ALTERNATIVE V_K GENE REARRANGEMENTS IN A MURINE B CELL LYMPHOMA

An Explanation for Idiotypic Heterogeneity

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The diversity of the antigen binding portion of the Ig molecule is generated through the recombination of multiple discontinuous gene segments (1, 2). The heavy chain variable (V) region coding sequence is formed when one of hundreds of V_H regions combines with one of 15–20 D regions and one of 4 J_H regions to create a complete VDJ segment (3, 4). Light chain recombination proceeds in a similar fashion between V_L and J_L segments (5). These events occur in a well-defined order with DJ_H rearrangement preceding V_H-to-DJ_H recombination (6, 7), followed later by V_L-to-J_L recombination. The exact site of recombination between these segments is imprecise, thus creating further diversity at the joints. Single nucleotides (N segments) may be added at the limits of the D region in heavy chain rearrangements (8, 9). These mechanisms, along with the association of different heavy and light chains, result in an expected antibody repertoire of $>10^{11}$ Ig molecules.

In recent years it has become apparent that rearranged VDJ segments are capable of undergoing further somatic variation. A major mechanism of generating further diversity is somatic point mutation within the V region coding sequence (1, 10). This process appears to be focused within and nearby the rearranged V region genes. Somatic point mutation of these genes has been estimated to occur at a rate as high as 10^{-3}/bp/generation (11, 12). It occurs during the late primary and during the generation of the secondary immune response (13). It may alter both antigen specificity and affinity (14). In addition to somatic mutation, less frequently implicated molecular events such as gene conversion or V_H region replacement may modify already expressed *Ig* genes (15–18). These processes appear to occur during specific stages of B cell differentiation and may be limited to certain B cell subsets (17).

The molecular basis of somatic diversification may be studied in greater detail by examining B cell tumors. Tumors that represent discrete stages or lineages of normal B cell development and have been adapted to growth in vitro should be good

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candidates for such studies. Recently we have described surface Ig^+ idiotype-negative (sIg^+, Id^-)[1] tumor variants in the murine lymphoma 38C13 (19). This B cell lymphoma bears surface Igκ but secretes little of this molecule, and therefore, is an excellent model for immunotherapy targeted against its Id. We generated a panel of syngeneic antiidiotype mAbs and have examined their antitumor effects (20, 21). We found minor tumor subpopulations that failed to react with one or another of the antiidiotype antibodies. Such variants could be selected either in vivo (19) or in vitro. Previously we reported biochemical and serologic differences between tumor variants (19). Antisera and mAbs were produced that distinguished each individual variant Id protein. The Ig produced by the tumor variants differed from one another in the migration of their κ light chain proteins on SDS-PAGE. We found that their κ light chain gene rearrangements were also different from one another and from the parental 38C13 lymphoma. Here we describe the molecular basis of this heterogeneity.

Materials and Methods

Tumor Cell Lines. The 38C13 murine B cell lymphoma of C_3H origin, and mAbs directed against its cell surface idiotypic determinants have been described in detail previously (20–23). Tumor cell lines were grown in RPMI 1640 medium (Irvine Scientific, Irvine, CA), supplemented with 12% FCS, penicillin, streptomycin (Sigma Chemical Co., St. Louis, MO), L-glutamine (Gibco Laboratories, Grand Island, NY), 10 mm Hepes (Gibco Laboratories), and 5×10^{-5} M 2-ME in a humidified 37°C, 5% CO_2 incubator. Id variants were selected by methods outlined earlier (19). Briefly, four different classes of tumor variants were isolated on the basis of surface Ig expression and binding to two different anti-Id antibodies, S1C5 and S5A8. Three of these variants were recovered from mice whose tumors regrew after therapy in vivo with S1C5. One of these variants, V1, retained surface Ig expression, but failed to bind both anti-Id mAbs. Variant V2 lost surface Ig expression altogether. Another variant, V3, was sIg^+, lost binding to antibody S1C5, but still bound antibody S5A8. A fourth variant, V4, was isolated after the 38C13 tumor was exposed in vitro to S5A8 coupled to recombinant A chain of ricin. This sIg^+ variant lost binding to antibody S5A8 but retained binding to S1C5. Binding in all these cases was assessed on whole cells by flow cytometry and confirmed by ELISA on Ig proteins isolated from the variant tumor cells (see below). These characteristics are summarized in Table I.

To recover large amounts of protein and mRNA for cDNA synthesis, variant tumor cell lines were fused to the nonsecreting mouse myeloma 8.653 as previously described (19, 23). Hybrids were selected with HAT medium. The absence of 2-ME prevented the growth of parental tumor cells. The resulting hybrids were screened for Ig production and were cloned by limiting dilution. Protein was isolated by affinity chromatography and anti-Id binding was examined by the ELISA method (19). In each case the Id determinants expressed on the tumor cell surface and by the isolated protein were identical.

Southern Analysis of Rearranged V_κ Genes. High molecular weight DNA was isolated from the original 38C13 lymphoma, the tumor variants V1–V4, their respective hybridomas, the P3x63 8.653 myeloma fusion partner, and C3H liver. 10 μg of DNA was digested with Bam HI restriction endonuclease (New England Biolabs, Beverly, MA) and loaded on a 0.8% agarose gel. Samples were transferred to an activated nylon filter (Genatran 45; Plasco Inc., Woburn, MA) according to the method of Southern (24). Filters were baked under vacuum at 80°C for 2 h. Prehybridization was carried out at 42°C for 6 h in 50% formamide, 3× SSC, 5× Denhardt's solution, 1 mM sodium pyrophosphate, and 100 μg/ml of denatured salmon sperm DNA.

Hybridization was performed under identical conditions with the exception of the addi-

[1] *Abbreviations used in this paper:* Id, idiotype; sIg, surface immunoglobulin.

TABLE I
mAb Reactivity of Tumor Subclones

Tumor	mAb		
	ακ	S1C5*	S5A8*
38C13	+	+	+
V1	+	−	−
V2	−	−	−
V3	+	−	+
V4	+	+	−

* Syngeneic antiidiotype mAbs raised against the 38C13 Ig protein.

tion of 8% Dextran sulphate and the radioactive probe. The filter was washed in 2× SSC 0.1% SDS at room temperature for 1 h followed by a more stringent wash in 0.1 × SSC/0.1% SDS at 62°C for an additional hour. Two probes were used to analyze κ rearrangements: a 500-bp Eco RI–Hpa I constant region cDNA clone and a Hind III 2.8-kb genomic clone encompassing the entire germline J_κ region (5). Probes were labeled to high specific activity using the random primer method (25).

Northern Analysis of TdT Expression. Poly(A)$^+$ mRNA from 38C13 and from EL-4 cells (a gift from D. Daney, Stanford University) was electrophoresed on a formaldehyde-agarose gel and transferred to a nitrocellulose filter as described (26). The filter was baked, prehybridized, hybridized, and washed as described for Southern hybridization. The mouse TdT probe (27) was a 1.8-kb insert in pUC13 and was a gift from N. Landau, University of California, School of Medicine, San Francisco, CA.

Cloning and Sequencing Variant V_H and V_κ Genes. cDNA libraries were made from each hybridoma generated from the sIg$^+$ tumor variants. Southern blot analysis of rearranged heavy and κ light chain genes showed identical rearrangements in both the parental tumor line and the representative hybridoma, which insured that fidelity of Ig gene structure was preserved through the fusion process (19). cDNA libraries were made using a highly efficient method described in detail previously (28). RNA was extracted as previously described (29) and poly(A)$^+$ RNA was selected over an oligo-dT-cellulose column (30). First strand cDNA was made using reverse transcriptase (Life Sciences, St. Petersburg, FL) and primers specific for the μ heavy chain (5′-CAGGAGACGAGGGGGAA-3′) and κ light chain (5′-TGGATGGTG-GGAAGATG-3′) constant regions just 3′ to the variable region. Second strand was synthesized with DNA polymerase I (New England Biolabs) and RNase H (Bethesda Research Laboratories, Gaithersburg, MD) (31). The resulting double-stranded cDNA was polished with T4 DNA polymerase (New England Biolabs) and ligated directly into a Sma I-digested (New England Biolabs), CIAP (Boehringer Mannheim Biochemicals, Indianapolis, IN)-treated (32), m13mp19 sequencing vector (33). JM101 bacteria were made competent by the Hanahan method and were transformed with the recombinant m13 (34). The resulting plaques were lifted onto nitrocellulose filters twice and filters were baked for 2 h at 80°C under vacuum. Filters were prehybridized, hybridized, and washed as described above for Southern hybridization. Filters were dried and exposed to XAR film (Eastman Kodak Co., Rochester, NY) for 1–6 h. Positive clones were identified and single-stranded sequencing templates were made by standard techniques (33). Clones were sequenced by the dideoxy method (35). If the isolated clones were in one orientation only, m13 replicative form was isolated and the insert was cloned in the opposite orientation (using flanking restriction sites Eco RI, Bam HI) into m13mp18.

V region-specific probes for plaque screening were obtained as follows. The 38C13 μ heavy chain gene had previously been isolated from a λgt11 total cDNA tumor library (36). An Eco RI/Sma I fragment encompassing the entire V region and a portion of CH1 was used to screen and isolate μ clones from each variant cDNA library. A 550 bp Hpa I/Eco RI V_κ region (37) was used as a probe for the isolation of κ clones from the variant tumor libraries.

Results

Tumor Variants Display Heterogeneous V_κ Rearrangements and Identical Reciprocal Product. We previously reported on Southern blot analysis of the rearranged V_κ alleles in these tumor variants using a constant region probe (Fig. 1 *A*) (19). Each tumor shares an identical pattern of rearrangement with its respective hybridoma and demonstrates that Ig gene structure was preserved through the fusion process. In addition, each hybrid retains 7.2-kb and 5.2-kb rearrangements inherited from the myeloma fusion partner 8.653. Uncloned 38C13 lymphoma showed a major 8.9-kb band along with three other less prominent rearrangements (8.2, 3.7, and 3.4 kb). Each variant displayed two rearrangements, some of which were also identified in the 38C13 tumor. The pattern of rearrangement differed among the tumor variants, although in some cases they appeared to share a single rearrangement. The same filter was washed and reprobed with a genomic fragment containing the J_κ region (Fig. 1 *B*). Each of the bands detected with the C_κ probe in the tumor DNA also hybridized to the J_κ probe with the exception of the lower faint rearrangement (3.4 kb) in the 38C13 tumor. Therefore, these bands represent V/J rearrangements linked to the κ constant region segment. In addition, a larger ~15-kb band was seen with the J_κ probe in 38C13 and in each tumor variant. This band did not hybridize to the constant region probe and indicates that a portion of the J_κ sequence was retained in the genome and was displaced from its normal association with C_κ. The constancy of this reciprocal gene product provides an independent confirmation of the clonal origin of these tumor variants. The 5.2-kb band that originated from the 8.653 fusion partner did not hybridize with the J_κ probe.

All Tumor Variants Contain Identical Heavy Chain V Region. Full-length V_H clones were isolated from the V1 and the V4 variant tumor cDNA libraries. A partial sequence from the V3 variant tumor was also determined. All of these sequences are identical (Fig. 2). Further comparison of those V regions with the published 38C13 sequence (36) demonstrated some minor differences. Therefore, the original 38C13 μ V region clone was resequenced in both orientations. It was identical to the variants described here and the previously reported 38C13 heavy chain sequence is now displayed in its corrected form (Fig. 2). This V_H gene, used by the 38C13 tumor and its variants, is most homologous to the previously reported germline VB6.5 gene of the T15 family derived from a mouse of the B10.P strain (38). The two genes differ from each other in three positions. These differences might be explained by a germline difference between the C3H and the B10.P mice or by somatic mutations of the expressed 38C13 V_H gene. However, since the parental V_H sequence and those of all the variants, which have been separated by years in culture, are identical, there is no evidence of somatic mutation in the tumor.

Thus, the original 38C13 tumor and the three sIg^+ variants are all clonally related. They use the same heavy chain V gene and have identical V/D and D/J joints. In addition, the V_HDJ sequences are all identical and, therefore, there is no evidence for somatic mutation of the heavy chain in this tumor. The differences in anti-Id binding in these variants (and thus the emergence of antibody resistant tumor subclones) are not explained by differences in the heavy chain. These findings corroborate the protein and *Ig* gene rearrangement analysis that we reported previously (19).

All Tumor Variants Use a κ Light Chain Gene Different from the Original 38C13. Full-

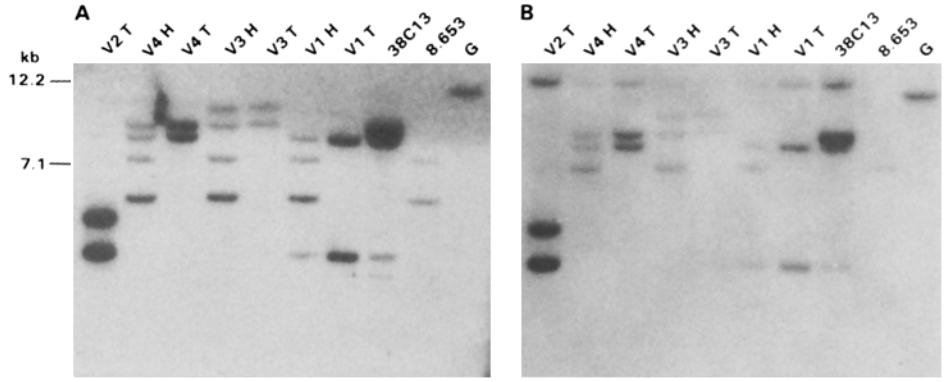

FIGURE 1. Southern blot analysis of L chain gene rearrangements. DNA was isolated from the 38C13 tumor, variant tumors (*T*) and respective hybridomas (*H*). The DNA was digested with Bam HI and probed with C_κ (*A*) and J_κ (*B*).

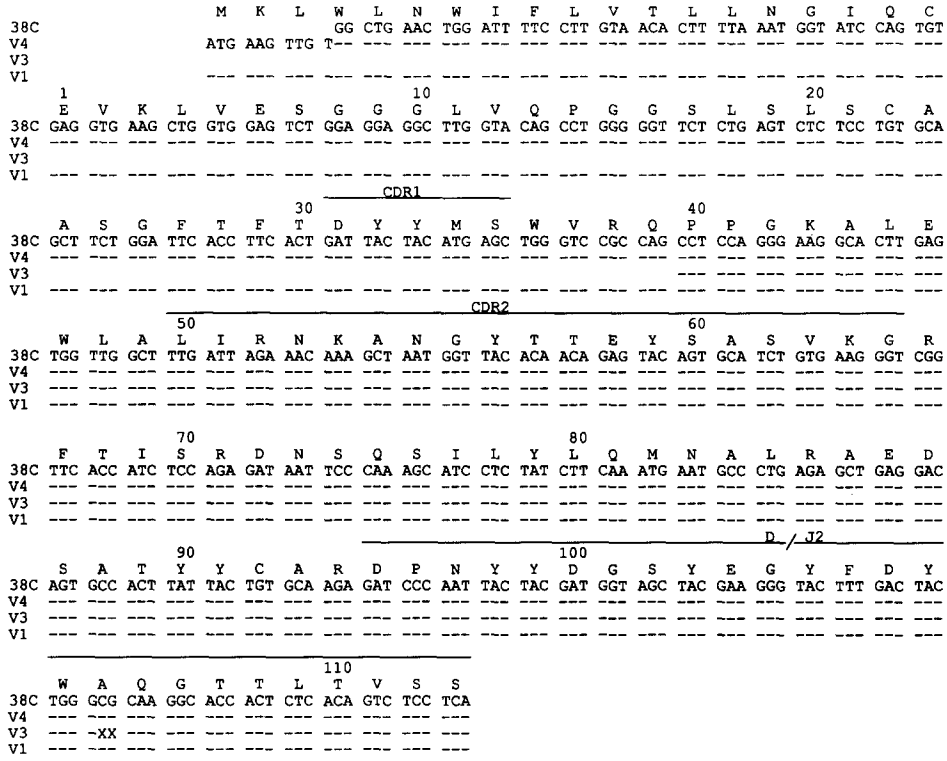

FIGURE 2. Nucleotide sequences of V_H DNA. The amino acid translation is given above the nucleotide sequences and numbered according to Kabat et al. (42). The complementarity-determining regions CDR1 and CDR2 and the D and J segments are indicated. X, ambiguous bases.

length κ V region clones were isolated from all sIg$^+$ tumor variants. The nucleic acid sequences are shown in Fig. 3 and the translated amino acid sequence is displayed in Fig. 4. All the v_κ genes expressed by the variants were similar to one another and appear to be representatives of the highly complex V_κ-Ox1 family (39). In fact, the sequence of V1 differs by only a single nucleotide from the previously reported BALB/C germline gene, R2 (39). Moreover the two κ light chain genes from tumor variants V3 and V4 were identical to each other in their V segments and their leader sequences. This V region gene differed by only five nucleotides from the previously reported NQ10.4.61 antiphenyloxazolone-producing hybridoma, de-

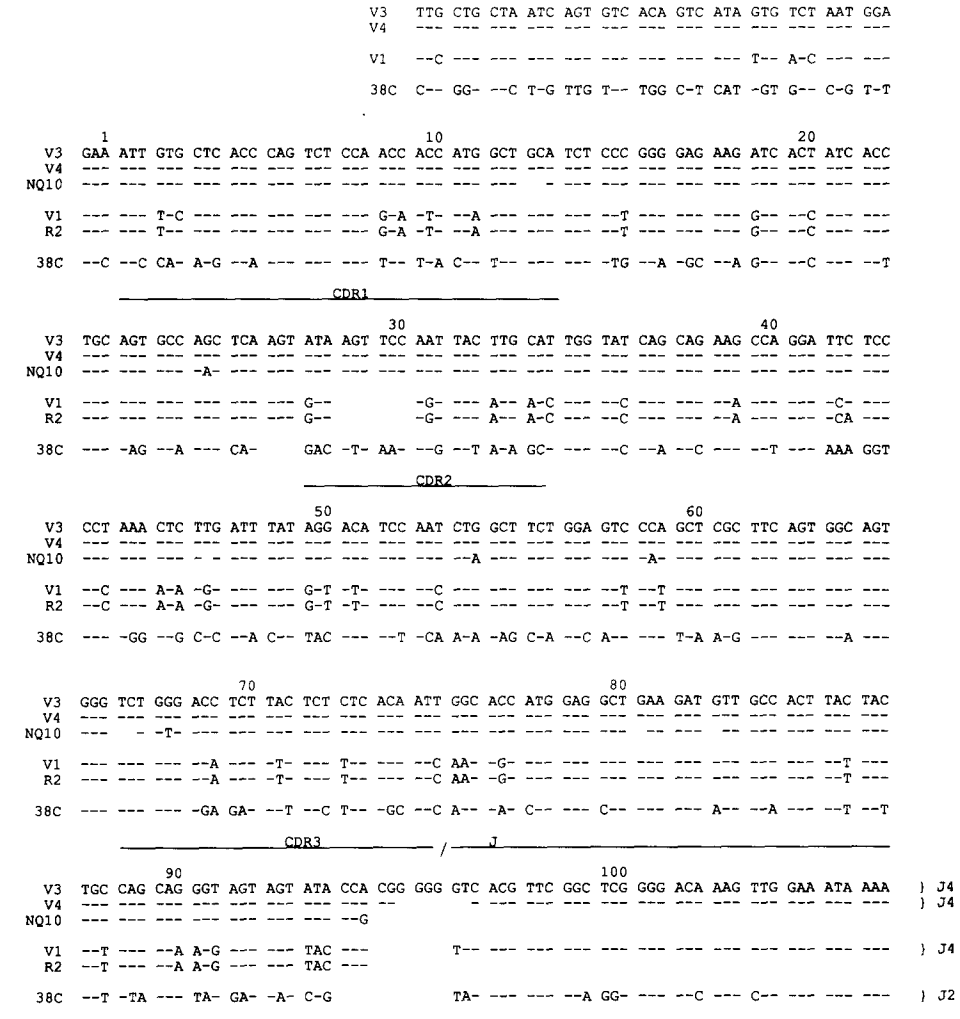

FIGURE 3. Nucleotide sequences of V_κ DNA. The nucleotides are grouped in codons and numbered according to Kabat et al. (42). The CDRs are indicated, as well as the J regions used by the 38C13 tumor (38C) and its variants. R2 and NQ10 (NQ10.4.61) are previously reported germline and mRNA sequences, respectively (39), and are both members of the V_κ-Ox1 family.

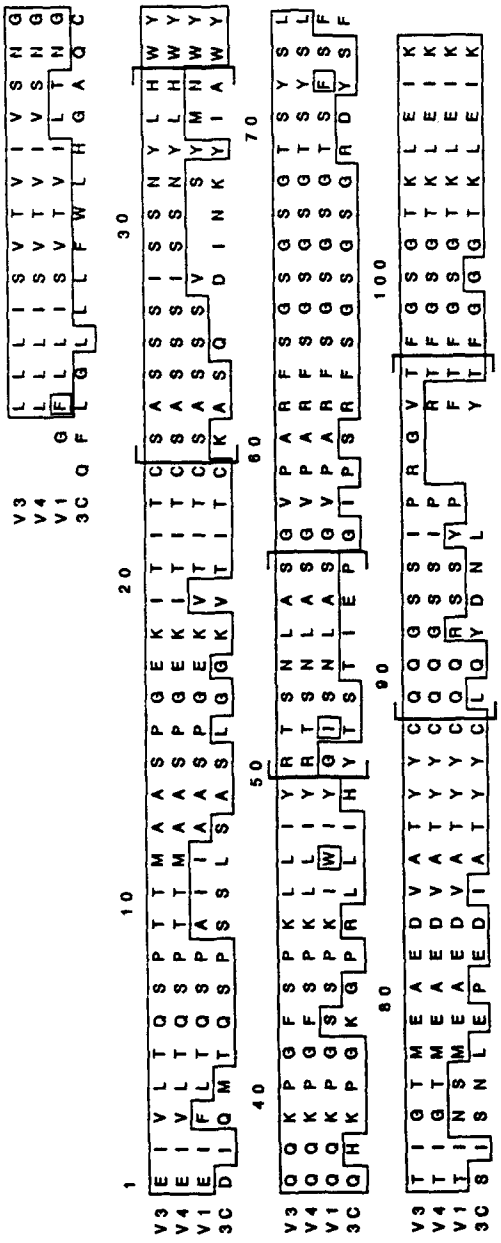

FIGURE 4. Amino acid translation of the V_κ tumor variants and the 38C13 tumor (3C).

rived from another member of the V_κ-Ox1 gene family (39). By comparison, the parental 38C13 tumor expressed a completely different V_κ gene, a member of the $V_\kappa 9$ family (40). All of the variants used $J_\kappa 4$. By comparison, the parental 38C13 tumor use $J_\kappa 2$.

Close inspection of the third hypervariable region reveals considerable variation in length among variants and 38C13. This is highly unusual since κ CDR3s tend to be constant in length (41), in contrast to the variation in length of the CDR3 region of Ig heavy chains (42). Fig. 3 illustrates the contribution of V region and J_κ segments to CDR3. In V3 and V4 there are additional GC nucleotides present at the V/J boundary. Such nucleotides are highly unusual for κ light chains and have only rarely been reported previously (41, 43, 44). Since tumors V3 and V4 share identical heavy chain sequences and V_κ regions, and both rearrange to J4, the variable addition of these extra nucleotides at the exact V/J recombination site is responsible for their different pattern of anti-Id binding.

38C13 Expresses Terminal Transferase. Poly(A)$^+$ mRNA from 38C13 cells and from EL-4 cells, which are known to express TdT, were electrophoresed on a formaldehyde-agarose gel and transferred to a nitrocellulose filter. The filter was probed with the mouse TdT probe (Fig. 5). Lanes *1* and *2* contain 0.5 μg and 1 μg of EL-4 mRNA. Lane *3* contains 8.5 μg of 38C13 mRNA. It is evident that 38C13 cells express TdT mRNA, albeit in ~1/10 of the level expressed in the EL-4 cells.

Discussion

In a previous report we described the isolation of variant tumor cells from the 38C13 B cell lymphoma after immunoselection with antiidiotype antibodies (19). These variant tumor cells were of two major types, those that had completely lost expression of Ig and those that continued the expression of Ig with an altered structure. Analysis of the Igs produced by these latter variants suggested that the μ heavy chains were similar to the wild-type tumor but that the κ chains were different in apparent molecular weight. Analysis of the *Ig* gene rearrangements in these cells suggested that the heavy chain genes expressed in these cells were identical but that the light chain genes were different. A number of possible explanations were considered for these differences in light chain structure. Among these were the possibility

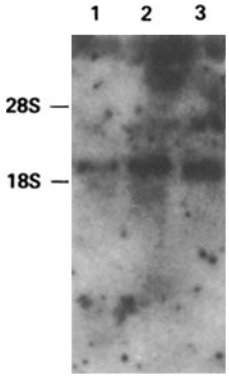

FIGURE 5. Northern blot analysis of TdT expression. mRNA from EL-4 and 38C13 cells were electrophoresed on a formaldehyde-agarose gel and transferred to nitrocellulose filter. The filter was probed with a mouse TdT probe (27). (Lanes *1* and *2*) 0.5 μg and 1.0 μg of EL-4 mRNA, (lane *3*) 8.5 μg of 38C13 mRNA. Markers indicate migration of 28S and 18S RNA.

of somatic point mutation, and continuing or alternative rearrangements at the light chain locus.

In the present report, we directly analyzed the molecular basis of the Ig heterogeneity in these tumor variants. cDNA was cloned for both the heavy chain and light chain genes from each of the variant tumor cells and the original wild-type cell population. Comparison of the heavy chain sequences showed that they were identical in all of these cells. This result establishes that the 38C13 tumor cell population is not undergoing V gene somatic mutation. In addition, the results indicate that the B cell that gave rise to the tumor had not mutated its heavy chain gene to any significant extent because it differed by only three nucleotides from a known germline V_H gene VB6.5 (38). Similar arguments can be made from the lack of somatic mutation in the light chain genes. The identity of the V_H sequences confirmed the previous suggestions from protein and Southern blot analysis that these tumor cells were all derived from a single original cell, that is that they are clonally related. Further confirmation of the clonality of the cell populations comes from analysis of light chain gene rearrangements using the J_κ region probe (Fig. 1 *B*). In addition to the bands that comigrated with those detected by the C_κ probe there was an additional band, detected with the J_κ probe, which was common to all of the variant tumor cells and to the parental cell. Rearranged fragments of DNA containing J sequences are known to be produced during the process of V/J joining when the V region used is in inverted orientation with respect to the J and constant region segments (45). Such reciprocal products of V/J joining can be retained in their original form (46).

From these data it appears that a plausable order of events in the cell that gave rise to this tumor were the rearrangement of a unique heavy chain V region gene followed by V_κ rearrangement on both alleles; either one of which could have given rise to a J_κ reciprocal product. The absence of a common C_κ band in tumor variants would then imply that subsequent rearrangements occurred at both κ alleles in progeny cells. Ongoing light chain rearrangements have been described previously in an Abelson leukemia virus-transformed B cell line. Lewis et al. (47) showed by Southern blot analysis that a rearranged κ allele can undergo further recombination. Recently an Ableson leukemia virus pre-B cell line has been described that is capable of proceeding to λ recombination (48). Future studies on the 38C13 tumor should be able to test the possibility that the recombinase system (49) may still be active in these cells and that ongoing κ light chain recombination may be occurring. If this were the case variant tumor cells should give rise to subsequent variants in culture. If so, it will be extremely interesting to determine whether the repetitive use of the V_κ-Ox1 gene family observed in the current set of data continues to occur.

Examination of the sequences of the productive V_κ genes revealed some extremely interesting phenomena. We noticed that the V region sequence of the variant V3 and the variant V4 were identical to each other and that each of these tumors had rearranged this identical V region gene to the same J4 segment. The only difference between these genes is in the length of their CDR3 region. Each of them has additional nucleotides composed mostly of Cs and Gs in the joint between the V and J segments. V3 has an unusually long CDR3 length composed of 11 amino acids. Although the addition of extra nucleotides at the V/D/J joint is commonly seen in heavy chain gene rearrangement, it has only rarely been observed in light chain genes. In general V/J recombination takes place between codon 95 of the V_κ segment and

the first codon, 96, of the germline J_κ segment. The exact splice junction may be located in either codon, but compensation occurs so that a CDR-3 length of nine amino acids results. However, additional nucleotides may be present 3′ to the germline V_κ segment and 5′ to germline J_κ located just adjacent to the respective conserved heptamer recombination sequence. In murine V_κ light chains one to two such nucleotides may be present and are usually Cs, sometimes As (44). These nucleotides have been shown to participate in V/J joining occasionally. Interestingly, Kaarttinen and Makela (41) have observed that a substantial portion of the murine $V_\kappa O_x$ response consists of light chains with extra long CDR-3 segments composed of 11 amino acids, similar to V3 reported here (41). They noted the presence of up to five extra nucleotides (mostly C) at the $V_\kappa J_\kappa$ joint and postulate that these are contributed by an unusually long string of germline nucleotides 3′ to V_κ before the heptamer. Up to seven nucleotides would have to be postulated to contribute to the extra segments observed in V3, and here they are mostly Gs instead of the more commonly observed Cs. Alternatively, the addition of extra nucleotides, so called N regions, in heavy chains is thought to be mediated by the action of the enzyme terminal deoxynucleotide transferase (TdT) (27). This enzyme has a preference for Gs and Cs and is thought to be inactivated by the time B cells begin to rearrange their light chain genes. Recently Heller et al. (43) described nucleotides not encoded by germline V genes or J regions at the site of V_κ/J_κ recombination in antigalactan antibodies. In addition, Klobeck et al. (44) also found a GC-rich region at the V_κ/J_κ joint in an aberrant rearrangement involved in a (2:8) translocation in a Burkitt's lymphoma cell line. Our finding of the message for TdT in the 38C13 tumor cells provides an explanation for the addition of N sequences at the V_κ joints in these cells and violates the notion that TdT is absent from cells of the fully differentiated B cell stage.

Since the V_κ/J_κ joints in variant V3 and variant V4 are different, these genes must have been created by independent joining events in the different subclones of this tumor, even though the same V gene was used. Interestingly, the V gene used by these two variant tumor cells is virtually identical to a gene used by a hybridoma with a binding activity for phenyloxazolone and is a member of the V_κ-Ox1 gene family (39). Inspection of the nucleic acid sequence of variant V1 shows that a different V_κ gene has been used by this variant, but that it is virtually identical to another previously reported member of the V_κ-Ox1 gene family R2 (39). All of these genes of variants V1, V3, V4, and the two previously reported members of the V_κ-Ox1 family are completely different from the gene used by the original 38C13 tumor cell, which is a member of a different V gene family. Therefore, it appears that this tumor has chosen alternative rearrangements at the V_κ light chain locus to create a number of different subclone variants of the prototype tumor cell, and that all of these variants have been created by the use of genes from the same V region family. It is possible that the repetitive use of the same gene family for rearrangements is based on the proximity of the V_κ-Ox1 gene family to the J region. Subsequent studies on the organization of the V_κ locus as well as that of the locus in the 38C13 tumor cell should be able to provide a test of this proximity hypothesis.

These studies on the sequence of the expressed V_κ genes in variants of the 38C13 tumor have provided an explanation for the idiotypic heterogeneity of the tumor population. As previously suspected from studies on the isolated light chain proteins and on Southern blot analysis of the light chain genes, the tumor heterogeneity

at the Id level is completely accounted for by variations in the amino acid sequence of the light chains. This variation can be extremely subtle. For instance, the entire difference between variant V3 and V4 is accounted for by three amino acids at the V/J joint of the light chain. Yet these two variants are easily distinguishable by a panel of syngeneic antiidiotype mAbs. It is clear from these studies that at some time in the history of this tumor alternative light chain gene rearrangements rather than somatic point mutation occurred and created Id diversity within the tumor cell population.

Summary

Idiotype variants of 38C13, a murine B cell lymphoma, have been isolated by immunoselection with antiidiotype mAbs. The V region genes for the κ light chains and μ heavy chains expressed by these tumor cells were sequenced and compared. There was no evidence for V region somatic point mutation in this tumor. However, while the heavy chain genes were all identical, the light chain genes were all different. The light chain genes of each variant were derived from the V_κ-Ox1 gene family and joined to $J_\kappa 4$, whereas the light chain gene of the parental tumor was derived from the $V_\kappa 9$ family and joined to $J_\kappa 2$. Two of the variants used the identical V_κ gene but differed by the inclusion of a variable number of additional nucleotides in the V/J joint. Thus, the idiotypic heterogeneity of this B cell lymphoma arises as a consequence of alternative light chain rearrangements rather than point mutation. This process repetitively uses members of the same V_κ gene family. Two of the variants use the identical V_κ and J_κ gene segments but differ by the presence of extra nucleotides at the V_κ/J_κ joint.

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