

MUTATIONAL HOT SPOTS IN Ig V REGION GENES OF HUMAN FOLLICULAR LYMPHOMAS

BY SHOSHANA LEVY, EILEEN MENDEL, SHINICHIRO KON,
ZAFRIRA AVNUR, AND RONALD LEVY

From the Division of Oncology, Stanford University School of Medicine, Stanford, California 94305

The phenomenon of somatic mutation in expressed Ig V region genes has been well documented in B cells participating in an immune response to several antigens in the mouse (1-6). In each case, the analyses were carried out by capturing individual B cells and expanding them in the form of hybridomas. V region genes expressed by these hybridomas were then sequenced. Because of the similarities of their DNA sequences, particularly at the V/D/J or V/J joints, multiple cells from an individual mouse could often be shown to be derived from a common precursor, and in such cases, a genealogical relationship between them could be inferred (2, 7, 8). Clonal expansion is accompanied by the progressive accumulation of point mutations in the expressed V genes. The rate of this mutational process has been estimated to be as high as 10^{-3} /bp per cell division (9). It is now clear that somatic hypermutation is restricted to certain phases of the immune response, particularly during the generation of the memory B cell. During the early primary response and during the late secondary response, a high rate somatic mutation does not occur (2, 8).

Human B cell lymphomas represent particular stages of B cell differentiation that have been clonally expanded during the malignant transformation (10). When V_H genes of such expanded clones from patients with follicular lymphoma were analyzed, they were found to be derived from the same original cell and to have undergone somatic mutation during clonal evolution (11, 12). The first analysis was done on a tumor that had regrown after initially responding to treatment by an anti-idiotypic antibody (13). Analysis of these V_H genes revealed extensive somatic mutations with an abundance of replacement mutations in complementarity-determining region 2 (CDR2)¹. A single mutation was identified as the probable cause for escape of the tumor from the effects of anti-Id antibody (11). However, it was clear that mutation had preceded any exposure to anti-Id. We proceeded then to analyze the V_H genes of cells derived from a pretreatment specimen of a second patient with nodular lymphoma whose tumor responded completely to anti-Id therapy. We found that this patient's V_H genes were similarly subject to a mutational process, but that none of the mutations interfered with the reactivity of the anti-idiotypic antibody used in therapy (12).

The process of somatic mutation in Ig V region genes is evidently a general phenomenon in human follicular lymphoma, since heterogeneous reactivity patterns with panels of anti-idiotypic antibodies have now been documented in the tumor

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

cell populations of a number of cases (14). In these tumors, it is not known if Ig V gene mutation is an ongoing process or if it happens at a defined time during the life of the clone and then ceases. However, the fact that V_H sequences from multiple individual cells of the tumor are all different from each other implies that mutation is ongoing. It is also unknown if the mutational process was activated by or selected on by an antigen.

We have now proceeded to study the V_L genes of the two tumors whose V_H genes were previously analyzed. Our intention was to determine if the V_L genes had also mutated, and if so, whether the V_H and V_L within the same cell had mutated at similar rates and at similar points in time. This comparison was possible because in each case, the V_H and V_L genes were derived from the same heterohybridoma cells. We find that light chain genes mutate independently of heavy chain genes and that certain positions within each gene may represent mutational hot spots.

Materials and Methods

Human Lymphoma Cells and Cell Lines. For patient PK, tumor cells were isolated from the peripheral blood during a leukemic phase of follicular small cleaved cell lymphoma (IgM- λ) and fused to NS-1/Ag-4 cells (15). Independent heterohybridomas secreting IgM- λ were derived from the PK tumor, as previously described (16).

For patient CJ, specimens from lymph nodes and spleen were obtained with a follicular small cleaved cell lymphoma (IgM- κ) and processed as previously described (11). Lymphoma cells were fused to the non-Ig-producing cell line K6H6-B5 (17) and expanded in the form of independent heterohybridoma clones each secreting IgM- κ derived from the CJ tumor as previously described (17, 18).

Nucleic Acid Isolation. Cytoplasmic RNA was isolated from heterohybridoma cells after lysis in 0.5% NP-40 in the presence of 10 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories, Gaithersburg, MD) in 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 5 mM MgCl₂, as previously described (11). Poly(A)-containing RNA was isolated by chromatography on oligo-dT-cellulose (Type 3, Collaborative Research, Lexington, MA) (19). DNA was extracted from lymphoma tissue and from heterohybridomas by using described procedures (11).

Construction and Screening of Genomic Library. DNA from the CJ tumor was digested to completion with Bam HI (New England Biolabs, Beverly, MA), subjected to electrophoresis on a preparative agarose gel, and a 7.5-kb fragment containing the rearranged κ gene was excised. DNA was electroeluted from the gel slice and ligated into the Bam HI site of the phage vector EMBL 3 (20). 10^6 in vitro packaged recombinants were screened (11) using the Eco RI fragment containing the human κ constant gene region (21) as a probe. Labeling and hybridization conditions were as previously described (11, 22). Phages that hybridized to the probe were plaque purified. The 7.5-kb insert was further digested with HindIII (New England Biolabs) to give a 5 and 2.5-kb fragment. The 2.5-kb piece was subcloned into M13mp19 and sequenced (23).

cDNA Cloning. The cDNA of the expressed human tumor V_L genes was obtained from individual heterohybridomas by using a rapid method for cloning and sequencing Ig V region genes. In this method, a primer homologous to the constant region is used to initiate first strand synthesis. The single-stranded cDNA is made double stranded and blunt ended by successive addition of enzymes to the same tube as described (24). The human κ primer used for cloning V_κ from patient CJ was 5'CAGATGGCGGGAAGATG 3' (21). The human λ primer used for cloning V_λ from patient PK was 5'GTGTGGCCTTGTTGGC 3' (25). The double-stranded blunt-ended cDNA was ligated directly into the SmaI site of M13mp19. The resulting M13 library was lifted onto nitrocellulose filters and screened (26). Hybridization probes were ³²P-labeled first strand cDNA of V_L (24) or, when available, a previously obtained cDNA clone labeled with ³²P by random hexamer priming (22).

cDNA Amplification. In some cases, a modification of the polymerase chain reaction (PCR) (27) was used to amplify cDNA. Double-stranded V_{λ} cDNA was obtained from PK heterohybridomas K6H6, K6F5, and K5G5, as described above. Amplification of the cDNA was carried out in a volume of 100 μ l amplification buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1.5 mM dNTPS, 5% DMSO, and 1 mM of primers). The primers used for this amplification included a sequence homologous to the λ constant region gene immediately 5' to the primer used to make the cDNA (5'pCGGGTAGAAGTCACT3') (25), and a sequence homologous to the leader region of the V_{λ} of the heterohybridoma K6H6. In each cycle of the PCR, the DNA was heat denatured (100°C for 5'), quickly chilled on dry ice, centrifuged for a few seconds, and preincubated at 42°C for 2'. 1 U of the large fragment of *Escherichia coli* DNA polymerase I (Klenow) (BRL), diluted to 2 U/ μ l in 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, was added, and the reaction was incubated for 2' at 42°C. After 14 cycles, the DNA was extracted with phenol/chloroform, then with ether, and was precipitated with ethanol. The pellet was dissolved in 50 μ l H_2O , an equal volume of 2 \times amplification buffer was added, and the amplification reaction was repeated for 15 more cycles. The amplified DNA was extracted with phenol/chloroform and then with ether and was precipitated with ethanol. The pellet was dissolved in H_2O and subjected to electrophoresis on a 4% Nusieve GTG (FMC) agarose gel. The major band of the expected size was removed, extracted with phenol, and then with ether, and was precipitated with ethanol. The amplified DNA was ligated into a blunt-ended M13 vector and isolated in the single-stranded form and sequenced.

Results

Sequences of V_L Genes. Each heterohybridoma captures the heavy and the light chain Ig genes expressed by an individual tumor cell. Four independent heterohybridomas were previously used to obtain the expressed V_H sequences from lymphoma cells of a patient (CJ) (11). In the present study, RNA from the same four heterohybridomas was used as a template for cDNA cloning of the expressed V_{κ} genes. In addition, a 7.5-kb Bam HI restriction fragment (11) containing a portion of the genomic rearranged V_{κ} gene was cloned directly from the tumor biopsy specimen. These five V_{κ} sequences are shown in Fig. 1. It is evident that these light chain genes are all derived from members of the same clone of cells because of the extensive homology of their V and J segments and their identical V/J joints. However, the sequences all differ from each other. The incomplete G1 sequence differs from clone 1H1 by one nucleotide. G1 was the genomic clone obtained by Bam HI digestion of tumor DNA. The Bam HI recognition site for this particular V_{κ} gene occurs, in bases coding for amino acids 57–59 (except for clone 1B11 that has mutated this site). The genomic sequences that flank the J region of G1 establish that the V region gene in this tumor has rearranged to J1 (28) (data not shown).

The germ-line version of this V region gene is not known, therefore, all the sequences are compared with a consensus sequence. The consensus sequence differs from the J1 germ-line gene in five positions, all of which represent silent mutations. The V region genes differ from each other by a number of silent mutations scattered throughout the gene. In addition, replacement mutations have occurred both in CDR2 and in CDR3. Two of the replacement mutations in CDR3 happen to be in a potential glycosylation site in amino acid positions 91–93. This potential glycosylation site is retained in spite of the replacement mutations, N-X-T to N-X-S.

Heterohybridomas obtained from the tumor of patient PK, previously used as a source of V_H sequences, were used here to obtain the V_{λ} sequences expressed by the same six individual tumor cells (Fig. 2). In this case, V region cDNA was either

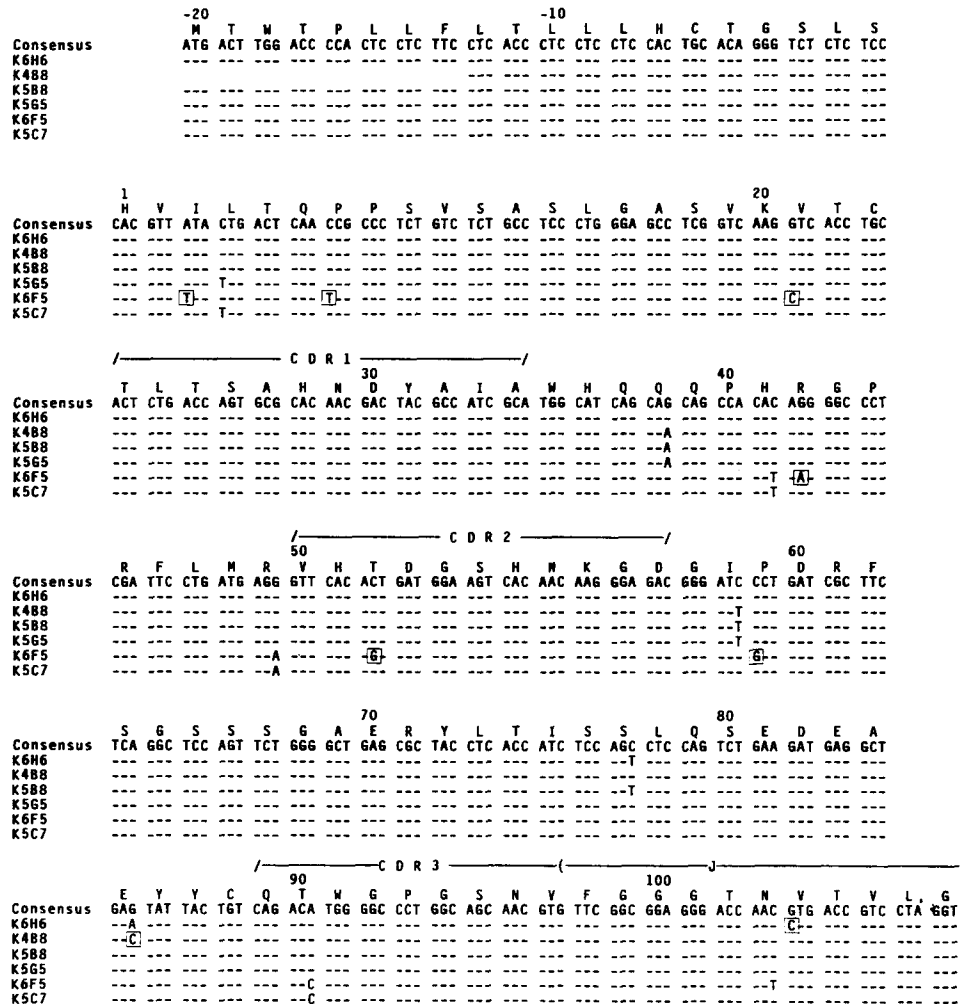


FIGURE 2. Nucleotide sequences of V_λ genes expressed by tumor PK. Sequences were derived from cDNA from independent heterohybridomas. See legend to Fig. 1 for details.

gion. We have previously reported (12) that the protein secreted by this heterohybridoma failed to react with two out of five anti-idiotypic antibodies. Moreover, heterohybridoma K6F5 exhibited a difference in the size of one of its rearranged λ gene Eco RI restriction fragments compared with the other heterohybridomas. All six heterohybridomas had in common an 8-kb Eco RI genomic fragment containing the Cλ2 and the Cλ3 genes (25). Whereas, all other clones had a second rearranged band of <6 kb, clone K6F5 had a second rearranged band of >16 kb (data not shown). Thus, the K6F5 cell differed from the other tumor cells at a site outside the V_λ coding region as well.

V_L and V_H Genes Mutate Independently. The analysis of V_L sequences from the two lymphomas revealed that the expressed V_L genes, like the respective V_H genes from

TABLE I
Summary of Mutations in Tumor C_J

V _κ Amino acid number	Base in consensus	Changed to	Position	Nature of mutation in heterohybridoma			V _H Amino acid number	Base in consensus	Changed to	Position	Nature of mutation in heterohybridoma		
				2A12	1H1	2C12					1B11	2A12	1H1
2	T	C	III		S	-8	G	A	I				R
16	A	T	III		S	20	C	G	III		S		
16	A	G	III		S	37	C	T	III			S	
30	C	T	III		S	49	C	G	III			S	
38	G	A	III		S	52A	A	G	I		R		S
40	T	A	III		S	52A	C	A	III			R	R
41	G	C	III		S	52C	C	G	II			R	R
56	T	A	III		S	54	T	A	III				R
58	C	T	III	R	S	58	A	G	III			R	
61	A	G	III		S	64	A	G	III				S
92	T	A	III	R	S	76	C	T	III			S	
93	C	G	II		R	77	C	T	II			R	
94	T	A	I	R	R	78	G	T	III			S	
100	G	A	III		S	89	G	C	III		S	S	
105	G	A	III		S	89	G	A	III			S	

The location of the mutations in V_κ and V_H (11) is given according to the amino acid number. Listed are the changes from consensus and their codon position in each heterohybridoma. R, replacement mutation; S, silent mutation.

the same cells (11, 12), had accumulated mutations. An attempt was made to relate the cells within each tumor to each other on the basis of the mutational events that had occurred in the two genes. A summary of the mutations in each V region gene is given in Tables I and II. The tables list the amino acid locations of the mutations, the bases that were changed, their codon positions, and the nature of the mutations (silent or replacement) as expressed in each heterohybridoma. The total number of mutations accumulated in each of the four sequences analyzed is extremely similar. Most of the mutations occur in the third codon position and are silent. The number of transversions and transitions in each of the sequences is also very similar. Table I summarizes the data obtained from V_{κ} and V_{H} of each of the heterohybridomas of patient CJ. It can be seen that two of the hybridomas, 2C12 and 1B11, have a number of mutations in common, both in V_{κ} and V_{H} . In addition to the shared mutations, the two hybridomas accumulated mutations that are unique. The number of these unique mutations is not always similar for V_{κ} and V_{H} in the same heterohybridoma; for example clone 2C12 has a single unique V_{κ} mutation but five unique V_{H} mutations. Similarly, sequences obtained for patient PK (Table II) show that clone K5G5, with a single unique mutation in V_{λ} , accumulated seven such unique mutations in V_{H} . Conversely, clone K6F5 accumulated seven unique mutations in V_{λ} and only three in V_{H} . It is interesting to note that common mutations are mostly silent, whereas many of the unique mutations are replacement mutations.

It is possible to construct separate genealogical trees for these lymphomas for each V region gene. However, the V_{L} and V_{H} trees derived from the same tumor cells cannot be superimposed. They diverge sometimes at branch point positions; for example, clones K4B8, K5B8, and K5G5 stem from the same node in the light chain, but only clones K4B8 and K5B8 are related in the heavy chain tree (Table I and Fig. 3). In addition, the trees cannot be superimposed because of differences in the number of the unique mutations and the distances of the tree branches for the two chains. This result suggested that the particular cells from which these sequences were derived were not related in any simple genealogy. Rather, within each set of sequences, common mutations may have occurred independently, at sites with a particular propensity to mutate (hot spots). One mechanism, suggested by Golding et al. (30) for mutation within Ig genes, is the misalignment of the template during DNA replication onto neighboring sequences, within the gene, which are almost exact direct or inverted repeats. The resulting mismatch on the complementary strand would then be corrected to produce a mutation. These authors have shown that Ig genes contain a high frequency of direct and inverted repeats, and that many mutations that have been reported could have arisen by such a mechanism. We have, therefore, examined the DNA sequences surrounding our putative mutations, with particular attention to the mutations that may have occurred repetitively. In Fig. 4, two examples of direct repeats are shown that could potentially have served as templates for two different sets of repetitive mutations in the PK V_{λ} gene set. A 10-nucleotide stretch beginning in codon 45 is repeated exactly, except for a T to C substitution downstream beginning in codon 57. A C to T mutation occurred at that position in three different cells from this tumor. Similarly, a stretch of nine nucleotides is repeated in a nested fashion between codons 75 and 80, with a single C to T change that could have templated that substitution, which occurred in two

TABLE II
Summary of Mutations in Tumor PK

V _λ Amino acid number	Base in con- sensus	Changed to	Position	Nature of mutation in heterohybridoma		V _H Amino acid number	Base in con- sensus	Changed to	Position	Nature of mutation in heterohybridoma	
				K6H6 K4B8	K5B8 K5G5					K6F5 K5C7	K6H6 K4B8
3	A	T	I		R	-2	G	C	III		R
4	C	T	I		S	3	A	G	III		S
7	C	T	I		R	12	A	G	III		S
21	G	C	I		R	12	A	T	III		S
38	G	A	III		S	15	G	A	II		R
41	G	T	III	S	S	18	T	G	III		S
42	G	A	II		R	35	C	T	III		S
49	G	A	III		S	40	C	T	II		R
52	C	G	II		R	49	G	C	III		S
58	C	T	III		S	77	G	C	III		S
59	C	G	I		R	78	T	G	I		R
77	C	T	III	S	S	97	G	A	III		S
85	G	A	III	S	S	98	G	A	I		R
85	G	C	III		R	98	G	T	II		R
90	A	C	III		S	98	G	A	III		S
103	C	T	III		S	100	G	A	I		R
104	G	C	I	R	R	112	C	T	III		S

The location of the mutations in V_λ and V_H (12) are given (see legend, Table I).

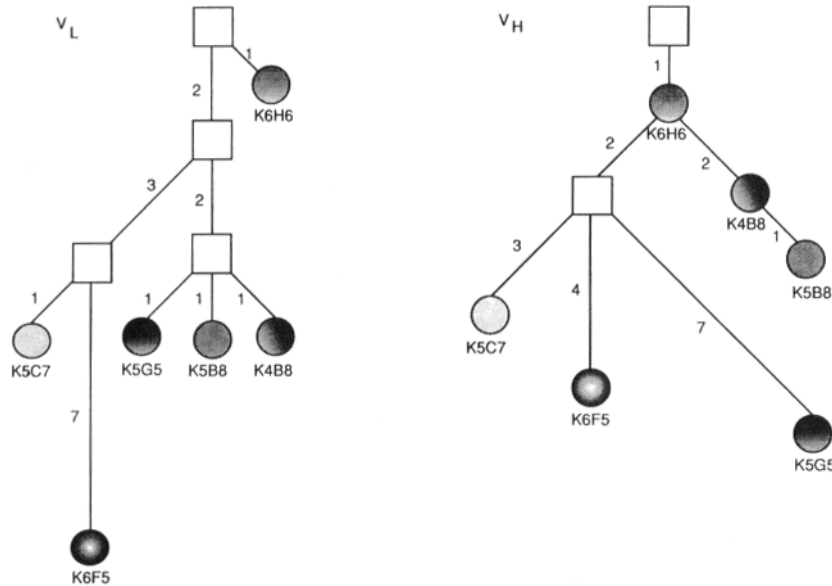


FIGURE 3. "Genealogic" trees of V_L and V_H genes expressed by six heterohybridomas obtained from the tumor of patient PK. The heterohybridomas are displayed as circles and hypothetical intermediates and precursors as squares. Distances between clones are proportional to the number of base mutations, as indicated along the lines. Notice that the V_L and V_H trees, derived from the same cells, cannot be superimposed.

different cells from this tumor. In Fig. 5, examples are shown of inverted repeats that could have served as templates for six different mutations in the CJ V_K genes. The examples shown in Figs. 4 and 5 are the only mutations within our data set that could have been explained by such a misalignment mechanism. Most of our cases of repetitive mutations do not have potential templates within the V region coding sequence.

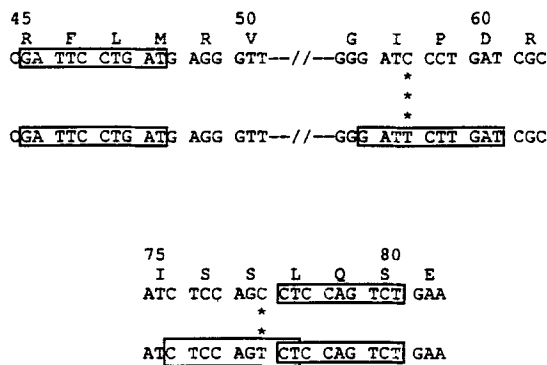


FIGURE 4. Direct repeats in the V_L gene of PK tumor. Two direct repeats are shown. The first in codons 45-48 (boxed) and repeated, except for one base, in codons 57-60 of the consensus (upper lane). Three of the V_L genes have mutated the C to a T (indicated by the asterisks), which results in an identical direct repeat in these genes (boxed in lower lane). The second repeat in codons 78-80 of the consensus (boxed in upper lane) is repeated in codon 75-78, except for one base. Two of the V_L genes have mutated a C to a T (indicated by asterisks), which results in an identical direct nested repeat in these genes (boxed in lower lane). Amino acid numbering was according to Kabat et al. (36).

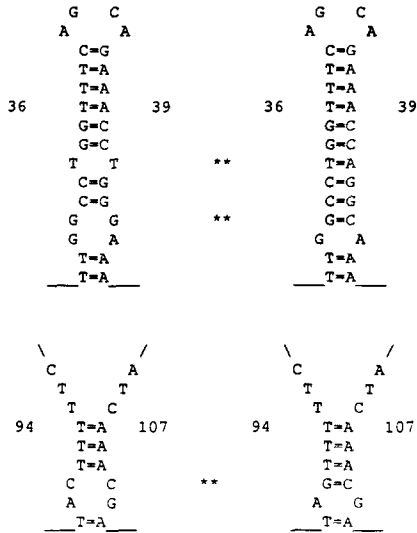


FIGURE 5. Inverted repeats in the V_{κ} gene of CJ tumor. Two inverted repeats are shown. The first is present in codons 33-42. The sequence on the left is the consensus sequence and on the right is the sequence in two of the V_{κ} genes that mutated a T to A and G to C as indicated by the asterisks. The second inverted repeat is present in codons 92-94 and 107-108. The sequence on the left is the consensus sequence and on the right is the sequence in two of the V_{κ} genes that mutated a C to a G as indicated by the asterisks. Amino acid numbering was according to Kabat et al. (36).

Another mechanism that can explain mutational hot spots involving the transition of C to T and G to A has been defined by Coulondre et al. (31). They have shown that when cytosine residues are methylated in the DNA, they can be converted to thymine by spontaneous deamination, resulting in a C to T change on one DNA strand, which upon replication can lead to a G to A change on the complementary strand. Table III contains a compilation of all the silent (nonselectable) mutations we observed in the V_L and V_H sequences from the two tumors we analyzed. The results are compared with those obtained by Li et al. (32), who analyzed mutations in mammalian pseudogenes, which presumably occurred in the germline over evolutionary time (meiotic mutation). Both sets of data display an excess of transitions over transversions with a predominance of C to T and G to A substitutions.

TABLE III
Comparison of Silent Mutations in Human V Regions to Meiotic Mutations in Pseudogenes

From	Somatic mutations (IgV silent)*				From	Meiotic mutations (pseudogenes)†			
	To:					To:			
	A	T	C	G		A	T	C	G
A	-	5.6	3.1	12	A	-	4.7	5.0	9.4
T	3.1	-	3.1	1.5	T	4.4	-	8.2	3.3
C	0	28	-	6.6	C	6.5	21	-	4.2
G	23	2.6	12	-	G	21	7.2	5.3	-

All possible base changes are listed, and the observed changes are shown as a percentage after correction for base composition according to the method of Gojobori et al. (35) and Li et al. (32). Total transitions are obtained by adding numbers along a diagonal from lower left to upper right and are listed in upper right; somatic, 66%; meiotic, 59%.

* Data from Figures 1, 2, and references 11 and 12. $n = 74$.

† Data on pseudogenes from Li et al. (32). $n = 587$.

Discussion

We have previously described extensive somatic mutations in the V_H genes expressed in human B cell lymphomas from two patients (11, 12). Here we examined the sequences of V_L genes expressed by the same cells from which the V_H genes were derived. As can be seen in Figs. 1 and 2, it is clear that these V_L genes had also undergone extensive somatic mutations. These data suggest that the mutational rate is high in these tumors and similar for both V_L and V_H genes, that silent mutations predominate, and that the underlying mutational process may involve hot spots.

Somatic mutation occurs at a high rate in Ig V region genes expressed by B cells participating in an immune response. Most of the documented examples represent situations in which inbred mice respond to a particular antigen by producing an immune response dominated by a unique idiotype. These recurrent idiotypes are encoded by particular germ-line genes for both the V_H and the V_L and, in some cases, use particular D and J segments (1-4, 6). Thus, the mutations occurring in these V region genes during clonal expansion can be determined precisely by reference to the known germ-line gene sequences. Some general conclusions from these experiments follows. (a) Both V_L and V_H genes in the same cells are subject to the mutational process. (b) Mutations that result in amino acid replacements tend to be found in CDR regions and can be correlated with improved antigen binding (1, 4, 6, 34). The occasional finding of identical replacement mutations in independent clones can thus be explained by the positive selective force of antigen. (c) Silent mutations are found as frequently in framework regions as in CDR regions and are not apparently under selective pressure. Identical silent mutations found in independent clones have been suggested to represent mutational hot spots (1, 4).

Genealogic trees have been constructed based on multiple identical V gene mutations shared by individual B cell clones (2, 7, 8). Such trees are obviously dependent on the assumption that the cells bearing the sequences originate from the same cell, which underwent rearrangement of a single functional V_H and V_L gene. For immune responses dominated by the use of particular V gene segments, such a demonstration is not always easy because multiple different B cells may use the identical gene segments. In the case of spontaneous human B cell tumors, it is usually clear that a single clone of cells is involved. The individual members of the tumor use not only the same V gene segments but also show identical V/D/J and V/J joints. In addition, the follicular lymphomas (the type of tumor analyzed here) all have a specific chromosomal translocation involving a gene called *bcl-2* on chromosome 18 and the Ig locus on the inactive allele of chromosome 14 (33). The analysis of evolution within these malignant human B cell clones is somewhat disadvantaged by the lack of information about the germ-line versions of the V genes in question, although the germ-line D and J segments are known. For this reason, one must use a consensus sequence as the frame of reference for the assignment of mutations. Our prior analyses of V_H sequences from two different tumors proposed a genealogic relationship between the members of each clone (11, 12). The current data on V_L sequences from these same cells can also be arranged into a plausible genealogic relationship for each tumor. However, no simple genealogic relationship can be made between the cells of each tumor that takes both the V_L and V_H sequences into account. Since these tumors contained $>10^{11}$ cells at the time of sampling, it is not surprising that the evolutionary relationship among a small number of cells is difficult to establish.

Also, the genealogic trees that can be constructed from the data have a preponderance of silent mutations at the branch points and coding mutations at the extremities. If the clone had indeed followed such a tree of evolution, it is difficult to explain why branches should occur in the absence of selective forces. Rather, it seems more likely that the repetitive silent mutations found both within the V_L and the V_H sequences are the result of sites that are especially prone to mutation, i.e., hot spots.

Berek and Milstein (4) found multiple examples of repetitive silent mutations among independent B cell clones responding to the antigen phenylloxazolone. For instance, in their data, as many as four pairs of identical silent changes were observed among 19 V_H sequences derived from different mice. Their statistical analysis showed this pattern of repeats to be exceedingly unlikely to occur by chance ($p = 0.011$). By comparison, we observe as many as five double and two triple repeats of silent mutations out of six V_L sequences from the PK tumor clone (Fig. 2 and Table II). Moreover, our data argues not just for hot spots but for hot specific events. That is, there must be a propensity to change not just a certain position but to make certain specific substitutions. One mechanism that has been proposed for the generation of mutations in Ig V genes involves the misalignment of the DNA template during replication due to neighboring direct or inverted repeat sequences that differ only slightly from the sequence being copied (30). Such a mechanism specifies not only the position of mutations, but also the specific change. In addition, it provides an explanation for multiple alterations of a gene as a result of a single mutational event. That is, the template being used can have several differences from the homologous sequence, and each of these can be simultaneously copied onto the new DNA strand. Indeed, potential templates were found within the coding region sequences for several of the repetitive silent mutations in our data (Figs. 4 and 5). In Fig. 5, an example of two substitutions within the same inverted repeat is shown. However, we found such repeats for only a minority of the hot spots we observed. It is possible that templates for more of the hot spots did exist further upstream or downstream beyond the coding regions that we analyzed here.

Additional clues about a possible mechanism of mutation in these cells come from an analysis of the chemical nature of the base substitutions. If mutation represented a random incorporation of inappropriate bases, transversions (purine \leftrightarrow pyrimidine) should be twice as frequent as transitions (purine \leftrightarrow purine, pyrimidine \leftrightarrow pyrimidine). In fact, this ratio is almost exactly reversed (Table III). On closer inspection, this excess of transitions is accounted for by the relatively high frequency of the changes of C to T and of G to A. The same pattern has been noted in mutations in a number of different pseudogenes over the evolution of species (32). The conversion of C to T can occur in DNA by deamination of methylated C residues. Indeed this reaction has been implicated in a mutational hot spot in the Lac Z gene in *E. coli* (31). If such a change occurred on the antisense strand, it would result in a change of G to A on the sense strand after subsequent replication. The possibility exists, therefore, that the mutational process could be targeted to certain positions by specific methylation events.

Mechanisms of mutation such as those mentioned above could, in principle, be tested by constructing substrate genes. Such genes containing specific internal templates or specific methylated cytosine residues could then be introduced into B cells undergoing mutations. B Cell lines at appropriate stages of differentiation, for in-

stance, those participating in the late primary or memory response to an antigen, could be used. Based on the data reported here, cell lines from patients with follicular lymphoma might also be suitable. The creation of such cell lines, therefore, remains high priority for the future.

Summary

The genes coding for the Ig light chains expressed in two cases of human follicular lymphoma were cloned and sequenced. In each case, multiple independent isolates of the tumor population were compared. Although each tumor represented a single clone of B cells with a unique V/J joint, different cells within each tumor had accumulated multiple point mutations in the V gene during clonal expansion. Most of the mutations observed were silent, but some resulted in amino acid replacements. Identical silent mutations were often observed in independent isolates of each tumor. By combining the current data with V_H sequences obtained previously from the same cells, it was apparent that the repetitive silent mutations could not be explained solely by a genealogic tree. Such mutations could represent hot spots whose tendency to mutate may be influenced by neighboring DNA sequences or by the methylation of specific cytosine residues.

Received for publication 14 March 1988 and in revised form 9 May 1988.

References

1. Malipiero, U. V., N. S. Levy, and P. J. Gearhart. 1987. Somatic mutation in anti-phosphorylcholine antibodies. *Immunol. Rev.* 96:59.
2. Claffin, J. L., J. Berry, D. Flaherty, and W. Dunnick. 1987. Somatic evolution of diversity among anti-phosphocholine antibodies induced with proteus morgani. *J. Immunol.* 138:3060.
3. Cumano, A., and K. Rajewsky. 1986. Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. 1986. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2459.
4. Berek, C., and C. Milstein. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* 96:23.
5. Caton, A. J., G. G. Brownlee, L. M. Staudt, and W. Gerhard. 1986. Structural and functional implications of a restricted antibody response to a defined antigenic region on the influenza virus hemagglutinin. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1577.
6. Wysocki, L., T. Manser, and M. L. Gefter. 1986. Somatic evolution of variable region structures during an immune response. *Proc. Natl. Acad. Sci. USA.* 83:1847.
7. Clarke, S. H., K. Huppi, D. Ruezinsky, L. Staudt, W. Gerhard, and M. Weigert. 1985. Inter- and Intracloal diversity in the antibody response to influenza hemagglutinin. *J. Exp. Med.* 161:687.
8. Siekevitz, M., C. Kocks, K. Rajewsky, and R. Dildrop. 1987. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell.* 48:757.
9. McKean, D., K. Huppi, M. Bell, L. Staudt, W. Gerhard, and M. Weigert. 1984. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. USA.* 81:3180.
10. Greaves, M. F. 1986. Differentiation-linked leukemogenesis in lymphocytes. *Science (Wash. DC).* 234:697.

11. Cleary, M. L., T. C. Meeker, S. Levy, E. Lee, M. Trela, J. Sklar, and R. Levy. 1986. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. *Cell*. 44:97.
12. Kon, S., S. Levy, and R. Levy. 1987. Retention of an idiotypic determinant in a human B-cell lymphoma undergoing immunoglobulin variable-region mutation. *Proc. Natl. Acad. Sci. USA*. 84:5053.
13. Meeker, T., J. Lowder, M. L. Cleary, S. Stewart, R. Warnke, J. Sklar, and R. Levy. 1985. Emergence of idiotype variants during treatment of B-cell lymphoma with anti-idiotype antibodies. *N. Engl. J. Med.* 312:1658.
14. Carroll, W. L., J. N. Lowder, R. Streifer, R. Warnke, S. Levy, and R. Levy. 1986. Idiotype variant cell populations in patients with B cell lymphoma. *J. Exp. Med.* 164:1566.
15. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511.
16. Brown, S., J. Dilley, and R. Levy. 1980. Immunoglobulin secretion by mouse X human hybridomas: an approach for the production of anti-idiotype reagents useful in monitoring patients with B cell lymphoma. *J. Immunol.* 125:1037.
17. Carroll, W. L., K. Thielemans, J. Dilley, and R. Levy. 1986. Mouse x human hetero-hybridomas as fusion partners with human B cell tumors. *J. Immunol. Methods*. 89:61.
18. Levy, R., and J. Dilley. 1978. Rescue of immunoglobulin secretion from human neoplastic lymphoid cells by somatic cell hybridization. *Proc. Natl. Acad. Sci. USA*. 75:2411.
19. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA*. 69:1408.
20. Frischauf, A. M., H. Lehrach, A. Poustka, and N. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. *J. Mol. Biol.* 170:827.
21. Hieter, P. A., E. E. Max, J. G. Seidman, J. V. Maizel, Jr., and P. Leder. 1980. Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. *Cell*. 22:197.
22. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.
23. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463.
24. Levy, S., E. Mendel, and S. Kon. 1987. A rapid method for cloning and sequencing variable-region genes of expressed immunoglobulins. *Gene*. 54:167.
25. Hieter, P. A., G. F. Hollis, S. J. Korsmeyer, T. A. Waldmann, and P. Leder. Clustered arrangement of immunoglobulin λ constant region genes in man. 1981. *Nature (Lond.)*. 294:535.
26. Benton, W. D., and R. W. Davis. 1977. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science (Wash. DC)*. 196:180.
27. Todd, J. A., J. I. Bell, and H. O. McDevitt. 1987. HLA-DQ β gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature (Lond.)*. 329:599.
28. Hieter, P. A., J. V. Maizel, Jr., and P. Leder. 1982. Evolution of human immunoglobulin κ J region genes. *J. Biol. Chem.* 25:1516.
29. Udey, J. A., and B. Blomberg. 1987. Human λ light chain locus: organization and DNA sequences of three genomic J regions. *Immunogenetics*. 25:63.
30. Golding, G. B., P. J. Gearhart, and B. W. Glickman. 1987. Patterns of somatic mutations in immunoglobulin variable genes. *Genetics*. 115:169.
31. Coulondre, C., J. H. Miller, P. J. Farabaugh, and W. Gilbert. 1978. Molecular basis of base substitution hot spots in Escherichia coli. *Nature (Lond.)*. 274:775.
32. Li, W.-H., C.-I. Wu, and C.-C. Luo. 1984. Nonrandomness of point mutation as reflected in nucleotide substitutions in pseudogenes and its evolutionary implications. *J. Mol. Evol.* 21:58.

33. Croce, C. M. 1987. Role of chromosome translocations in human neoplasia. *Cell*. 49:155.
34. Rajewsky, K., I. Förster, and A. Cumano. 1987. Evolutionary and somatic selection of the antibody repertoire in the mouse. *Science (Wash. DC)*. 238:1088.
35. Gojobori, T., W.-H. Li, and D. Graur. 1982. Patterns of nucleotide substitution in pseudogenes and functional genes. *J. Mol. Evol.* 18:360.
36. Kabat, E. A., T. T. Wu, H. Bilofsky, and M. Reid-Miller. 1983. Sequences of Proteins of Immunological Interest, U.S. Public Health Service, Bethesda, MD.