Restricted Immunoglobulin V<sub>H</sub> Region Repertoire in Chronic Lymphocytic Leukemia Patients With Autoimmune Hemolytic Anemia

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Between 10% and 25% of chronic lymphocytic leukemia (CLL) patients have episodes of autoimmune hemolytic anemia (AIHA) during the course of their disease. The anti-erythrocyte autoantibodies in most cases are polyclonal and express a different heavy chain isotype than the malignant clone, indicating that they are secreted by normal autoreactive B lymphocytes. To further investigate the pathogenesis of the AIHA in CLL, we analyzed the Ig heavy (H) chain variable region genes expressed by leukaemic cells from CLL patients with and without AIHA. Two VH genes were preferentially expressed by the leukaemic cells in the CLL cases with AIHA and were present in 9 of the 12 investigated cases. The 51p1/DP-10 gene was expressed in 5 of these cases and was absent in the control group of 12 consecutive CLL cases without AIHA, whereas the DP-50 gene was present in 4 CLL-AIHA cases and only once in the control CLL group. A strikingly similar H-chain CDR3 region that contained a single reading frame of the DXP4 DH gene segment, an N-encoded proline at the D<sub>H</sub/>J<sub>H</sub> boundary, and a tyrosine-rich region encoded by the J<sub>S</sub> gene segment was observed in all four CLL-AIHA cases. The preferential expression of two VH gene segments and a particular CDR3 region by the leukaemic cells of patients with AIHA suggests that the antibodies produced by the CLL cells are directly involved in the pathogenesis of the hemolytic anemia.

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MATERIALS AND METHODS

Patients. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from 12 CLL patients with direct antiglobulin test (DAT) positive warm AIHA (8 from Macedonia, 2 from Italy, and 2 from the United States) and from 12 consecutive CLL patients that were consistently DAT negative throughout their disease (four from Macedonia and eight from Italy). The diagnosis in all patients was established according to the criteria of the International Workshop on Chronic Lymphocytic Leukemia and all patients were followed for at least 4 years before the blood sampling. Informed consent was obtained in all cases.

Sequence analysis of CLL VH regions. Total cellular RNA was isolated by the procedure of Chomczynski and Sacchi. One microgram of RNA was reverse transcribed (RT) using oligo-DT and the GeneAmp RNA/PCR kit (Perkin Elmer Cetus, Norwalk, CT), following the procedure recommended by the manufacturer. The entire RT sample was next subjected to 35 cycles of PCR with 50 pmol of V<sub>H</sub> and C<sub>H</sub> specific oligonucleotide primers. The C<sub>H</sub> primer 1 (5' GTCCTGTGCGAGGCAGCCAA 3') was from the first exon of the heavy chain constant <i>μ</i> gene (codons 139 to 145), and was used either with hFW1 (5' AGGTGCAGCTGGA(T)G(C)AGT(G)-T(G)GG 3'), from a conserved sequence in FW1 located between codons 1 and 8, or with an equimolar mixture of six family specific primers from the leader sequences of the V<sub>H</sub> gene segments (VF1: 5' CCATGGACTGGACCTGGA 3'; VF2: 5' ATGGACATATTTGT(G)GG 3'; VF3: 5' ATGGGAGTATTGGCTGAGC 3'; VF4: 5' ATGAAACACCTGTTGTCCTT 3'; VF5: 5' ATGGGCATCCAAGCCATCTT 3'; VF6: 5' ATGGACATATTTGT(G)GG 3'; all from reference 13). The PCR reactions were carried out with 1

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Submitted March 27, 1995; accepted December 15, 1995.

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minute of denaturation at 95°C, 1 minute of annealing at 62°C, and 1.5 minutes of extension at 72°C in a Perkin Elmer Cetus thermal cycler. The amplified DNA fragments were purified by electrophoresis from 1.2% agarose gels. The recovered DNA fragments were ligated in the Sma I site of pUC18 (Pharmacia LKB, Uppsala, Sweden) and used to transform Escherichia coli strain DH5α. Clones were picked randomly, and a double-stranded DNA template was prepared and sequenced using the T7 Sequencing Kit (Pharmacia LKB).

Nucleotide sequence data were analyzed and comparisons were performed with the Genetics Computer Group, Inc (Madison, WI) software package and the Genbank (Los Alamos, NM) and EMBL (Heidelberg, Germany) databases. The assignment of DH and JH gene segments was done by comparison with published germ-line sequences, according to the criteria used in reference 15. Statistical analysis was performed by the Chi-square test using Microsoft Excel software (Microsoft Corporation, Redmond, WA).

RESULTS

Cloning and sequencing of VH genes. The gene segments encoding the heavy chain variable regions of the CLL IgGs were amplified by RT/PCR from 12 patients with AIHA and from the 12 DAT-negative controls using a Cμ primer and either a degenerate primer from FW1 (homologous to published gene segments from the Vμ1, Vμ3, Vμ4, and Vμ5 families) or an equimolar mixture of six family specific primers from the Vμ leader exon. Six clones were sequenced from each patient and the obtained sequences were always identical. Either direct sequencing or IgM gene fingerprinting was also performed to confirm that these sequences belonged to the malignant clones. The sequences of the VH gene segments from the 24 CLL patients are shown in Fig 1. Four different VH gene segments were identified in the CLL-AIHA group (Fig 1A). The CLL cells in five patients (HA-1, HA-2, HA-3, HA-5, and HA-9) expressed the VH1 family gene 51p1 in germline configuration. The VH genes in four other cases were identical or highly homologous to the Vμ3 family member DP-50 (HA-4, HA-7, HA-8, and HA-R1). The remaining three CLL-AIHA cases expressed VH genes identical to the Vμ3 family member DP-47 (HA-6 and HA-VG) or the Vμ1 family member DP-25 (HA-10). The VH sequences in the control CLL group were homologous or identical to nine different VH genes that belonged to the Vμ4 family (Vμ4.21 in CL-P and CL-G, Vμ4.11 in CG-6, and Vμ4.18 in CG-2), Vμ3 family (DP-29 in CG-1 and CG-7, DP-35 in GP-4 and CL-D, DP-47 in GP-3, and DP-50 in CLJ) and Vμ1 family (DP-8 in GP-1 and DP-15 in CLO) (Fig 1B). Only three of these sequences were in germline configuration, whereas the others shared 87% to 99.3% nucleotide sequence homology with published VH gene sequences. Although we cannot exclude that some of these VH gene segments belong to unknown members and/or polymorphic variants of previously described members of the Vμ1, Vμ3, and Vμ4 gene families, the overall distribution of replacement and silent mutations with a high R/S ratio in CDR1 and CDR2 (4.23) and a low R/S ratio in FW1, FW2, and FW3 (0.9) is strongly indicative of somatic mutation and selection. This is further supported by the fact that most of these sequences also contained mutations in the JH gene segments, whereas no mutations were present in the JH gene segments that were associated with germ-line VH genes.

Analysis of the DH and JH gene segments. Biased DH gene segment usage was also seen in the CLL-AIHA group, with preferential expression of DXP family members (CLL-AIHA: 7/12 patients, control CLL: 1/12 patients, P = .009) (Fig 2). Interestingly, 4 VH domains (patients HA1, HA2, HA5, and HA8) contained a strikingly similar CDR3 region characterized by a DXP4 encoded DFWSGY motif, an N-nucleotide encoded proline, and a JH6 encoded stretch of tyrosines (Fig 2A). This CDR3 region was associated with the 51p1 gene in three cases and the DP-50 gene in one case. A similar CDR3 region containing SGY and the JH6-encoded tyrosines was present in patient HA-10. The JH6 gene segment was preferentially expressed in the CLL patients with hemolytic anemia (7 of 12 cases), whereas DH1 and JH1 gene usage appeared random in the control CLL group (Fig 2B).

DISCUSSION

In a recent review of VH gene usage in CLL, Schroeder and Dighiero showed that the B-CLL clones express a diverse VH gene repertoire. Out of the approximately 50 known germline VH genes, at least 27 were present among the 75 analyzed CLL sequences. At the same time this analysis showed that four VH gene segments (51p1, Vμ4.21, Vμ4.18, and Vμ251) are overrepresented in the CLL VH repertoire, accounting for approximately 50% of the CLL VH domains. This apparent skewing in VH gene segment use and the finding that CLL cells frequently produce polyreactive autoantibodies suggests that the malignant clones in CLL are selected by self-antigens that are preferentially recognized by VH domains encoded by particular gene segments. In the case of the Vμ4.21 and Vμ251 genes this conclusion is further supported by the frequent finding of somatic mutations indicative of an antigen driven response, as was also observed in a subset of our patients, and by the association of the Vμ4.21 gene segment with autoantibodies that react with the erythrocyte I/i blood-group antigens.

We now show that the antibodies produced by the leukemic cells from CLL patients with AIHA are preferentially encoded by the 51p1 and DP-50 VH genes. These two VH genes were found in 9 of the 12 CLL-AIHA H-chain sequences, and only once in the control group of CLL patients that had not developed AIHA before the sampling. Chi-square analysis showed a significant correlation between the expression of these VH genes and the occurrence of AIHA (P < .001), indicating that the 51p1 and DP-50 gene have been selected because of particular binding specificities that can contribute to the development of AIHA.

The 51p1 gene was present in approximately 12% of the CLL VH sequences from the survey of Schroeder and Dighiero. An even higher frequency of 18% has been reported by the group of Kipps, which investigated 51p1 usage in a similar number of CLL cases. Taken together, these two studies suggest that the expected frequency of 51p1 in CLL is approximately 15%, which is significantly lower than the frequency we observed in the CLL-AIHA patients (42%, P
Fig 1. Nucleic acid sequences of the rearranged CLL V\textsubscript{H} genes from the 24 investigated patients. The nucleotide and deduced amino acid sequences of the most homologous germline V\textsubscript{H} genes are shown above the CLL sequences. The name of each CLL sample is indicated at the beginning of the sequence. Dashes indicate nucleotide identity. Nucleotide differences shown in bold result in amino acid changes. (A) Sequences of V\textsubscript{H} gene segments obtained from the CLL patients with AIHA. (B) (see pages 3872 and 3873) Sequences obtained from the control group of consecutive CLL patients without AIHA.
A significant correlation between \( V_H \) gene usage and AIHA was also observed for the DP-50 gene, which was not present among the 75 surveyed sequences from reference 17, but accounted for 33% of the CLL-AIHA sequences \( (P < .001) \). Interestingly, this VH gene has also been found in 5 of 14 investigated human monoclonal anti-Rh(D) antibodies, indicating that it is also overrepresented in the human anti-Rh(D) response.\(^{24}\)

The \( V_H \) domains in four of the CLL-AIHA patients contained a strikingly similar CDR3 region that contained a DFWSGYXP motif (where \( X \) corresponds to L or Y) encoded by a single reading frame of the DXP4 gene segment and N-nucleotides from the DH-JH junction (Fig 2A). This motif was followed by a long stretch of tyrosines encoded by the JH6 gene segment. A similar incomplete motif (SGY) was encoded by the DK1 gene in patient CLL130 and was also associated with the \( J_d \) segment. Although at present we cannot conclude that the Igs containing these conserved structures will bind the same antigen(s), this assumption is not unlikely considering recent findings that autoreactivity of CLL antibodies might be a selected specificity determined by the association of certain \( V_H \) genes and CDR3 regions.\(^{24,25}\)

A similar restriction of the antibody repertoire has been observed in the autoimmune response to RBC antigens in the New Zealand Black inbred strain of autoimmune mice. This strain is considered a mouse model of human CLL since it is characterized by pauciclonal proliferations of CD5\(^+\) B cells that occur during aging and by frequent occurrence of AIHA.\(^{26}\) The anti-erythrocyte antibodies in this strain are directed mainly against antigens that are exposed on the
surface of intact mouse RBC, such as the erythrocyte band 3 protein. These antibodies are polyclonal, are usually IgG, and are encoded by a variety of \( V_{H} \) gene segments that have undergone somatic mutation.\(^{27} \) However, a subset of the anti-erythrocyte antibodies react only with RBC antigens that become exposed after treatment with proteolytic enzymes such as bromelain. Interestingly, these antibodies are produced by CD5\(^+ \) B cells and are encoded by only two nonmutated \( V_{H} \) gene segments (\( V_{H}11 \) and \( V_{H}12 \)), which are overrepresented in the \( V_{H} \) repertoire of mouse CD5\(^+ \) B cells, but are infrequently expressed by conventional mouse B cells.\(^{38,39} \) The function of these anti-bromelain treated RBC autoantibodies is still unknown, but a role in the clearance of senescent erythrocytes has been suggested.\(^{30} \)

The antibodies produced by the CLL cells could have a similar specificity and could promote the binding of RBCs, sensitized by polyclonal IgG antibodies, to the Fc receptors on macrophages. In a parallel study we have found that a subset of CLL cells produces secretory \( \gamma \)-chain transcripts, which in two of the CLL-AIHA patients were predominantly of the \( \gamma 2 \) and \( \gamma 3 \) \( H \)-chain isotypes (D.G.E., M.I., F.D.B., and O.R.B., manuscript in preparation). Antibodies of the IgG3 subclass seem to play an important role in the initial adherence of sensitized erythrocytes to macrophages because of their greatest affinity for the Fc\( \gamma RIII \).\(^{7} \) Alternatively, the CLL antibodies might not bind directly to RBC antigens, but could represent second antibodies that bind to the Fc portion of the anti-RBC antibodies. Our initial experiments with a recombinant Fv antibody that corresponds to the CLL Ig of patient HA2 show low-affinity reactivity against a number of self antigens including human IgG. Finally, the CLL antibodies could have antiidiotype activity and could stimulate normal autoreactive B cells to produce anti-RBC antibodies. Antiidiotypic IgG antibodies that crossreact with some anti-Rh(D) antibodies have been detected in eluates from sensitized RBCs and have been implicated in the pathogenesis of AIHA.\(^{31} \)

In conclusion, we have shown that the Ig \( V_{H} \) domains expressed by the leukemic cells from CLL patients with AIHA are preferentially encoded by the 51p1 and DP-50 \( V_{H} \) gene segments in association with a particular CDR3 region. Further studies should determine whether this information can also be used as a prognostic factor to define a subset of CLL patients at an increased risk to develop autoimmune hemolytic anemia.

**NOTE ADDED IN PROOF**

We have more recently determined the rearranged \( V_{H} \) gene sequence in three additional CLL patients with AIHA. The 51p1 \( V_{H} \) gene encoded the CLL Ig in two of these cases, which raised the frequency of 51p1 \( V_{H} \) rearrangements in CLL-AIHA to 47% (7/15 cases).

**ACKNOWLEDGMENT**

We thank Dr F. Franzén for his help in collecting some of the samples and Dr S. Anand for critical reading of the manuscript.

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