Differential Usage of an Ig Heavy Chain Variable Region Gene by Human B-Cell Tumors

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A monoclonal anti-idiotypic antibody has been raised that recognizes Igs with heavy chains encoded by a member of the V_{H}4 family, the V_{H}4-21 gene segment. The idiotope (Id) is detectable on a high percentage of early B cells in fetal spleen, and is expressed by certain autoantibodies, particularly cold-reactive anti-red blood cell antibodies. Therefore, it was of interest to investigate usage of this V_{H}4 gene by neoplastic B cells; 81 chronic lymphocytic leukemias (CLLs) involving CD5+ B cells and 62 B-cell lymphomas of varying histologic type have been analyzed. The Id was expressed by only 3 of 81 (3.7%) of the CLLs, indicating a relatively low usage by these tumors. In contrast, the Id was expressed by 9 of 62 (14.5%) of the lymphomas across a range of histologic types, indicating a differential use of the V_{H}4-21 gene among B-cell neoplasms. For three of the Id-positive lymphomas, each of a different histologic class, the nucleotide sequence of the tumor-derived V_{H}4 gene was determined; the V_{H}4-21 gene was identified, as expected. The sequence from the CLL was identical to the germline sequence, and the marginal zone lymphoma showed only 3 nucleotide changes, 2 of which gave rise to amino acid substitutions. In contrast, the sequence from the follicular lymphoma showed 29 nucleotide changes giving rise to 14 amino acid substitutions, which were scattered among the CDR and FW regions.

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categories of B-cell tumors for Id expression, anticipating that it would be found to be preferentially expressed by those tumor cells that are thought to arise from the early CD5+ lineage, ie, CLL.

MATERIALS AND METHODS

Cell suspension analysis. Blood was obtained from patients with CLL and from two patients with lymphoma; lymphocytes were separated using Ficoll-Hypaque. Cells were then suspended in warm medium (RPMI 1640, HEPES buffered) containing fetal calf serum (FCS; 10%), streptomycin (0.1 mg/mL), and penicillin (60 μg/mL) and washed twice. Cell surface antigens were analyzed in the FACS-SCAN (Becton Dickinson Lab Systems, Mountain View, CA) by indirect immunofluorescence using monoclonal anti-Ig reagents as described and anti-CD5 (OKT1; ATCC, Rockville, MD), with fluorescein isothiocyanate (FITC)-labeled sheep antimouse IgG (Sigma Chemical Co, Poole, Dorset, UK) for detection. In one case of lymphoma (JJ), frozen cells were cytotoxic interfered and analyzed by the sensitive single-stage alkaline phosphatase-antialkaline phosphatase (APAAP) technique, with sheep antimouse Ig as second bridging antibody followed by complexes of AP with its mouse antibody. The rat anti-idiotypic antibody, 9G4, which detects the V4-21-associated Id, has been described. It is of the IgG2a subclass, and was used with a control subclass-matched rat antibody; detecting antibody was FITC-sheep antimouse IgG selected for cross-reactivity with rat IgG.

Tissue section analysis. Frozen tissue sections obtained from reactive lymph node, or from lymph node, spleen, and lung from patients with lymphoma were used to assess expression of Id. Cases of B-cell lymphoma were obtained from the frozen tissue files of the Department of Pathology, University of Southampton, and the original histologic diagnosis, made according to the Keil classification, was confirmed by independent review. Cryostat sections (5 μm) were air-dried at room temperature and stored at −70°C until required. Immediately before staining, the sections were fixed in acetone for 10 minutes and then stained by an indirect immunoperoxidase technique using HRP-rabbit antimouse IgG or HRP-rabbit antirat IgG, followed by development of reaction product by diamobenzidine tetrahydrochloride.

A diagnostic monoclonal antibody (MoAb) panel, containing antibodies against CD37, CD19, CD22, CD5, and CD10, in conjunction with antibodies against Ig heavy and light chains, was used to confirm the presence of a B-cell tumor and allowed identification of areas of tumor involvement. Morphologically identified tumor cells were considered positive if a clear membrane immunoperoxidase reaction product could be seen in the frozen section.

Patients. Patients with a clinical diagnosis of CLL were identified from the routine immunology laboratory; those with a white blood cell count (WBC) of greater than 10^10/L and with tumor cells expressing the CD5 antigen were included in the study.

Three patients with Id-positive tumor cells were selected for nucleotide sequence analysis; each of these represented a distinct category of B-cell neoplasia. The first patient, KR (a 69-year-old woman) was a typical long-standing case of CLL, having a lymphocytosis (8 × 10^9/L) comprising small λ-positive monomorphic lymphocytes. The second patient was a 63-year-old man, JJ, who presented in 1986 with a lymphocytosis and an enlarged spleen that was removed surgically. The histologic diagnosis was of a lymphocytic lymphoma with greater than 90% tumor cells in the involved spleen and bone marrow. However, a more recent assessment of the histologic appearance of the spleen has led to an assignment to the category of marginal zone lymphoma. The blood lymphocyte count was 7.5 × 10^9/L, of which the majority (>95%) were λ-positive tumor cells. These cells were stored frozen in liquid nitrogen for further analysis. The third patient, PS (a 43-year-old woman), presented in 1987 and had a lymph node removed surgically; the histologic diagnosis was of follicular lymphoma involving cells of centroblastic and centrocytic morphology. The patient also had a lymphocytosis (3.7 × 10^9/L) consisting of greater than 90% κ-positive tumor cells; treatment with several courses of chemotherapy failed to control disease and tumor cells in the blood were at 4.5 × 10^9/L when collected for the study.

Establishment of cell line from JJ. Active EBV was harvested from the marmoset cell line B95/8 and filtered (0.45 μm) before use. Blood lymphocytes from patient JJ were suspended in B95/8 culture fluid at 2 × 10^8 cells/mL. After incubation for 1 hour, cells were centrifuged, resuspended in medium, and plated at 5 × 10^5 cells/well in 200 μL medium in a 96-well plate. After a minimum of 4 days, cells were monitored for growth before transfer. When in flasks, supernatant was taken for assay of IgM and Id by enzyme-linked immunosorbent assay (ELISA) as described, and cells were examined for clonality by staining for Id using the single-stage APAAP technique.

Cloning and sequencing of the productively rearranged V_{H} gene. For the V_{H} of the EBV line established from JJ, the method of cloning and sequencing has been described in detail. For patients KR and PS, a more rapid method of direct sequencing from cDNA was used. Total RNA was isolated from ≥5 × 10^6 tumor cells (>95% of the blood lymphocyte population) using RNAzol B (Cinna Biotech laboratories Inc, Houston, TX) and was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and a Not-I-d(T)_{14} primer (Pharmacia LKB, Uppsala, Sweden).

One-twentieth of the cDNA was amplified by polymerase chain reaction (PCR) using an oligonucleotide primer specific for the V_{H} heavy chain leader (5'-ATGAAACACCTGTGGTTCTT) and a C_{α} primer (5'-CGAGGGGGAAAAGGGTTGG). Amplified products were electrophoresed through a 1.5% agarose gel and purified using GeneClean II (Bio 101 Inc, La Jolla, CA). Purified DNA (100 ng) was sequenced directly by the dideoxy chain termination method with T7 DNA polymerase (Pharmacia) and 100 ng of the relevant PCR primer. PCR amplification and sequencing was performed three times.

RESULTS

Phenotypic analysis of blood cells from patients with CLL. Samples of blood lymphocytes from patients with a diagnosis of CLL and WBC of greater than 10^10/L were analyzed in the FACS-SCAN. All expressed CD5, and those that were also clearly positive for membrane Ig were selected for further study, giving a total of 8. The surface Ig was identified as IgM (43) or IgM (38), and of these, 2 IgM and 1 IgM also expressed the V_{H}4-21-associated Id. The overall percentage of positiveness of the Ig-expressing tumor cell populations in CLL was therefore 3.7% (Table 1).

Expression of Id in normal lymphoid tissue. The fact that the Id is expressed by ~3% of B cells in normal lymph nodes has been described, and the detailed distribution of those cells in a typical follicle of a normal lymph node is shown in Fig 1. Staining with anti-CD22, a pan-B-cell antigen (Fig 1A), shows a high density of B cells in the mantle zone, with a mixed population containing some B cells in the follicle center. Figure 1B then shows a similar distribution for the Id-positive cells; there is a clear subpopulation of Id-positive B cells in the mantle zone, comprising a small percentage of the total B-cell population. However, Id-positive cells are not confined to the mantle zone, because there are also some scattered cells visible within the follicle center.
Expression of Id in B-cell lymphoma. The incidence of Id positivity among lymphomas of various histologic type is also shown in Table 1. In each case, the Id was expressed by the morphologically identifiable tumor cell population, and there were 9 of 62 clear positives (14.5%), which is significantly higher than that for CLL \((1.1 > P > .05\) by \(\chi^2\) analysis). Although numbers in each histologic category are too small to detect any significant differences, there is an indication of a low incidence in lymphocytic lymphoma (0 of 9 positive), consistent with the similarity of this neoplasm in morphology and behavior to CLL.\(^{11}\) Expression of CD5 was heterogeneous among the lymphocytic lymphomas, with 6 of 8 being positive (1 case was not evaluable).

An example of the pattern of staining in the follicle center of a lymph node infiltrated with Id-positive tumor is shown in Fig 2. Again, the pan-B-cell marker CD22 has been used to stain the total B-cell population (Fig 2A), and the positive cells (marked with arrows), identifiable by darkly stained surface membranes, are clearly visible among a background of T cells, with the morphology of the stained population being strongly suggestive of tumor cells. This feature is also seen in Fig 2B using the anti-Id antibody that stains the large pleomorphic tumor cells (marked with arrows) in a pattern similar to that with anti-CD22, suggesting that most of the B cells are neoplastic.

The phenotypic analysis of the Id-positive cases, including those from the CLL group (Table 2), indicates that there was a mixture of \(k\) and \(\lambda\) light chain types and that all identifiable heavy chains were \(\mu\). In one case of \(\lambda\)-expressing high-grade lymphoma, technical reasons prevented allocation of the heavy chain class. All the cases of Id-positive CLL were CD5\(^+\), but the Id-positive lymphomas were all CD5\(^-\) (Table 2).

Analysis of tumor cells from patients KR, JJ, and PS. The phenotypic profiles of the tumor cells from the three patients available for nucleotide sequence analysis, KR, JJ, and PS, are included in Table 2 and are indicated by an asterisk. For patient KR (CLL), the blood lymphocytes included 73% of IgM\(k\)-positive cells, all of which were positive for both Id and CD5; the majority of the remaining lymphocytes (22%) were T cells. For JJ (marginal zone lymphoma), the blood lymphocytes that were used for genetic analysis had a profile similar to that of the splenic tumor cells, with the majority (>95%) of cells expressing Id-positive IgM\(k\), but no CD5, and few detectable T cells. After transformation with EBV, the same IgM\(k\)Id profile was maintained in greater than 95% of the cells in the line, and transformed cells secreted IgM\(k\) that was all idiotypic by ELISA,\(^{16}\) consistent with a clonal population. For patient PS (follicular lymphoma), the Ig phenotype of tumor cells in the lymph node sections was IgM\(k\) and CD5\(^-\) (Table 2); again, the neoplastic blood lymphocytes used for genetic analysis were of the same phenotype being greater than 90% positive for Id-positive IgM\(k\).

Molecular analysis of expressed \(V_{\mu}\) genes of KR, PS, and JJ. The nucleotide sequence of the \(V_{\mu}\) region of the IgM from patient KR indicates that it is identical to the germline \(V_{\mu}4\)-21 gene sequence (Fig 3). The \(V_{\mu}\) sequence of JJ is also derived from the \(V_{\mu}4\)-21 germline gene, and there are only three mutations. However, as shown in Fig 4, two of the

![Fig 1](https://www.bloodjournal.org/.../stevensonetal.jpg) Expression of 9G4 Id by normal B cells in a reactive lymph node. (A) B-cell population stained with anti-CD22. (B) Id-positive cell population stained with anti-Id (9G4). Positively stained cells are indicated by arrows. Original magnification X 450.
three mutations give rise to amino acid substitutions, i.e., a glycine to aspartic acid in CDR1 and a lysine to arginine in FW3. Interestingly, the same two substitutions are found in the PS V<sub>H</sub> (Fig 4), and the gly to asp substitution via the same nucleotide change has been observed in four other

IgMs derived from V<sub>H</sub>4-21, all of which had cold agglutinin activity; however, it is not mandatory for such specificity. The V<sub>H</sub> sequence of PS is also similar to the V<sub>H</sub>4-21 germ-line gene, but in this case there is only 90.0% homology with 29 nucleotide differences, giving rise to 14 amino acid substitutions. Among the recently updated analysis of the V<sub>H</sub>4 family, the sequence of PS lies within the VS8 group, which also includes V<sub>H</sub>4-21. However, homology with the other members of the group is less than 90% and the V<sub>H</sub>4-21 gene is the closest match. The D segments of KR and JJ are both long (Fig 4) and J<sub>H</sub>6 is used in each case. In fact, KR has a stretch of 26 nucleotides homologous to the DXP4 germline gene and JJ has a stretch of 23 nucleotides homologous to the DLR3 germline gene, with flanking sequences showing shorter sequence homologies to two other D segments. In contrast, the D segment of PS is a short sequence with a stretch of 7 nucleotides homologous to the DM2 germline gene. In all three cases, there are probable N-terminal additions.

**DISCUSSION**

Anti-idiotypic antibodies have been used for more than 20 years to identify common structures in the variable regions of Ig molecules. The advent of monoclonal anti-Ids...
allowed more precise localization of the sequences involved in Id expression, and such reagents have proved useful for investigations of the clonal Igs characteristic of B cell lymphomas, and of the oligoclonal autoantibodies synthesized in autoimmune diseases such as systemic lupus erythematosus (SLE). The monoclonal anti-idiotypic antibody that we raised in 1986 has proved particularly useful because it recognizes an Id that is expressed by Igs with heavy chains encoded by a defined VH gene, VH4-21, a member of the VH4 family. The Id has been reported to be expressed by 13 of 13 Igs that use this gene, and we have since found it to be present on a further 29 of 30 sequenced Igs with heavy chains encoded by the VH4-21 gene (unpublished observations). In contrast, it is not expressed by other sequenced Igs encoded by different members of the VH4 family, or by Igs from other VH families.

Another interesting point is that the Id is expressed by autoantibodies that are specific for the I/α carbohydrate antigen on the RBC surface, the so-called cold agglutinins, and the monoclonal anti-idiotypic antibody to be present on a further autoantibodies that are specific for the I/i carbohydrate antigen.23 Studies on fetal spleen showed that the Id was expressed early in development, being identifiable on ~6% of B cells at 20 weeks of gestation. The Id was also detected on cord blood lymphocytes, in which it appeared to be preferentially expressed by CD5+ B cells. This finding of expression of an Id, known to be associated with both cold agglutinins and anti-DNA autoantibodies, by immature CD5+ B cells led us to anticipate that the Id would be present on CD5+ neoplastic cells found in CLL, thereby supporting the proposed link between this normal cell population and the cells found in CLL. In fact, it is known that a significant proportion of such tumors synthesize autoantibodies, particularly rheumatoid factor. The proposal that CLL could represent the neoplastic counterpart of immature B cells found in fetal spleen has been supported by the finding of common high-frequency cross-reactive Igs,26 some of which have been located to usage of certain V-genes, particularly VH4, VH5, and VH6. However, the finding in this report is that expression of the Id associated with a member of the VH4 family, VH4-21, which
is expressed by ~6% of fetal B cells, is found on only 3 of 81 (3.7%) cases of CD5+ CLL. This indicates a consistency between the use of the VH4-21 gene by early B cells and its incidence in CLL. However, usage in CLL is low in comparison with other VH genes such as the 5-1p1 VH1 gene, which is associated with the G6 idiotype. For G6, although expression in the fetal spleen (6.9%) is comparable with our results for the VH4-21-associated Id, expression by cases of CLL is high (20%). Loss of Id by mutation is an unlikely explanation for this relatively low level of expression because VH genes in CLL tend to be unmutated; also, the Id appears to tolerate quite extensive mutations without loss (Pascual et al. and unpublished observations).

In contrast to CLL, the VH4-21 gene is used by neoplastic B cells that secrete IgM with cold agglutinin activity and these tumors represent a significant proportion (13 of 105, ie, ~12%) of the IgM-secreting neoplasms known as Waldenstrom’s macroglobulinemia. Because the tumor cells tend not to be in the blood, there is less information on their CD5 status; however, in our preliminary studies using double staining for Id and CD5, we have found that the majority of cases (7 of 8) do express CD5 (unpublished observations).

The relatively high incidence of use of the VH4-21 gene in this group of tumors could indicate that they develop from a normal, possibly CD5+ B cell that is distinct from that which gives rise to CLL. Our findings that the gene appears to be switched on after certain common infections such as EBV suggest that those postinfective IgM-secreting cells could represent the population that undergoes neoplastic transformation. The frequency of Ig class switching events among this normal B-cell population is not yet clear; certainly the VH4-21 gene can be found in IgG, but a search of our myeloma IgG protein panel found 0 of 55 positive for Id, indicating a low use of the gene in plasma cell tumors.

With regard to expression of the 9G4-associated Id in normal lymphoid tissue, we have shown already that it has a wide distribution. Within the lymph node there is a clear population of Id-positive B cells in the mantle zone, but there is also expression by some of the cells in the germinal center. This distribution is consistent with the specificity of the antibody for an Id that is effectively a V-region subgroup and appears to hold for the VH4 Id that has a restricted specificity within the VH4 family.

The finding of 9 of 62 Id-positive cases among the lymphomas indicates that the VH4-21 gene is used at about the same frequency as in Waldenstrom’s macroglobulinemia, and more commonly than in CLL (11 > P > 0.05 by 2-by-2 analysis). Previously, we found the Id-positive population of normal lymph node to represent 3.2% ± 2.4% of the B cells; this would suggest that the incidence of 14.5% in B-cell lymphomas reflects a preferential use of the VH4-21 gene. It will be of interest to expand the investigation of the various histologic types of lymphoma to see if there is any heterogeneity of expression among the groups. In a previous study of low-grade follicular lymphoma, usage of VH4 families by tumor cells was comparable to that by normal B cells; however, use of individual members of the VH4 family appeared asymmetric, with 3 of 8 of the tumors of the 11 to 14 member VH4 family being assigned to VH4-21.

To verify the association between Id expression and usage of the VH4-21 gene, we investigated the nucleotide sequences of randomly chosen B-cell tumors from the panel. Tumor-derived material was available from three patients, one a typical CLL and two having lymphomas of different histologic categories. For this particular VH4 gene, we have the advantage first, that it has a sequence that is quite distinct from other members of the VH4 family and second, that it has a very low degree of polymorphism, which allows recognition of mutations from the germline gene. In fact, in a recent study of a random series of 11 Id-positive clones established from six patients with infectious mononucleosis, we have found the VH4-21 to be in germline configuration and to be identical in nucleotide sequence.

The sequences obtained from the three tumors were closely related to the VH4-21 germline gene, which has a characteristic sequence particularly in FW1. In fact, that from patient KR was typical of the pattern found in CD5+ CLL in being unmutated from germline. Little is known concerning VH4-gene usage in marginal zone lymphoma, but it was interesting to note a very low level of mutations in this CD5+ tumor. In contrast, the sequence of the VH4 gene from the follicular lymphoma PS showed 29 mutations, of which 18 were replacements. This result is consistent with previous findings in follicular lymphoma, and may reflect the fact that normal B cells undergo hypermutation in the follicle center. In fact, in a recent report of a case of follicular lymphoma involving the VH4-21 gene, a similar number of mutations from germline (23) was found. However, if an analysis of the pattern of mutations in the two cases is made, there are differences, in that the changes in patient PS do not show a high replacement:silent ratio in the complementarity determining regions (CDRs) that may result from antigen selection. For patient PS, the analysis of mutations was performed in the same way. To assess the incidence of replacement and silent mutations, each change was assumed to have occurred independently and was designated as R or S and located in the CDRs or in framework regions (FWRs). The majority of the mutations (23) are located in FW regions, particularly FWR3, and the numbers of replacement and silent mutations in the FWRs (13R and 10S) and in the CDRs (5R and 18S) are not sufficiently different from those expected from a random distribution and composition (FWRs: 14R and 8S; CDRs: 4R and 3S) to indicate a role for antigen selection. This finding contrasts with the published case in which there were 23 mutations, with a concentration of replacement mutations (11) in the CDRs compared with 4 expected. Clearly, more sequences of the VH-genes of tumor cells are needed and the relatively non-polymorphic VH4-21 gene is ideal for investigating this crucial point. At present, it is difficult to assess the role of chemotherapy in inducing mutations, because the patient in the previously reported study and in the current study had both received chemotherapy. It will be of interest in the future to analyze V-gene sequences from patients at presentation. Even if chemotherapy is not a perturbing factor, it is possible that the situation in the lymph node is complicated in that the hypermutation mechanism may be activated in a B cell, but, after a neoplastic event, the tumor cell may or may not be influenced by antigen.
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