurs (54). In the case presented here, the lymphoma had different histologic appearances at diagnosis and recurrence, and genetic analysis demonstrated the emergence of new subpopulations representing divergent clonotypes. The analysis also suggests that these clonotypes were present early in the evolution of the clone, and were not the result of genetic changes accumulated during later stages of tumor progression, as has been demonstrated for chromosomal changes in many hematopoietic malignancies (55). The histologic and clonotypic changes are two manifestations of heterogeneity within the malignant clone, but cannot be connected in a simple way. Numerous factors unrelated to Ig function may contribute to the emergence of such new malig-

nant subpopulations. For instance, heterogeneity of expression of a molecule responsible for drug resistance may permit a small subpopulation to survive chemotherapy which eliminated the majority of the malignant clone (56, 57). Intraclonal heterogeneity in mitotic rate might also result in differential susceptibility to chemotherapy. From the data presented here, we can draw the conclusion that the observed histologic changes and shift in banding pattern on Southern analysis are due to the emergence of a divergent subpopulation of the original malignant clone rather than a second, new malignant clone, as has been suggested in some studies of B cell neoplasms (17, 40). In this diffuse lymphoma, as in one previously reported case of follicular lymphoma (18), only sequence analysis provided definitive evidence of clonality.

Antigen Driven Clonal Selection

We advanced the hypothesis that this lymphoma arose from a preexisting autoreactive B cell clone which had been expanded under the influence of the autoantigen, and furthermore, that the malignant clone continued to be influenced by autoantigen after transformation. To support this pathogenetic model, we sought evidence of antigen mediated clonal selection by applying the probability analysis of (37) to the observed pattern of nucleotide mutations in the divergent subpopulations of this lymphoma. Each base difference between VH1GRR and the expressed V_H genes was characterized as causing an amino acid replacement or as silent, and as occurring in a CDR region or a framework region, and these results are shown in Fig. 7.

Because N4 is the most divergent clonotype, analysis of N4 for evidence of clonal selection should reflect selective forces acting over the entire evolution of the clone. Although the fraction of replacement mutations expected at random is about 0.75 (58), N4 contains more silent than replacement mutations. When the probability model is applied to the pattern of mutations in N4, the null hypothesis, that the distribution of mutations in N4 occurred by chance, is rejected with a confidence of $p < 0.003$. There are significantly fewer replacement mutations than would be expected at random. This result suggests that clonal selection against replacement mutations, or "negative selection", has influenced the pattern of divergence in this neoplastic clone. By "negative selection" we mean selection against mutations which would disrupt Ig structure, reduce affinity for antigen, or result in loss of specificity or idiotype. The pattern of mutations in N4 suggests that clonal selection against replacement mutations is a plausible explanation for the observed conservation of specificity and idiotype in this neoplasm.

In principle, both positive and negative selection may occur during the evolution of an immune response, both contributing to the development of high affinity and narrow specificity. "Positive selection", selection in favor of mutations which might increase affinity for antigen, can be inferred when there is an excess of replacement mutations in CDR regions. Of the shared mutations which occurred in the early expansion of the clone, 4/6 are replacement mutations of which 3/6 occur within CDR regions. Only six mutational events occurred in the early phase, too few to provide statistical evidence for positive selection. The distribution of replacement mutations in the early phase, however, differs markedly from the pattern in N3 and N4, where silent mutations predominate. The change in the pattern of mutation suggests that positive selection may have influenced the early phase of this immune response, while the later phase was dominated by negative selection. The concept of positive selection followed by negative selection, which has also been proposed in models of murine antihapten responses (11, 59), is consistent with our hypothesis that this autoreactive clone was preferentially expanded before neoplastic transformation.

Our previous work with this model system defined a mechanism which explains the association of an autoantibody mediated human disease with a B cell clonal disorder. The current findings from longitudinal analysis of V_H gene changes in this system demonstrate a pathologic feature of B cell neoplasia, clonotypic shift. Shifts in the predominant V_H clonotype coincided with recurrences of the lymphoma over a 4-yr period. Clonotypic shift may be a molecular correlate of the emergence of a subpopulation which has other variant characteristics, for example, a different histologic appearance or increased drug resistance. Clonotypic shift differs from the conventional view of tumor progression, because diversity of clonotypes is generated early in the clonal evolution, whereas the commonly observed genetic changes, such as chromosomal abnormalities, are thought to accumulate during the later stages of the disease.

The findings from this longitudinal analysis also substantiate our hypothesis that autoantigen played a role in the pathogenesis of this autoreactive B cell clone. Analysis of the relevant germline and expressed V_H sequences allowed us to construct a genealogic tree representing evolutionary changes in the B cell clone. The pattern of nucleotide substitutions suggested, first, that the clone may have been preferentially expanded prior to transformation and second, that the clone established and maintained idiotypy and specificity, at least in part, by a process of clonal selection mediated by autoantigen.

This enlarged model for the association of autoimmunity with B cell clonal disorders would predict that autoreactive clones might in general be expanded relative to clones with other specificities, providing larger "targets" for stochastic transforming events.

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