AUTOANTIBODY-ASSOCIATED *K* LIGHT CHAIN VARIABLE REGION GENE EXPRESSED IN CHRONIC LYMPHOCYTIC LEUKEMIA WITH LITTLE OR NO SOMATIC MUTATION

Implications for Etiology and Immunotherapy

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Chronic lymphocytic leukemia (CLL),[1] the most common leukemia, may be considered a malignancy of the CD5 (Leu-1) B cell. The small lymphocytes that accumulate in the peripheral blood of affected patients generally coexpress both sIg and the pan-T cell surface protein CD5 (1–3). Patients with CLL may have seemingly paradoxical, yet coincident, immunologic defects of impaired humoral immunity and augmented autoimmune reactivity (4). Furthermore, it has been reported that a high proportion of CLL cells express surface IgM (sIgM) with rheumatoid factor (RF) autoantibody reactivity (5). In contrast to other leukemias, the incidence of CLL is not increased after ionizing radiation, but rather displays a familial tendency, suggesting that genetic factors possibly contribute to the pathogenesis of this disease (6). Recently, genetic factors have been found to influence the proportions of circulating lymphocytes that coexpress CD5 and sIg in normal adults (7). Such normally occurring CD5 B cells have been implicated in the regulation of Ig production by other B cells, and as an important source of IgM autoantibodies (8–11). Understanding the nature of Ig variable region gene expression in CLL may provide insight into the physiology of autoantibody production by the CD5 B lymphocyte.

Recently, we discovered that a surprisingly high proportion of patients with CLL have malignant cells reactive with a murine monoclonal antiidiotypic antibody, 17.109 (12). This antibody, prepared against the IgM RF cryoglobulin, SIE, recognizes a κ light chain–associated crossreactive idiotype (CRI) present on over one-third of all human IgM RF paraproteins isolated from unrelated patients with Waldenstrom's macroglobulinemia or cryoglobulinemia (13–15). In contrast, the 17.109 antibody does not react substantially with either normal pooled Ig or circulating B lymphocytes from normal adults (12, 15). However,

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[1] *Abbreviations used in this paper:* CDR, complementarity determining region; CLL, chronic lymphocytic leukemia; CRI, crossreactive idiotype; RF, rheumatoid factor; sIg, surface Ig.

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in a survey of >30 patients, 5 of the 20 (25%) κ light chain–expressing CLL reacted with this antibody (12).

To determine the precise molecular basis for the 17.109-CRI expression in CLL, we performed nucleic acid sequence analyses of the κ light chain variable regions expressed by 17.109-reactive leukemic B lymphocytes from two unrelated individuals. We found striking homology between the κ variable region genes (V_κ genes) expressed by these leukemic B cells. Furthermore, these expressed V_κ genes are homologous to a nonrearranged V_κ gene that previously had been isolated from placental DNA and theorized to encode κ variable regions of IgM autoantibodies (16). The implications of these findings relative to the physiology of the CD5 B cell, and the etiology and potential immunotherapy of CLL are discussed.

Materials and Methods

Cells. Leukemic cells were isolated from the peripheral blood of two patients (HAH and HIC) with sIgMκ CLL previously noted to react with the 17.109 antibody (12). Before RNA isolation, a sampling from the cells was tested for expression of κ sIg bearing the 17.109-CRI, using R-phycoerythrin-conjugated 17.109 (PE-17.109) and fluorescein-conjugated murine mAb specific for the constant portion of the human κ or λ light chain (12).

RNA Sequencing. Polyadenylated RNA was isolated on oligo-dT–cellulose from total RNA extracted from 17.109^+ CLL using 6 M guanidium isothiocyanate (17, 18). Oligonucleotides dCTCACAGATGGCGGGAAGATGAA, dAACCTGTCTGGGATGC, or dGGGTGCCTGGAGACTG, corresponding to the κ constant region (positions 116–124), third (positions 58–63) or first (positions 6–11) variable region frameworks, respectively, were used to prime cDNA synthesis of 10–12 μg of polyadenylated RNA for sequencing by the dideoxynucleotide chain-termination method using α-[^{35}S]ATP, α-[^{32}P]CTP or α-[^{32}P]ATP (New England Nuclear, Boston, MA) as described (19, 20). Different radiolabeled nucleotides and reaction mixtures were used to resolve sequencing ambiguities.

cDNA Isolation. 4 μg of polyadenylated RNA were used to construct a cDNA expression library of $>2.6 \times 10^6$ independent recombinants in λgt10 as described (21). Recombinant λ phage plaques lifted onto nitrocellulose were hybridized with ^{32}P-labeled synthetic oligonucleotides specific for the constant and variable region of the Ig κ light chain using conditions described previously (12, 21). Desired recombinant phage were isolated to homogeneity. The insert DNA was purified and then ligated into the polylinker site of a pUC19 plasmid (Bluescribe; Vector Cloning System, San Diego, CA) as described (22).

DNA Sequencing. *Escherichia coli* strain JM83 was transformed with the ligated plasmid DNA using the procedure of Hanahan (23). We identified colonies with plasmids containing the desired recombinant insert and extracted the plasmid DNA for dsDNA sequencing as described (24). Oligonucleotides used in mRNA sequencing were each used to prime DNA synthesis for sequencing using α-[^{35}S]CTP (New England Nuclear) as described (20, 25). Computer programs of the University of Wisconsin Genetics Computer Group were used to analyze sequence data and to prepare figures on a Digital VAX750 computer.

Oligonucleotides. Oligonucleotides were as described (12). Labeling the 5′ ends of the oligonucleotides with γ-[^{32}P]ATP was achieved using polynucleotide kinase (26).

Results

Dual immunofluorescence staining of the CLL cells from patients HAH and HIC demonstrated that all leukemic cells expressed CD5, sIg, and the 17.109 idiotope (Fig. 1). The staining intensity of HAH with PE-17.109 was ~10-fold greater than the PE-17.109 staining intensity of HIC. In dual immunofluorescence studies, however, the relative staining intensity of PE-17.109 was propor-

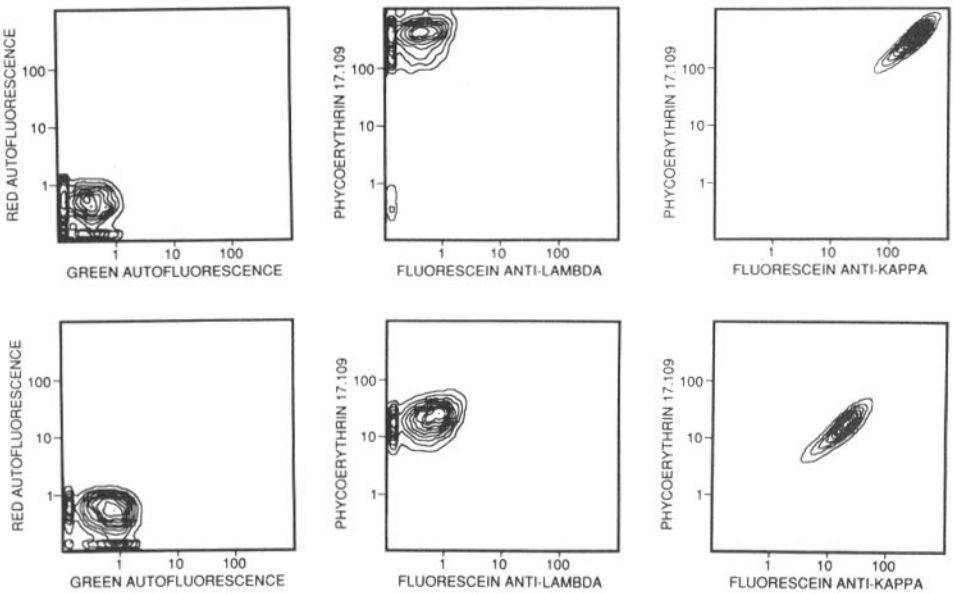

FIGURE 1. Dual immunofluorescence detected at 572 nm (red, ordinate) and 535 nm (green, abscissa) of peripheral blood lymphocytes from patient HAH (*top row*) or HIC (*bottom row*). Depicted are the contour histograms of the autofluorescence of HAH and HIC cells (*left*), of cells stained with PE-17.109 and FITC-(IgG1 anti-λ light chain) (*middle*), or PE-17.109 and FITC-(IgG1 anti-κ light chain) (*right*).

tional to that of an anti-κ constant region FITC-labeled mAb. Thus, quantitative differences in the staining intensity of HIC and HAh with the 17.109 antibody were a function of the relative amounts of sIg expressed and not of qualitative differences in the ability of the 17.109 antibody to bind to the different cell populations.

Nucleic acid sequence analyses of polyadenylated RNA isolated from the CLL cells of each patient demonstrate that the κ variable regions are highly homologous (Fig. 2). Complete resolution of the nucleic acid sequence of the V_κ RNA derived from HIC was possible through repeated sequencing reactions using different oligonucleotide primers. Because of limiting quantities of polyadenylated RNA, multiple sequencing reactions of the RNA from HAH using each of the synthetic oligonucleotides were not possible. The nucleic acid sequence derived from priming reactions with the oligonucleotide complementary to the sense strand of the κ constant region (positions 116–124) revealed one base difference in the HAH sequence from that of HIC at position 93 in the third complementarity determining region (CDR3), resulting in a structurally conservative amino acid substitution of Thr for Ser. Despite different reaction mixtures, the sequence of both RNAs could not be resolved for one base in the codon for the amino acid at position 81, numbering according to Kabat et al. (27). This is consistent with the two RNAs having similar secondary structure through sequence homologies. From the data in Fig. 2, it can be deduced that the leukemic cells from both patients use a nonmutated $J_\kappa 1$ segment in their light chain RNA

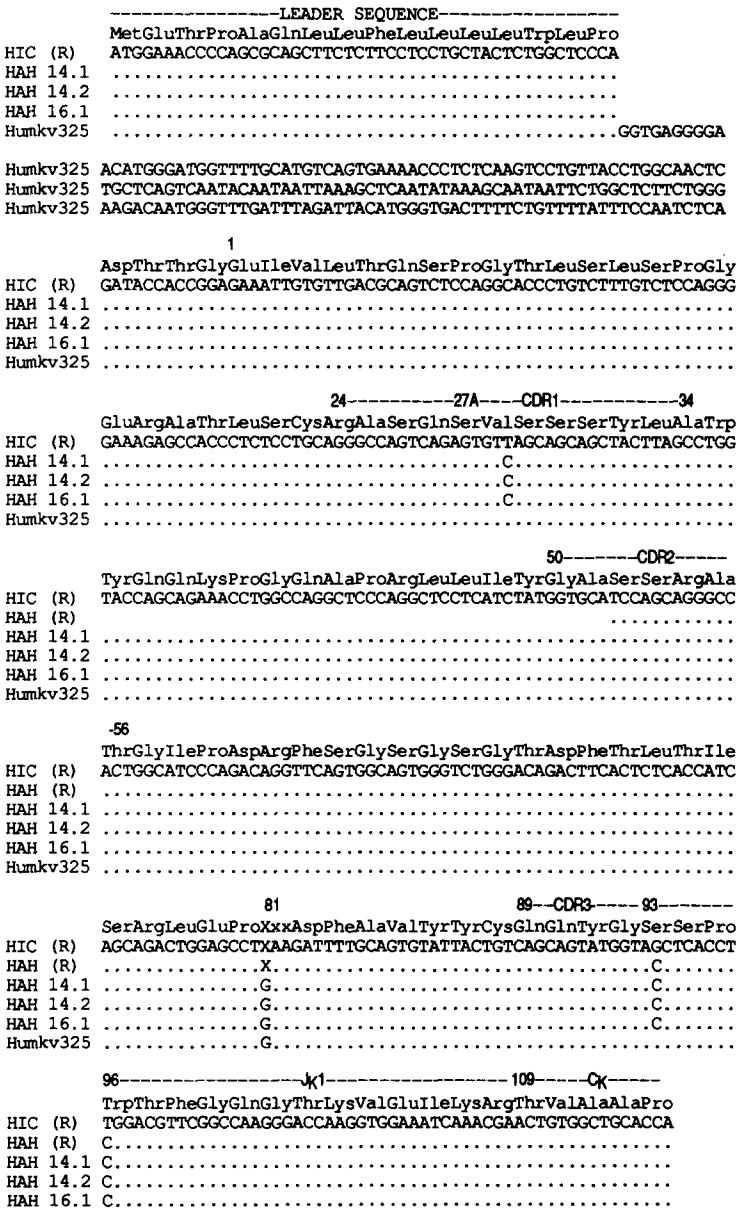

FIGURE 2. Nucleic acid sequence comparison of 17.109-reactive CLL (HIC and HAH) and *Humkv325*. Listed to the left are the names of the nucleic acid sequences. Sequences derived from RNA sequencing are listed by the name followed by an *R*. Sequences *HAH 14.1, HAH 14.2* and *HAH 16.1* are independent κ light chain cDNA clones derived from HAH polyadenylated RNA. Below the nucleic acid sequence of HIC are depicted nucleotides at positions where there exists differences in the compared sequence. Dots indicate sequence homology, *X* indicates sequence base ambiguity. Genomic bases of *Humkv325* between the leader sequence and the coding region that are deleted during mRNA processing are listed without comparison to the other sequences. Above the nucleic acid sequence is depicted the three-letter code of the deduced amino acid sequence for HIC. The deduced amino acid sequence of HAH is identical to that of HIC except at position 93, where HAH encodes Thr instead of Ser, and at position 96, where HAH encodes Arg instead of Trp. HAH encodes Glu at position 82, where the sequence of HIC could not be resolved with certainty. Listed above the deduced amino acid sequence are amino acid position numbers according to Kabat et al. (27) and descriptions indicating the position of the leader sequence, the CDR1, CDR2, and CDR3 and the J region. These sequence data have been submitted to the EMBL/Gen Bank Data Libraries under the accession number Y00648.

transcripts (28). Differences between HIC and HAH at position 96 may be attributed to junctional diversity.

To ascertain the complete nucleic acid sequence of the expressed light chain variable region of HAH and to evaluate for potential V_κ RNA transcript heterogeneity, we constructed an expression library in λgt10 of cDNA generated from the polyadenylated RNA of HAH. $>10^6$ independent recombinant phage plaques from a nonamplified cDNA library were screened using ^{32}P-labeled oligonucleotides specific for the κ constant region (12). Nitrocellulose filter–immobilized DNA from several independent plaques hybridized intensely with this probe. Such plaques were isolated to homogeneity and their insert DNA was subcloned into the pUC19 plasmid vector for dsDNA sequence analyses.

Three independent full-length κ light chain cDNA clones were analyzed. The complete nucleic acid sequences of the V_κ regions of each of these isolates were identical, consistent with the Ig RNA transcripts in the population of leukemic CLL cells having limited heterogeneity (Fig. 2). These sequences agreed with the RNA sequence data of HAH, and demonstrated >99% nucleic acid sequence homology with the V_κ mRNA sequence expressed by leukemic cells from HIC.

The V_κ sequences expressed by HIC and HAH show striking homology with a nonrearranged V_κ gene recently isolated from placental DNA, designated *Humkv325* (or V_κRF) (16). The coding region of *Humkv325* is identical to the elucidated cDNA V_κ gene sequence of HIC and is >99% homologous to that of HAH (Fig. 2). In addition, the deduced leader sequences of all three V_κ genes are identical. Such sequence homology strongly argues that the leukemic cells of HIC and HAH express *Humkv325* with little or no somatic mutation.

Comparison of the deduced amino acid sequences of the CLL κ light chains with the reported κ light chain variable region sequences of several 17.109^+ RF paraproteins isolated from patients with Waldenstrom's macroglobulinemia demonstrates close homology (Fig. 3) (27, 29–33). The V_κ gene encoded κ light chain variable region of the immunogen used to induce the murine 17.109 antibody, SIE, differs from the deduced HIC or HAH sequence by two or three V_κ gene–encoded amino acids, respectively, this representing >97% sequence homology. Even greater homology is noted between the deduced cDNA sequences and V_κ sequences of other 17.109^+ IgM RF paraproteins, GLO, CUR, and GAR (Fig. 3). The amino acid sequence encoded by the V_κ gene for each of these paraproteins differs from HAH only at position 93, these paraproteins having Ser at this position rather than the structurally similar Thr. In none of the sequenced 17.109^+ κ light chains does there appear a Thr at this position. However, Thr is found at position 93 in a few other sequenced V_κIII proteins not tested for reactivity with 17.109 (27). Thus we can not exclude the possibility that the Thr at position 93 of HAH results from genetic polymorphism in the *Humkv325* gene rather than from a point mutation in the Ser codon.

The J regions of most 17.109^+ κ light chain paraproteins are probably encoded by the same J_κ segment used by HIC and HAH, namely J_κ1 (27–33). The exceptions are proteins GOT and GAR, which most likely use the structurally similar J_κ2 segment, and KAS, which most likely uses J_κ4. Differences between these proteins at position 96 reflect junctional diversity occurring during the rearrangement of the V_κ gene with the J region. Whether there is an obligatory

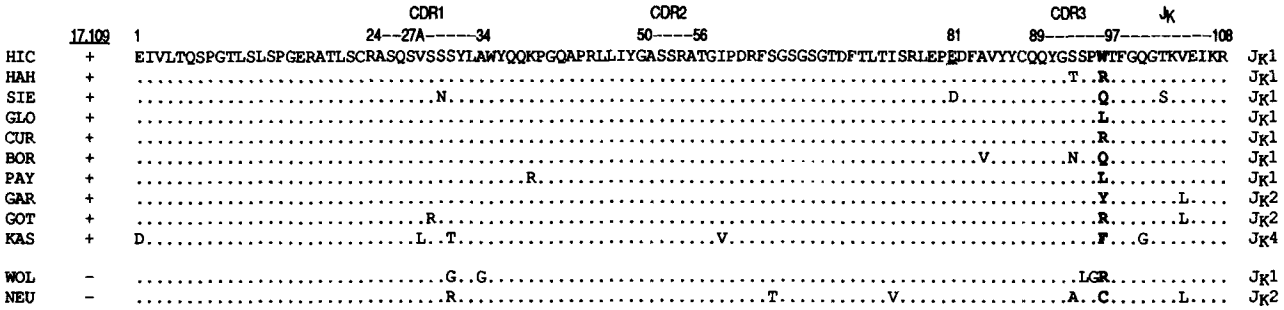

FIGURE 3. Comparison of the deduced κ variable region sequences of 17.109-reactive CLL (HIC and HAH) and κ light chains from RF paraproteins tested for reactivity with 17.109 (5–8, 21). Listed to the left are the names of the amino acid sequences. Below 17.109 is listed the reactivity of the protein with 17.109, a (+) indicating reactivity and a (–) indicating no reactivity (3, 4). The complete amino acid sequence of HIC is depicted in the single-letter code and numbered according to Kabat (27). Although this sequence could not be resolved at position 81 (underlined residue), the data are consistent with there being an E (Glu) at this position. Below the sequence of HIC are depicted the amino acids of the other sequences only at positions where they differ. Dots indicate sequence homology. The amino acid letter at position 96 is depicted in bold print. Above the sequences are descriptions indicating the position of CDR1, CDR2, and CDR3 and the J region. The proposed J segment used to encode the fourth framework region of each sequence is listed at the end of each sequence.

structural requirement for the sequences to use either $J_\kappa 1$, $J_\kappa 2$ or $J_\kappa 4$ to react with the 17.109 antibody cannot be determined. The sample bias also may reflect the preferential usage of the different J_κ segments by the malignant B cells expressing *Humkv325*.

Comparison of the deduced amino acid sequences of the V_κ genes expressed by 17.109-reactive CLL with the structurally related but 17.109^- κ light chains of IgMκ RF paraproteins WOL and NEU (29–32), reveals amino acid differences in the first and third CDR (Fig. 3). In addition, NEU differs at two positions in the second framework. Of note, both 17.109^- κ light chains have structurally nonconservative differences in the third CDR. NEU has Ala instead of Thr or Ser at position 93, and Cys in lieu of Arg at position 96. Although WOL has Ser at position 93, it has nonconservative differences at positions 94 (with Leu instead of Ser) and 95 (with Gly instead of Pro). Despite sharing 95% overall homology with the deduced amino acid sequences of HIC and HAH, the structural changes resulting from these third CDR differences may account for the inability of isolated NEU and WOL κ chains to react with the 17.109 antiidiotypic antibody. These data also serve to emphasize that even minor differences in the primary sequence encoded by *Humkv325* can result in loss of light chain reactivity with the 17.109 antibody.

Discussion

This study demonstrates that the presence of a major CRI in human CLL is secondary to expression of the same V_κ gene that has been highly associated with IgM autoantibodies. Previously, we noted in a study of >30 patients with CLL that one-quarter of the κ light chain–expressing CLL possessed a CRI recognized by the 17.109 mAb (12). Here we present sequence analyses of κ light chain polyadenylated RNA or cDNA derived from two unrelated patients with CLL bearing the 17.109-CRI. The results reveal striking sequence homology between the expressed V_κ genes and a V_κ gene previously isolated from placental DNA, designated *Humkv325* (or V_κRF) (16). We conclude that a relatively high percentage of patients with CLL have leukemic cells that express the *Humkv325* gene.

Estimates of the number of distinct V_κ genes in man range from 25 to 50 (34–38). To date, a total of 34 V_κ genes have been distinguished (33–43). Of these, 8 belong to the V_κIII subgroup (43, 45). Only one of these, *Humkv325*, has nucleic acid sequence homology with the V_κ gene expressed by leukemic cells from HIC. Moreover, only *Humkv325* can encode the κ light chain paraproteins that are recognized by 17.109 without sequence permutations. Thus, the high frequency at which 17.109 is noted to react with leukemic cells from unrelated patients with CLL argues that antibody V genes are expressed nonstochastically in this disease.

The repeated use of *Humkv325* in CLL raises the possibility that the variable region protein encoded by this gene may be related to the leukemogenic process. The 17.109-CRI has been associated with multiple different IgM autoantibodies, including antibodies reactive with low-density lipoproteins, intermediate filaments, and autologous erythrocytes in addition to RF (13, 15, 29–32, 46–50). Furthermore, the sIg on a high proportion of CLL have been reported to have

such RF activity (5). It is possible that B lymphocytes with self-reactive sIg may be stimulated to divide in the absence of exogenous antigen, thereby increasing their likelihood for chance transformation. Also, autoreactive sIg may serve to focus a transforming agent onto the cell, for example, by binding either a leukemogenic virus or an antigen–antibody complex containing such a transforming agent (51). In this light, it is noteworthy that a recently elucidated amino acid sequence of the κ light chain variable region from a human anticytomegalovirus (CMV) IgG_κ shares extensive homology with the deduced amino acid sequences of HIC and HAH, and probably is encoded by the same V_κ gene (52).

Alternatively, the high frequency of 17.109-reactive CLL may reflect a nonstochastic use of the *Humkv325* gene by the physiologic counterpart to the CLL B cell, namely the CD5 B cell. These cells constitute a minor lymphocyte subset that has been shown to be enriched for cells synthesizing IgM autoantibodies (31, 32). Consistent with this notion, we have identified hybridomas generated from autoreactive CD5 B cells that express the 17.109-CRI and the *Humkv325* gene (unpublished observations). If CD5 B cells express sIg with low-avidity antiself reactivity, they may be stimulated constitutively throughout the life span of the individual. Thus, these cells may serve as a renewing stem cell population for B lymphocytes that somatically diversify their expressed Ig V genes during immune responses to exogenous antigens. Alternatively, CD5 B cells may represent a distinct cell lineage that uses autoreactive sIg to focus immune complexes and debris from senescent cells onto accessory cells. In either case, selective pressure would be exerted on V genes that may encode antibodies with such autoreactive specificities. Furthermore, such antibody V genes may be expressed preferentially in early B cell ontogeny when CD5 B cells constitute a major lymphoid subpopulation (53, 54).

Our studies also indicate that the malignant B cells from patients with CLL constitute a relatively homogeneous population that have undergone little or no Ig V gene somatic mutation. This stands in marked contrast to the relatively high rate of somatic mutation detected in human B cell follicular lymphomas that do not express the CD5 antigen (55–59). Heterogeneity in the idiotype(s) expressed by these latter tumors has confounded attempts at passive immunotherapy with solitary antiidiotypic antibodies, as idiotype variants arise at high frequency (56–59). Such idiotypic heterogeneity in follicular lymphomas also is noted before therapy with antiidiotypic antibodies (55). Levy and his colleagues (56, 59) have demonstrated that such idiotypic heterogeneity within a given follicular lymphoma results through an ongoing process of somatic mutation in both expressed heavy and light chain V genes. In contrast, the correlation between the cell surface levels of 17.109-CRI and κ light chain on leukemic cell populations from patients HIC and HAH argues against extensive idiotypic heterogeneity in CLL. Indeed, sequence comparisons of κ light chains tested for reactivity with 17.109 indicate that subtle changes in the primary sequence often result in the complete loss of idiotypic reactivity (Fig. 3). Furthermore, the V_κ genes expressed by the leukemic cells from HIC and HAH have a paucity of conservative or silent nucleic acid base substitutions. To be sure, the data presented do not exclude the possibility that Ig V gene somatic mutation may occur in CLL, this producing idiotype variants within a CLL population that may expand under selective pressure. Such idiotype variants also may become

evident after immunotherapy with antiidiotypic antibodies. However, the homogeneous fluorescence staining with the 17.109 antiidiotype and the identical V_κ region nucleic acid sequences of three independent κ cDNA clones derived from the leukemic cells of HAH is consistent with the notion that the V genes expressed by a given CLL population have limited heterogeneity.

Our results have important implications for immunotherapy of CLL (and perhaps Waldenstrom's macroglobulinemia). A disease derived from a minor B cell subset expressing sIg that uses a restricted set of Ig V genes with minimal somatic mutation should be particularly amenable to passive immunotherapy with antiidiotypic antibodies. Furthermore, it may be anticipated that other CRIs will be found that are highly associated with this malignancy. The further identification and characterization of such conserved CRIs is essential and would facilitate the generation of batteries of mAbs suitable for treating large numbers of CLL patients.

Summary

Recently the minor B cell subpopulation that expresses the CD5 (Leu-1) antigen has been implicated as a source of IgM autoantibodies. Chronic lymphocytic leukemia (CLL), the most common leukemia in humans, represents a malignancy of small B lymphocytes that also express the CD5 antigen. However, little is known concerning the antibody variable region genes (V genes) that are used by these malignant CD5 B cells. We have found that a relatively high frequency of CLL patients have leukemic B cells with surface immunoglobulin (sIg) recognized by 17.109, a murine mAb specific for a κ light chain associated crossreactive idiotype (CRI) associated with rheumatoid factor and other IgM autoantibodies. Flow cytometric analyses revealed that the relative expression of the 17.109-CRI by circulating leukemic B cells was directly proportional to the levels of sIg κ light chain, indicating that there exists stable idiotype expression in the leukemic population. To examine this at the molecular level, the nucleic acid sequences encoding the Ig κ light chains of two unrelated patients with CLL bearing sIg with the 17.109-CRI were determined. Analyses of multiple independent κ light chain cDNA clones did not reveal any evidence for sequence heterogeneity in the CLL cell population. Furthermore, the nucleic acid sequences expressed by the leukemic cells of these two patients were identical or very homologous to a germline V_κ gene isolated from placental DNA, designated *Humkv325*, or "V_κRF" because of its association with IgM autoantibodies. This study suggests; (*a*) that the malignant $CD5^+$ B lymphocytes in CLL use the same V_κ gene that has been highly associated with IgM autoantibodies and (*b*) that the expression of V genes is stable in CLL, in contrast to other B cell malignancies examined to date. We propose that many CLL cases represent malignancies of autoreactive CD5 B cells that use a restricted set of conserved V genes. This property may render CLL particularly amenable to immunotherapy with antiidiotypic antibodies.

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