

Original Articles**B-Cell Monoclonal Lymphocytosis and B-Cell Abnormalities in the Setting of Familial B-Cell Chronic Lymphocytic Leukemia**

Gerald E. Marti,^{1*} Patricia Carter,¹ Fatima Abbasi,¹ Glennelle C. Washington,¹ Nisha Jain,¹ Vincent E. Zenger,¹ Naoko Ishibe,² Lynn Goldin,² Laura Fontaine,² Nancy Weissman,² Maria Sgambati,² Guy Fauget,³ Pablo Bertin,⁴ Robert F. Vogt, Jr.,⁵ Barbara Slade,⁶ Philip D. Noguchi,¹ M. A. Stetler-Stevenson,⁷ and Neil Caporaso²

¹Flow and Image Cytometry Section, Laboratory Stem Cell Biology, Division of Cell and Gene Therapies, Center for Biologics Research and Evaluation, Food and Drug Administration, Bethesda, Maryland

²Genetic Epidemiology Branch, National Cancer Institute, National Institute of Health, Rockville, Maryland

³Veterans Administration Medical Center, Medical College of Georgia, Augusta, Georgia

⁴Laboratorio de Hematologia-Oncologia, Escuela de Medicina, Pontificia Universidad Catolica de Chile, Santiago, Chile

⁵Immunology Section, Clinical Biochemistry Branch, Center for Environmental Health, Centers for Disease Control, Atlanta, Georgia

⁶Division of Health Studies, Health Investigations Branch; Agency for Toxic Substances and Disease Registry, Atlanta, Georgia

⁷Clinical Flow Cytometry Section, Laboratory of Pathology, Division of Clinical Sciences, National Institutes of Health, Bethesda, Maryland

Background: Among all hematologic malignancies, B-cell chronic lymphocytic leukemia (BCLL) has the highest familial clustering (three- to sevenfold increase), strongly suggesting a genetic component to its etiology. Familial BCLL can be used as a model to study the early pathogenesis of this disease.

Methods: We examined nine kindreds from the National Cancer Institute's Familial BCLL Registry, consisting of 19 affected members with BCLL and 33 clinically unaffected first-degree relatives. Flow cytometric immunophenotyping to detect a B-cell monoclonal lymphocytosis (BCML) was performed. Monoclonality was confirmed by polymerase chain reaction analysis of whole blood DNA. Cell cycle analysis for aneuploidy was conducted.

Results: In all affected individuals, we observed the classic BCLL CD5/CD19/CD20/CD23 immunophenotypic patterns. Six of the 33 unaffected individuals (18%) had evidence of BCML. Additional individuals (13/33, 39%) showed some other abnormality, whereas 14 individuals (42%) were normal. Based on an estimated prevalence of 0.7% for BCML in the general population, the finding of six subjects (18%) with clonal abnormalities in this relatively modest sample was significantly greater than expected (i.e., 18% vs. 0.7%, $P < 5.7 \times 10^{-9}$).

Conclusions: Individual components of BCML and other B-cell abnormalities were observed in almost half of the apparently unaffected individuals. Our findings suggested that BCML may be an early detectable abnormality in BCLL. The spectrum of some of these observed abnormalities suggested the involvement of different B-cell subpopulations or different pathways in clonal evolution. Population-based, longitudinal studies will be required to determine the incidence of BCML and other B-cell abnormalities and their relation to disease progression in BCLL and other closely related B-cell lymphoproliferative disorders. *Cytometry Part B (Clin. Cytometry) 52B:1–12, 2003.* Published 2003 Wiley-Liss, Inc.[†]

Key terms: familial B-cell chronic leukemia; flow cytometry; immunophenotyping; cell cycle analysis; CD5; CD20; B-cell monoclonal lymphocytosis; κ/λ ; absolute lymphocyte count

B-cell chronic lymphocytic leukemia (BCLL) is the most common hematologic malignancy in adults in the Western world and accounts for 30% of all leukemias (1). Its incidence increases logarithmically from age 35 years, does not plateau, and has a median age of onset of 65 years (2). Of all the hematologic malignancies, BCLL shows the highest familial clustering, suggesting a genetic compo-

*Correspondence to: Gerald E. Marti, M.D., Ph.D., Chief, Flow and Image Cytometry Section, Laboratory Stem Cell Biology, DCGT, CBER/FDA, NIH Building 29B, Room 2NN08, 8800 Rockville Pike, Bethesda, MD 20892.

E-mail: gemarti@helix.nih.gov

Received 31 July 2001; Accepted 11 November 2002

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.b.10013

ment to its etiology (3-9). More than 80 pedigrees have been reported in the past seven decades describing the familial clustering, with first-degree relatives of affected individuals exhibiting a two- to sevenfold increase in risk over the general population (10-15).

Neither the basis for the increased risk of familial chronic lymphocytic leukemia (CLL) nor the presumed environmental determinants of sporadic BCLL are known. Although characteristic cytogenetic findings are often observed, to date no specific gene has been identified (16). Other B-cell malignancies have associated precursor states, i.e., monoclonal gammopathy of unknown significance (MGUS) in multiple myeloma (MM) (17,18). This entity is recognized in 3% of the elderly population, and 25% of individuals with MGUS progress to MM or other lymphoproliferative disorders or diseases (LPD) over a 10-year period (17). We propose that an analogous condition, termed B-cell monoclonal lymphocytosis (BCML), exists in relation to BCLL and other closely related B-cell LPDs.

Cross-sectional studies have been conducted to evaluate a variety of laboratory markers in communities near hazardous waste sites. In the course of this work, a recurring pattern of abnormalities similar to those seen in BCLL was noted. The term BCML refers to a pattern of B-cell monoclonal abnormalities suggestive of, but not fulfilling, the minimal criteria for the diagnosis of BCLL. In 1995, a U.S. Public Health Service Workshop on determining the role of environmental exposures as risk factors for B-cell LPDs was held at the Centers for Disease Control and Prevention. Among 6,000 individuals studied, the overall prevalence of BCML (defined by κ/λ analysis or a CD5 B-cell lymphocytosis) among participants 40 years or older was 11 in 1,499 (0.7%) (19-23). On the basis of recommendations made at that workshop in terms of the definition of BCML, a follow-up study confirmed the same level of BCML (24,25).

Given this background, we speculated that BCML might also be increased in unaffected individuals in kindreds with familial BCLL. The Genetics Epidemiology Branch of the National Cancer Institute (NCI) has identified and characterized familial BCLL for more than 30 years (26,27). Because unaffected members in kindreds with familial BCLL might be at higher risk for the development of overt disease and to gain insight into the pathogenesis of this disorder, we investigated the presence of BCML in familial BCLL kindreds. By using the NCI Familial BCLL Registry, we identified kindreds with two or more living affected individuals in addition to unaffected individuals for detailed study. In this article, we report our observations and interpretations concerning B-lymphocyte subset analysis in unaffected individuals.

MATERIALS AND METHODS

Study Design

We examined the lymphocyte populations by flow cytometric immunophenotyping, cell cycle analysis, and an assay based on polymerase chain reaction (PCR) for immunoglobulin gene rearrangement in affected and unaf-

ected family members of nine kindreds enrolled in the NCI Familial BCLL Registry. BCML was defined as a flow cytometric pattern indistinguishable from BCLL except that the absolute lymphocyte count was fewer than 5,000 cells/ μ l or as a detectable population of CD5⁻ monoclonal B cells. This study was approved by an Institutional Review Board, and informed consent was obtained from all subjects in this report. Nine kindred with at least two living affected subjects with BCLL and one or more living first-degree relatives from the NCI registry were available for study.

Sample Collection, Clinical Information, and Pedigree Description

Blood samples in heparin or ethylene-diaminetetra-acetic acid were collected from all family members at outpatient clinic visits and processed soon after collection but usually within 12 h. In some cases, blood samples were obtained from local physicians, shipped overnight, and processed within 24 h of collection. An attempt was made to examine and follow all affected individuals and their first-degree relatives. Clinical information was obtained as previously described (26). The diagnosis of BCLL was established by standard criteria and confirmed by flow cytometric immunophenotyping and review of peripheral blood films in affected individuals (28-30). Individuals who met the minimum criteria for diagnosis of BCLL at any time during the period of observation were classified as affected. Nine kindred including 52 individuals affected with BCLL and unaffected were examined. Individuals examined for this report were identified by a combination of the kindred number and an assigned letter. Selected representative pedigrees are presented in Figures 1-6.

Flow Cytometry: Immunophenotyping and Cell Cycle Analysis

Whole blood lysis preparation, flow cytometric immunophenotyping, and cell cycle analysis were performed as previously described (31-38). Flow cytometric immunophenotyping was performed by two- and three-color immunofluorescent staining using the combinations of directly conjugated reagents, as shown in Table 1. Blood samples from patients with known sporadic BCLL were used as positive controls for affected members of the families. Samples from blood bank donors and unaffected spouses were used as controls for unaffected members. CellQuest (Becton-Dickinson Biosciences [BDB], San Jose, CA) was used to acquire and analyze the flow cytometric immunophenotyping data. FACSComp (BDB) and Quick-Cal (Flow Standards, Inc., San Juan, PR) microbead standards were used to validate instrument performance and linearity. Software-derived percentages of G₀/G₁, S-phase, and G₂/M cells, their corresponding mean channels and coefficients of variation, and the presence or absence of aneuploidy or tetraploidy were determined with ModFit software (Verity Software House, Topsham, ME).

All monoclonal antibodies were obtained from BDB, except anti-CD22 phycoerythrin (PE), were obtained from Caltag (Burlingame, CA). Three sets of light-chain reagents

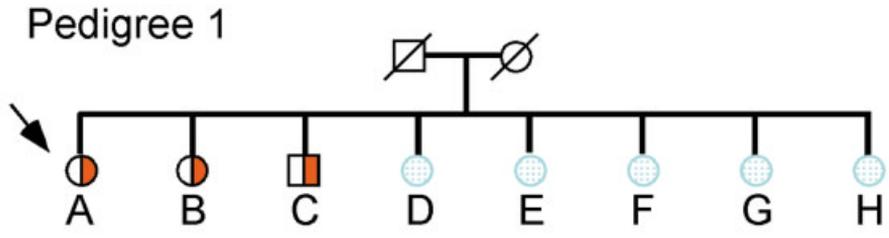
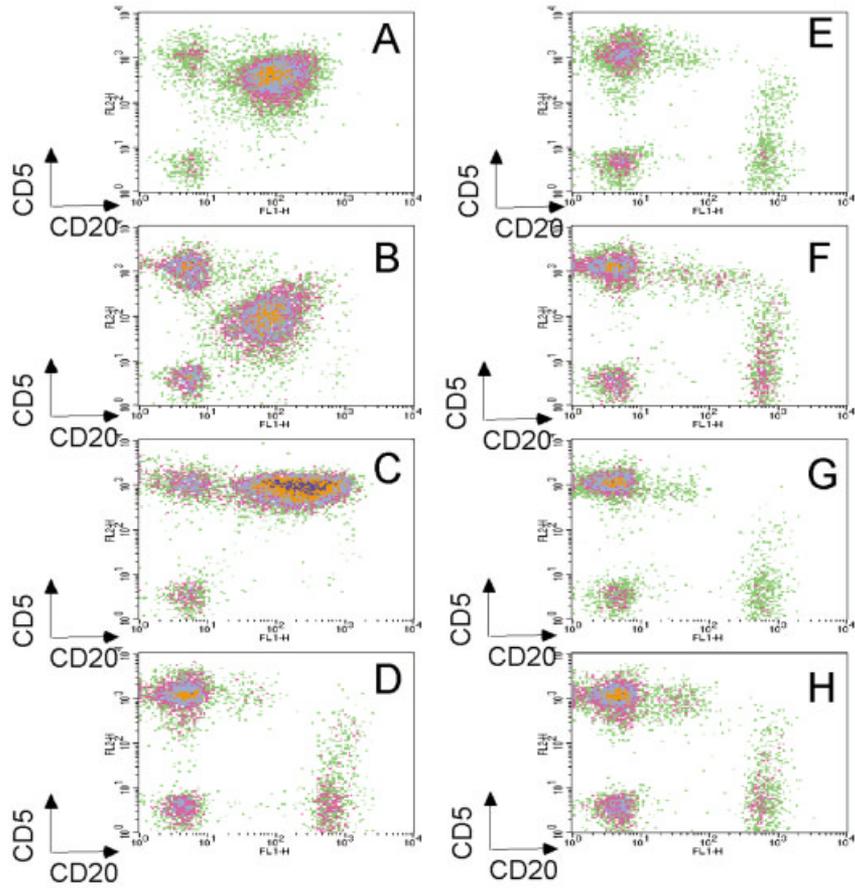
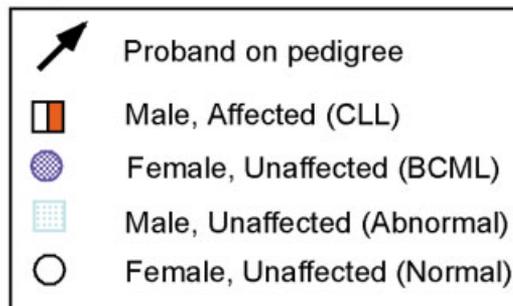


FIG. 1. Pedigree 1 consists of eight siblings, three of whom are known to be affected (one male and two females; A–C). The other five siblings (D–H) are normal by physical examination but had different flow cytometric abnormalities. Originally, two were identified as being affected (1-A untreated and 1-C previously treated). At the time that these flow cytometric studies were first conducted, individual 1-B had an absolute lymphocyte count (ALC) of fewer than 5,000 cells/ μ l and was monoclonal by polymerase chain reaction (PCR). The other five siblings were normal by physical examination and ALC. Subsequent values for individual 1-B were in excess of 5,000 cells/ μ l, and for this reason this patient was designated as having B-cell chronic lymphocytic leukemia (BCLL) rather than B-cell monoclonal lymphocytosis (BCML). One unaffected sibling (F) had an unusual distribution of CD20/CD5 cells. Flow cytometry showed a continuous distribution of CD5⁻ B cells, CD5⁺ B cells, and CD20/CD5 B cells with decreasing CD20 expression. Two of the three siblings (G and H) had normal κ/λ immunoglobulin surface expression and normal polyclonal PCR pattern but showed distinct evidence of a tetraploid population by cell cycle analysis (data not shown). Two-parameter histograms show CD20 fluorescein isothiocyanate on the x axis and CD5 phycoerythrin on the y axis.



Legend



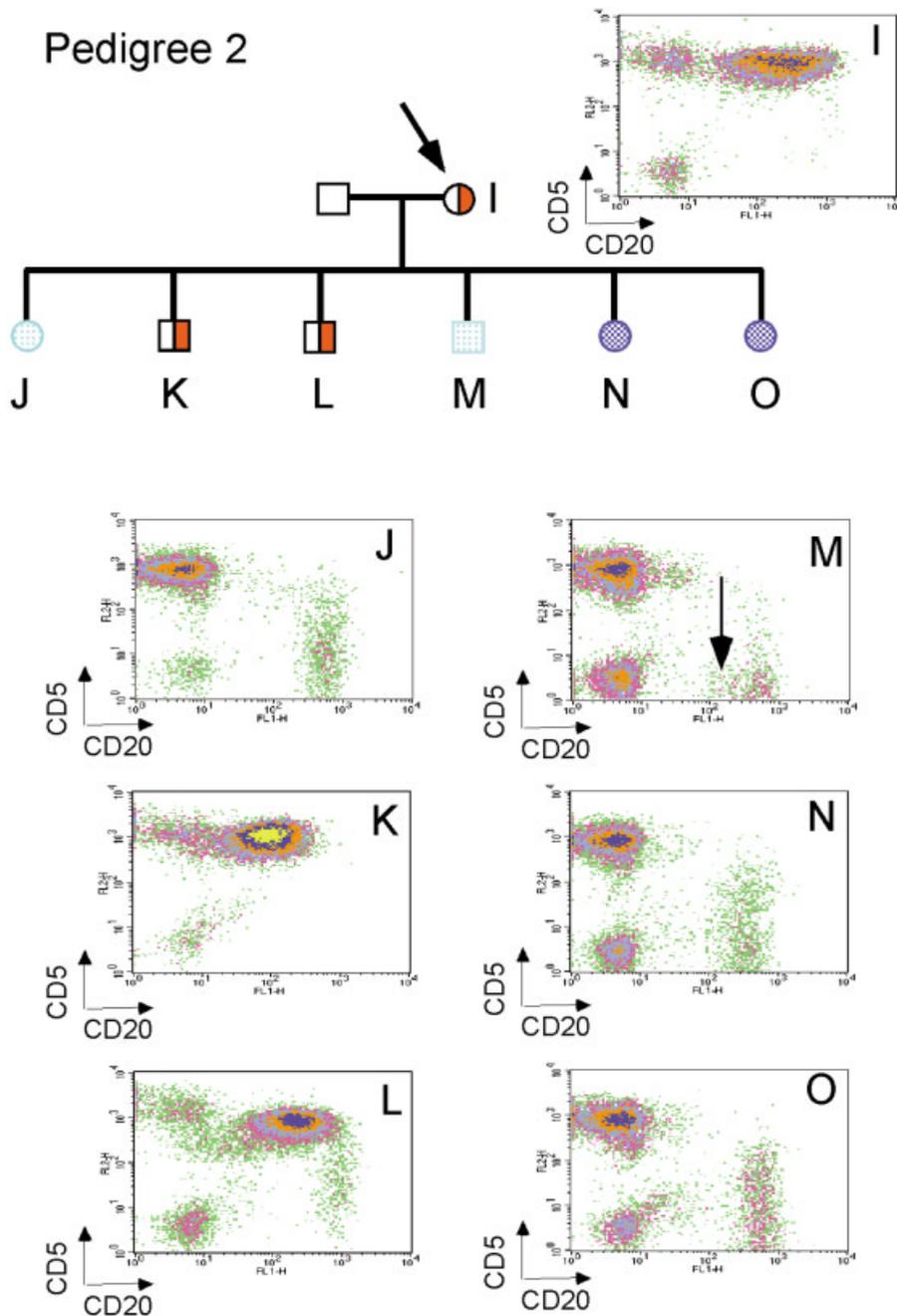


Fig. 2. Pedigree 2 is a family of seven (affected parent and six siblings). The mother (I) is the proband, and two male siblings (K, L) have BCLL. The other four siblings (three females and one male; J, M, N, and O) were clinically normal. Sibling J showed no abnormalities. The immunophenotypic patterns for the proband and the two affected siblings are typical for BCLL. The one unaffected male sibling (M) had a small abnormal CD20-dim subpopulation (arrow). Sibling O had 45% CD5⁺ B cells that expressed κ light-chain surface immunoglobulin. Sibling N had evidence of a κ ⁺ CD5 B-cell clone via a clonal search with an absolute B-cell count of 399 cells/ μ l. In addition, in this individual, the CD5 B-cell lymphocytosis showed a 64-channel decrease in its CD20 Fluorescence Intensity (FI) compared with the CD5⁻ B cells. Siblings N and O have B-cell monoclonal lymphocytosis. The two-parameter histograms show CD20 fluorescein isothiocyanate on the x axis and CD5 phycoerythrin on the y axis. The κ/λ analysis of CD19⁺ cells is not shown.

were used to establish monoclonality in BCLL. The κ/λ antibodies (Table 1, tubes 9–11) were obtained from BDB, Caltag, and Dako (Carpinteria, CA), respectively. In some cases, light-chain restriction could be ascertained only with the use of two additional tubes: anti- κ fluorescein isothiocyanate (FITC) combined with anti-CD22 PE and anti-CD20 peridinin chlorophyll protein (tube 13) and anti- λ FITC combined with anti-CD22 PE and anti-CD20 peridinin chlorophyll protein (tube 14). In these two separate tube comparisons, the fluorescence intensities of anti- κ FITC and anti- λ FITC could be compared directly for

an assignment of monoclonality. These κ/λ reagents were obtained from Kallestad (Austin, TX).

PCR Analysis

PCR amplification and immunoglobulin (Ig) gene product analysis using DNA isolated from fresh or frozen blood or gradient density purified peripheral blood mononuclear cells were done according to the methods of McCarthy et al. (39) and Griesser et al. (40). The finding of one discrete band was assigned as monoclonal. Several isolated, denser bands on a polyclonal background were

Pedigree 3

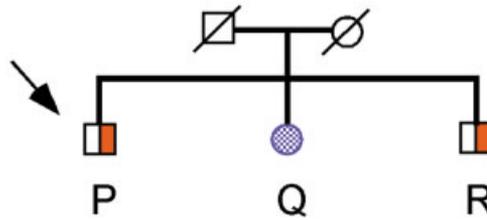
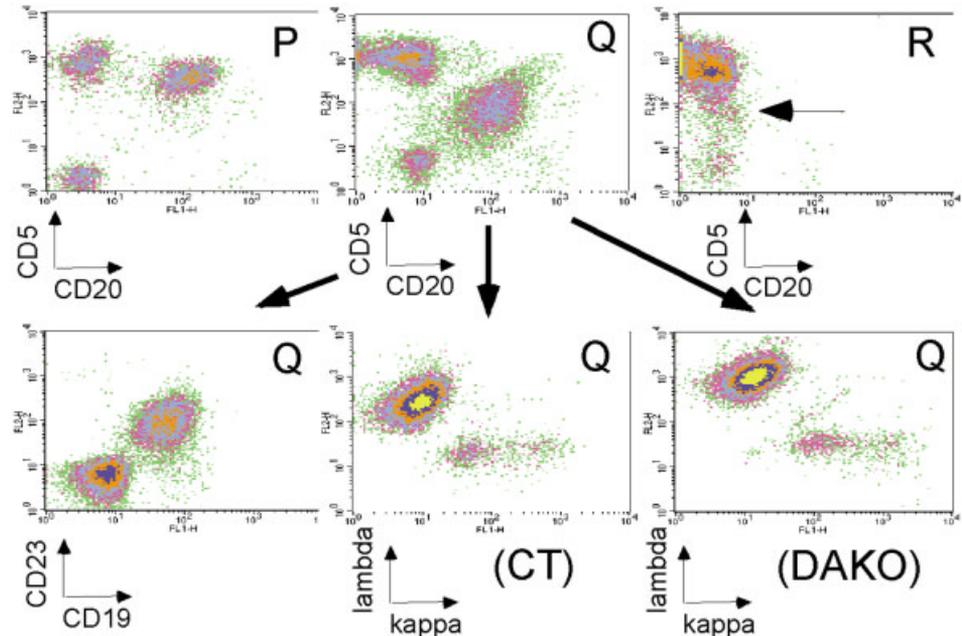


FIG. 3. Pedigree 3 consists of three siblings. One male sibling (R) had treated B-cell chronic lymphocytic leukemia with Richter's transformation and underwent an autologous peripheral blood stem cell transplantation. Presently he has minimal residual disease. His older brother (P) had an untreated small lymphocytic lymphoma with subleukemic involvement. A third sibling (Q) had an unremarkable history and normal physical examination and screening laboratory values. This unaffected sibling was found to have persistent, unequivocal, CD5⁺, B-cell monoclonal lymphocytosis (BCML). For the upper three panels, the two-parameter histograms show CD20 fluorescein isothiocyanate (FITC) on the x axis and CD5 phycoerythrin (PE) on the y axis. The lower left-hand panel shows BCML expression of CD23 PE on the y axis and CD19 FITC on the x axis. The middle and right two lower panels show the anti- κ FITC and anti- λ PE distributions for the two sets of light-chain reagents. In both cases, the light-chain analysis was based on CD19 tricolor (Tago) or CD19 Cy5 (Dako) respectively. CT, Caltag.



designated as an oligoclonal pattern. Results that appeared as a series of smooth, multiple bands (ladders) were designated as polyclonal. Controls used included a monoclonal B-cell line JVM-2 (obtained from Melo et al. [41]) and blood bank donors.

Statistical Analysis

P values were calculated with Poisson distribution statistics, which assumes that the outcome (BCML) is a rare event (11/1,449, 0.7%). We used SAS 6.12 (Cary, NC) to compute the probability of observing the number of BCML cases among unaffected first-degree relatives.

RESULTS

General Observations

Nine kindreds were selected for study from the NCI Familial BCLL Registry that included affected subjects and clinically unaffected first-degree family members who were available for flow cytometric and other studies. Individual immunophenotypic results are presented on representative pedigrees (Figures 1-6). Results from the analysis of all 52 individuals in these nine pedigrees are

presented in Table 2, which lists 19 affected (i.e., met standard diagnostic criteria for BCLL) and 33 unaffected (i.e., normal physical examination and screening laboratory values) subjects. Of these 33 individuals, 19 had one or more abnormal findings and are discussed in detail.

Immunophenotypic, Cell Cycle, and PCR Patterns in Affected and Unaffected Individuals With Familial BCLL

Affected individuals. All individuals classified as affected fulfilled minimum NCI-defined criteria for BCLL in terms of absolute lymphocyte count, blood film morphology, bone marrow involvement, and flow cytometric findings at some time during the period of observation. However, at the time the immunophenotyping was conducted, the absolute lymphocyte counts in individuals 1-B, 1-C, and 3-P was fewer than 5,000 cells/ μ l. Subsequently, absolute lymphocyte counts were more than 5,000 cells/ μ l in individual 1-B. Individual 1-C had been treated previously, and individual 3-P was diagnosed as having small lymphocytic lymphoma (SLL). The finding of a BCML in the setting of SLL was not unexpected. The affected son in pedigree 9 (9-YY) had an absolute lymphocyte count of

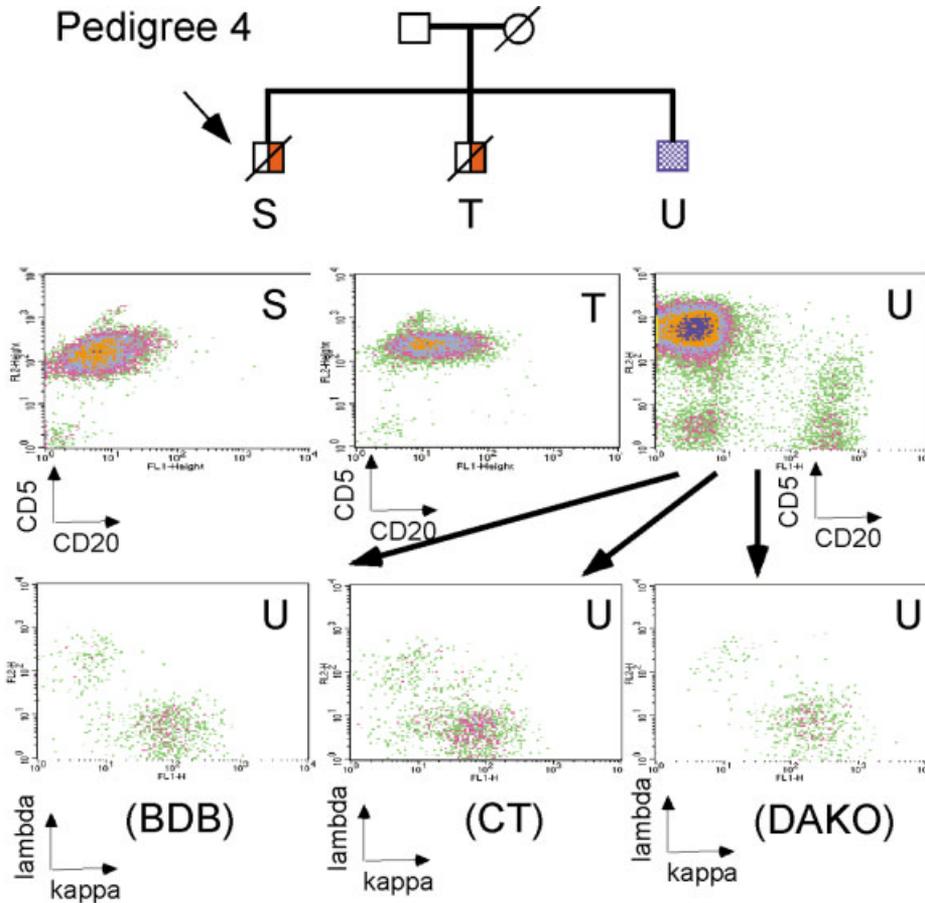


Fig. 4. Pedigree 4 consists of three male siblings. When first seen, B-cell chronic lymphocytic leukemia was confirmed immunophenotypically in the two affected brothers (S and T). A review and comparison of the blood films showed that the third, unaffected, clinically normal brother (U) had a small population of similar, abnormally appearing, lymphoid cells. His κ/λ analysis for all three light-chain reagents are shown in detail. The three lower panels show the anti- κ fluorescein isothiocyanate (FITC) on the x axis and anti- λ phycoerythrin (PE) on the y axis; see Table 1 for a listing of the three fluorochromes used with CD19. All three reagents demonstrate a monoclonal excess. In the three upper panels, the two-parameter histograms show CD20 FITC on the x axis and CD5 PE on the y axis. The mother is reported to have died from acute leukemia, type unspecified. BDB, Becton-Dickinson Biosciences; CT, Caltag.

500 cells/ μ l, with normal hemoglobin and platelet count. Although most of his B cells expressed the CD5 marker, there was no evidence of monoclonality. He also had a relative increase but normal number of natural killer cells, which was consistent with not only a hematologic but also a flow cytometric remission. In addition to the diagnostic light-chain restriction, there was a characteristic pattern of CD19, CD20, and CD5 coexpression in BCLL and all expressed CD23 and were negative for CD10. All cases of monoclonality were confirmed with the PCR assay. Examples of oligoclonality or biconality were not seen in any of the affected individuals in this report. With regard to cell cycle analysis, and consistent with a previous report, aneuploidy was not observed (21).

Thus, among the affected subjects, six exhibited a flow cytometric pattern indistinguishable from BCML. Individual 1-B had a transient BCML followed by BCLL, whereas individual 1-C in the same kindred had minimal residual disease. In a separate family, individual 3-P had SLL with BCML, but this was not unexpected. His sibling's (individual 3-R) BCML was after treatment, with minimal residual disease exhibiting a BCML pattern. Individual 5-V had BCML in the setting of a spontaneous remission (13). Individual 9-YY had a hematologic and flow cytometric remission. However, most of his B cells expressed CD5,

and he had a relative increase but normal natural killer cell numbers. His PCR pattern also showed a restricted polyclonal ladder pattern.

Unaffected individuals. Analysis of the 33 unaffected individuals showed 14 individuals who were normal in all of the assays used. The abnormal findings involving the remaining 19 individuals follow.

1. Six of the 33 unaffected and apparently clinically normal individuals (2-N, 2-O, 3-Q, 4-U, 8-QQ, and 9-XX) in these nine kindreds had BCML. The expression of monoclonality by flow cytometric analysis of light-chain restriction was confirmed by PCR in individuals 3-Q, 8-QQ, and 9-XX. The other three subjects had a light-chain-restricted B-cell subpopulation by flow cytometric analysis (not always seen in the overall κ/λ ratio) accompanied by an oligoclonal (2-O) or a polyclonal (2-N and 4-U) pattern on PCR accompanied by a normal absolute B-cell count. These six individuals were designated as having flow cytometric BCML.

2. Four individuals (1-E, 1-F, 2-J, and 5-X) showed oligoclonality by PCR analysis. Individual 1-F also had an unusual L-shaped distribution of CD20/CD5 cells with an elevated absolute B-cell count, i.e., an oligoclonal B-cell lymphocytosis.

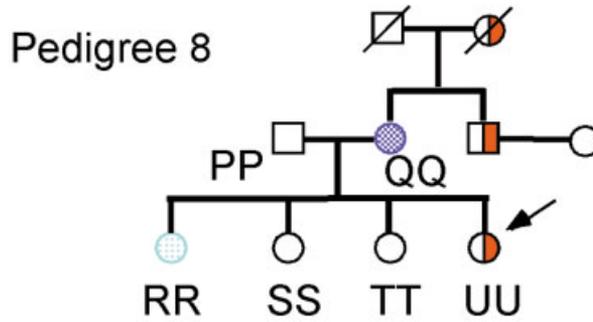
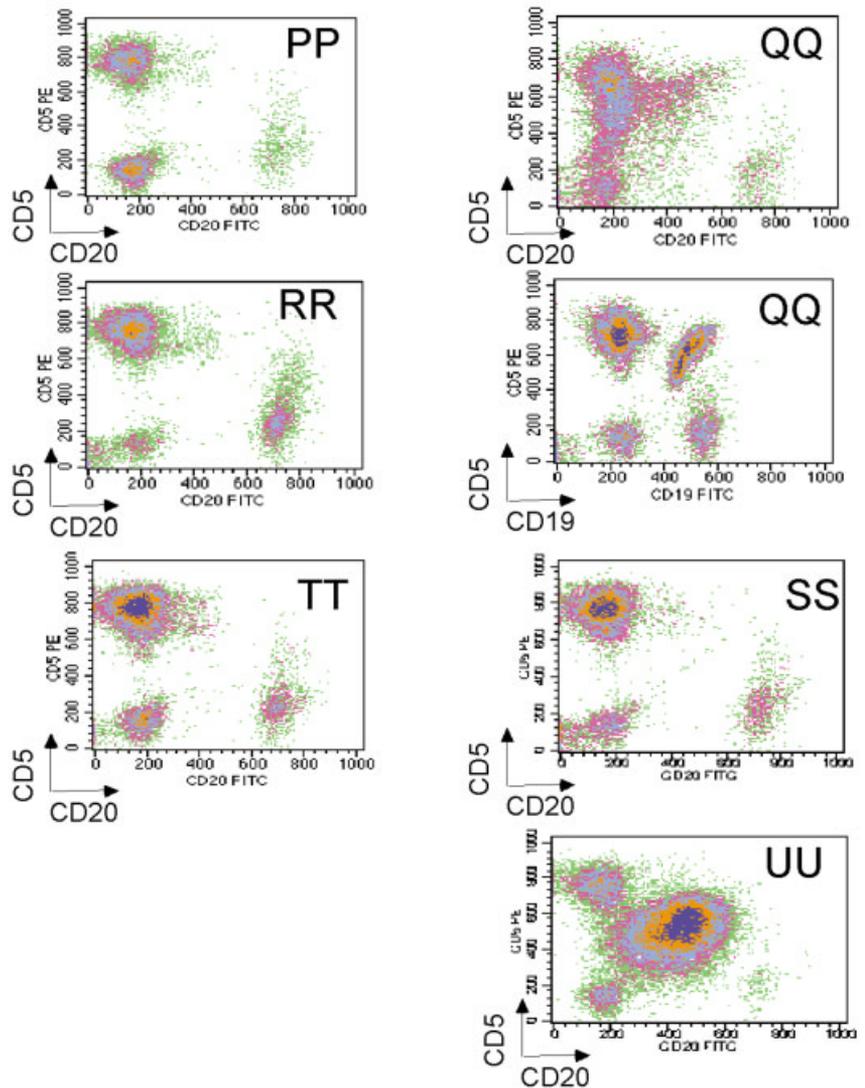


Fig. 5. Pedigree 8 consist of three generations: the proband, a maternal uncle, and the grandmother. In this kindred, the mother (QQ) is believed to be an obligate carrier for a putative genetic marker of B-cell chronic lymphocytic leukemia (BCLL) and in fact has a CD5⁺ B-cell monoclonal lymphocytosis. Records on the grandmother in the first generation were not available, and her CLL was self-reported by the proband (UU). Medical records on the uncle in the second generation confirmed a diagnosis of CLL. The two-parameter histograms show CD20 fluorescein isothiocyanate on the x axis and CD5 phycoerythrin on the y axis. Because the CD20/CD5 pattern of the mother (QQ) is diffusely complex, her CD19/CD5 profile is shown directly beneath her CD20/CD5 profile (right-hand column, second panel). One sibling (RR) has a polyclonal B-cell lymphocytosis.



3. Individual 2-M demonstrated an abnormal flow pattern consisting of a small polyclonal population of CD20/CD5 B cells (91 cells/ μ l) and had a second subpopulation of CD5⁻ cells with decreased CD20 fluorescence intensity (see arrow in Fig. 2).

4. Siblings 1-G and 1-H had normal κ/λ immunophenotypic surface expression, normal CD20/CD5 expression patterns, and normal polyclonal ladders by the Ig gene rearrangement assay but showed distinct evidence of a tetraploid population by cell cycle analysis.

Pedigree 9

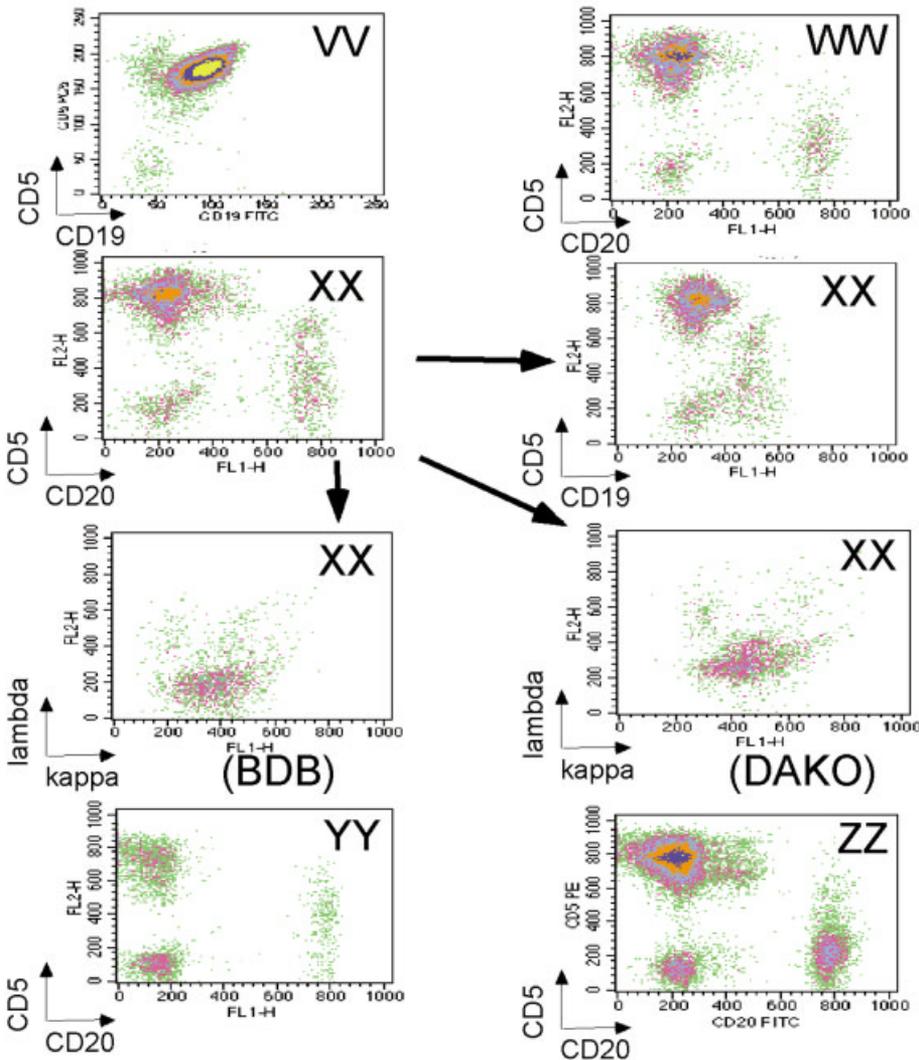
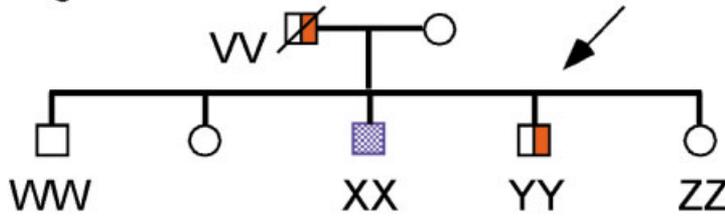


FIG. 6. Pedigree 9 consists of an affected parent (father, VV) and an offspring (son, YY). Before the death of the father, a blood sample was obtained. The son was diagnosed and treated at another medical center. Blood samples on three of four remaining siblings (WW, XX, and ZZ) were drawn and shipped to the National Institutes of Health by overnight mail, but blood films and complete blood cell counts were not available for this pedigree at the time of flow cytometric immunophenotyping. The son had a hematologic, flow cytometric, and molecular remission, but the relative number of CD5 B cells is increased. Of three unaffected siblings (WW, XX, and ZZ) tested, WW and ZZ were normal. Individual XX had B-cell monoclonal lymphocytosis (BCML). The two-parameter histogram for VV shows CD19 phycoerythrin (PE) on the x axis and CD5 PE on the y axis. There are four histograms for sibling XX with BCML. In the left column, second panel and the right column, second panel, the two-parameter histograms show CD20 FITC on the x axis and CD5 PE on the y axis on the left, and the right shows CD19 PE on the x axis and CD5 PE on the y axis. The anti- κ FITC and anti- λ PE gated on CD19 peridinin chlorophyll protein and CD19 Cy5 are shown in the bottom panels in each column, respectively.

5. Individuals 7-MM, 7-NN, 7-OO, 9-WW, and 9-ZZ showed a relative CD5 B-cell lymphocytosis.

6. Individuals 1-D and 8-RR showed an isolated polyclonal B-cell lymphocytosis.

7. Individual 7-LL appeared to have an early T-cell proliferation (data not shown).

Individuals 5-Y, 5-Z, 5-AA, 6-DD, 6-EE, 6-FF, 6-GG, 7-HH, 7-II, 8-PP, 8-SS, and 8-TT were normal. Thus, several unexpected patterns were seen in these 19 unaffected individuals including a CD5⁺ or CD5⁻ BCML with Ig rearrangement, oligoclonality with or without a B-cell lymphocytosis, and tetraploidy. The most expansive and intriguing pattern

Table 1
Reagent List for Familial Immunophenotyping Panel*

Tube 1	Unstained		
Tube 2	CD45 FITC	CD14 PE	
Tube 3	CD3 FITC	CD19 PE	
Tube 4	CD3 FITC	CD16+CD56 PE	
Tube 5	CD4 FITC	CD8 PE	
Tube 6	CD19 FITC	CD5 PE	
Tube 7	CD20 FITC	CD5 PE	
Tube 8	CD19 FITC	CD23 PE	
Tube 9	Anti- κ FITC	Anti- λ PE	CD19 PerCP
Tube 10	Anti- κ FITC	Anti- λ PE	CD19 TC
Tube 11	Anti- κ FITC	Anti- λ PE	CD19 RPE-Cy5
Tube 12	CD10 FITC	CD34 PE	CD19 PerCP
Tube 13	κ FITC	CD22 PE	CD20 PerCP
Tube 14	λ FITC	CD22 PE	CD20 PerCP

*FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; TC, tricolor; RPE-Cy5, tandem conjugate.

was an L-shaped distribution connecting all of these subpopulations (see individual 1-F). The pattern shown by individual 8-QQ was equally interesting. Both patterns suggested different pathways or different sets of normal B-cell subpopulations that may undergo early neoplastic transformation.

Statistical Analysis

The Poisson distribution was used to find the probability of observing 6 in 33 individuals with BCML in our sample. Assuming that the population prevalence of BCML was 0.7%, the probability of observing 6 of 33 BCML individuals was highly significant ($P = 5.7 \times 10^{-9}$).

DISCUSSION

We found BCML in 6 of 33 (18%) unaffected individuals with familial BCLL. We defined BCML for the purpose of this study to be the flow cytometric detection of any monoclonal B-cell population in the absence of a history of B-cell leukemia or other related LPD. The definition of BCML has gone through some variation since its earliest description (21-23,25). Initially, we recognized the abnormal CD20/CD5 pattern in normal individuals and to this was added the conventional serologic detection of light-chain restriction (21). Given that κ/λ analysis may be difficult in the setting of reduced-surface Ig, three sets of light-chain reagents were used, and monoclonality was further supplemented on the basis of Ig gene rearrangement. However, the PCR detection of monoclonality on non-enriched B-cell preparations is limited by the polyclonal background and the consensus primers. Considerations of percentages and absolute numbers of B cells and CD5 B cells were added for screening purposes (23).

The appearance of an L-shaped pattern for the CD20/CD5 expression was a new finding in this study and may be related to the collection of a substantial number of cells in our study. Of equal interest was the presence of CD19⁺, CD20⁻, CD5⁺ B cells in BCML subject 8-QQ. These findings suggested to us multiple B-cell subsets for the origin and development of the CLL clone. In previous reports, we suggested a pathway for the clonal evolution involving

progression from a negative CD5 BCML, to a CD5 CD20 bright BCML, to a CD5 CD20 dim BCML (42-44).

In the largest, cross-sectional, population study of B-cell monoclonality in subjects older than 40 years, the Agency for Toxic Substances and Disease Registry (ATSDR) found evidence of a BCML in 8 of 891 (0.8%) individuals and 3 of 608 (0.5%) individuals in the test (area around Superfund waste sites) and control sites, respectively (22). A follow-up study confirmed the existence and identical level of BCML (25). In addition, BCML was observed in a sample of normal blood bank donors at a low level (3/656, 0.46%) (45). In the present study, BCML was found in first-degree relatives of patients with familial BCLL at a considerably higher level of 18%.

Since we began our studies, Rawstron et al. (46) identified the prevalence of the monoclonal CLL-like and monoclonal non-CLL-like immunophenotypic cells in a series of 910 hospital outpatients who had normal hematologic parameters and no history of malignant disease and found 3.5% and 1%, respectively. In a companion study involving 59 healthy first-degree relatives of patients from 21 CLL families, Rawstron et al. (47) reported the detection of CLL-like immunophenotypic cells in 8 of 59 relatives (13.5%), representing a highly significant increase in risk ($P = 0.00002$) for the presence of this immunophenotype. Although our controls differed in that they used an outpatient clinic population and we used a cross-sectional population-based control, we found that the incidence of a BCML is significantly increased in first-degree relatives in familial CLL. Rawstron et al. suggested that the presence of a CD5⁺ BCML or CLL-immunophenotype represents a surrogate marker of carrier status in CLL families. They further speculated that the existence of a CD5⁻ BCML suggests a common genetic predisposition to the development of B-cell malignancies.

The relation of BCML to BCLL in the familial and sporadic BCLL population settings are unknown. There are few reports suggesting the range of possible outcomes. Faguet et al. (48) identified a series of 39 referred patients with a slight increase in their absolute lymphocyte count. They reported that 24 of the 39 patients (62%) had an abnormal B-cell clone. Over 5 years, one-third (8/24) progressed to clinical BCLL. This finding established that at least some patients with BCML progress to BCLL within 5 years. A median of 10 years is required for MGUS to convert to MM (17,18). Further studies are needed to determine the ultimate outcome of BCML in the familial and sporadic settings and to better understand the factors controlling this progression.

In addition to the potential relation of BCML to CLL and LPD, a benign monoclonal lymphocytosis should be considered. A benign monoclonal lymphocytosis indistinguishable from BCLL has been reported (49-53), and Wang et al. reported patients with a stable, CD5⁻ lymphocytosis (52). More recently, Byrd (54) raised the practical issue of long-term, serial follow-up to determine the relation of BCML to CLL in the same manner that MGUS was followed. In all likelihood, BCML is the cellular equivalent of MGUS (17,18). If this is so, BCML should increase with

Table 2
Lymphocyte Subsets, κ/λ Analysis, and PCR Status*

IND	WBC	ALC	%T	AbT	%B	Ab B	%NK	AbNK	%CD5	ACD5	κ/λ ratio	PCR	Cat
1-A	77.5	54.2	5	2,710	93	50,400	2	1,084	95	47,880	90.5	MC	1
1-B	16.5	4.29	23	986.7	70	3,430	10	429	70	2,401	4.2	MC	1
1-C	16	3.2	3	96	85	2,720	15	480	85	2,312	88	MC	1
1-D	7.3	2.44	63	1,537	22	537	15	366	36	193	1.62	PC	4
1-E	6.5	1.66	56	929.6	20	332	21	348.6	47	156	1.14	OLC	4
1-F	7.75	2.99	56	1,674	29	867	15	448.5	57	494	2.3	OLC	4
1-G	7.23	2.36	66	1,558	12	283	16	377.6	33	93	1.42	PC/TET	4
1-H	4.99	1.89	60	1,134	13	246	20	378	27	66	2.26	PC/TET	4
2-I	15.1	9.2	34	3,128	61	5,612	5	460	100	5,612	34.8	MC	1
2-J	6.41	1.96	79	1,548	12.6	247	8.3	162.68	41	101	1.0	OLC	4
2-K	17.4	1.39	8	111.2	90	1,251	1	13.9	100	1,251	93.3	MC	1
2-L	9.01	5.67	10	567	83	4,706	8	453.6	10	470	36.6	MC	1
2-M ^a	4.09	1.3	69	897	7	91	24	312	20	18	1.95	PC	4
2-N ^b	9.4	2.22	69	1,532	13	399	18	399.6	46	168	1.29	PC	3
2-O	3.6	1.57	70	1,099	18	172	11	172.7	45	78	13.3	OLC	3
3-P	6.7	2.81	30	843	51	1,433	19	533.9	98	1,404	IND	MC	1
3-Q	8.7	3.32	38	1,262	45	1,494	14	464.8	96	1,434	0.1	MC	3
3-R	6.7	1.3	85	1,105	7	91	5	65	70	64	79	MC	1
4-S	42.5	38.2	2	764	95	36,300	0	0	100	36,300	λ	MC	1
4-T	27.7	22	88.3	19,426	8.1	19,400	0	0	98.6	19,150	λ	MC	1
4-U	6	1.6	79.5	1,272	10.3	165	1.8	28.8	53	87	0.3	PC	3
5-V	4.4	1.3	80.1	1,041	6.8	139	10.7	139.1	34	47	3.2	PC	1
5-W	33.1	23.9	15.5	3,705	75.8	18,116	0.8	191.2	75.8	18,116	κ	MC	1
5-X ^c	4.4	1.27	65	822.3	13	164	22	278.3	9	15	6.7	OLC	4
5-Y	4.5	1.82	91.4	1,663	4.9	89	2.8	50.96	25.9	47	1.2	PC	2
5-Z	6.5	2.4	81.5	1,956	10.6	122	5.1	122.4	35	54	1.7	PC	2
5-AA	6.5	2.3	84	1,932	5.9	136	8.4	193.2	44	60	1.2	PC	2
6-BB	87.1	69.7	7.4	5,158	86.7	60.4	0	0	89	53.7	77	MC	1
6-CC	8.7	6.7	10.5	703.5	87.2	5,842	0	0	99	5,842	77	MC	1
6-DD	10	2.3	66	1,518	7.8	179	25.4	584.2	18	32	1.8	ND	2
6-EE	4.6	2	81.5	1,630	8.5	170	9	180	24.7	42	1.6	PC	2
6-FF	4	1.5	60	900	12	180	26.5	397.5	53.2	95.4	1.0	PC	2
6-GG	5.4	2.3	73.3	1,686	13.6	312	12.3	282.9	38	119	1.2	PC	2
7-HH	7.7	2.2	77	1,694	10.7	235	8.8	193.6	53	99	1.6	PC	2
7-II	8.6	2.1	86	1,806	8.1	170			33	56	1.6	PC	2

*Abbreviations used are IND, Individual; WBC, white blood count; ALC, absolute lymphocyte count; %T, percent CD3positive T cells; AbT, absolute CD3positive T cell count; %B, percentage of CD19positive cells; AbB, absolute CD19positive B cell count; %NK, percentage NK cells; AbNK, absolute NK cell count, %CD5, percentage CD5positive B cells; ACD5, absolute CD5 B cell count; κ/λ ratio, kappa bearing cells/lambda bearing cells; PCR: Analysis of heavy chain immunoglobulin rearrangement (MC-monoclonal, PC-polyclonal, OLC-oligoclonal, Tet-Cell Cycle Analysis showed tetraploidy, Indt, indeterminate); Cat, Category (1-Affected, 2-Unaffected (Normal), 3-Unaffected (BCML), 4-Unaffected (Abnormal). Color code: red is affected; blue is unaffected and normal; lavender is unaffected but abnormal.

Normal ranges for percentage and absolute values (95% confidence level) were obtained from the Clinical Immunology Laboratory, CC, NIH. They are CD3positive 60.9–84.0% and 832–2028 cells per μl ; CD19positive 5.7–16.2% and 84–378 cells per μl ; CD20positive 4.8–15.9% and 88–330 cells per μl ; CD4positive 32.6–58.9% and 480–1339 cells per μl ; CD8positive 17.8–46.7% and 351–911 cells per μl ; CD5positiveCD20positive 1.3–10.3% and 24–222 cells per μl ; and CD3negativeCD16positive CD56positive NK cells, 6.5%–29.5% and 120–490 cells per μl . WBC 4,300–9,200 cells per μl and lymphocytes 17–41% with an ALC of 1,173–2640 cells per μl .

(a) Individual. 2-M: Abnormal CD20 subpopulation see arrow in figure.

(b) Individual. 2-N: The value 46% CD B cells was determined using CD20/CD5 reagent configuration, while 62% was obtained using CD19/CD5 configuration (not shown). Of further interest, the geometric mean channel value for CD20 FITC in the CD20/CD5 configuration showed a value of 260 for CD20 positive CD5 negative cells and a value of 196 for CD20 positive CD5 positive cells. This represents a 64-channel shift in these two populations. Upon a clonal search this subject had a small cluster of kappa bearing cells even though the overall kappa lambda ratio was normal. Fukushima et al. (59) found no such abnormalities in 104 normal individuals studied.

(c) Individual 5-X: In the kappa lambda analysis there is a small population of B cells negative for kappa and lambda expression.

(d) Individual 7-LL: Has an extra population of CD5positive T Cells and his CD3positive NK cells are elevated at 20%. This is consistent but not diagnostic of an early T cell lymphoproliferative disorder.

(e) Individual 8-QQ and individual 8-UU: kappa lambda expression was not only indeterminate tubes #9–#11, but the CD19positive B cells from these two individuals did not express these kappa lambda reagents, i.e., they were double negatives. However, in tubes #13 and #14, kappa light chain overexpression was noted in both of these individuals. This is a strong argument for the use of pattern recognition rather than the solitary use of a kappa lambda ratio. However, monoclonality based on a PCR analysis of Ig heavy chain rearrangement clarifies this situation.

(f) BCML individuals 8-QQ and 9-XX on PCR had a single band on a polyclonal background.

(g) Individuals 8-RR, 8-SS and 8-TT reacted only with two of the kappa lambda analyses; tubes #9 and #11 while tube #10 was negative.

(h) The PCR pattern on the affected individual 9-YY with hematological and flow cytometric remission shows a restricted polyclonal ladder.

Table 2
Lymphocyte Subsets, κ/λ Analysis, and PCR Status* (continued)

IND	WBC	ALC	%T	AbT	%B	Ab B	%NK	AbNK	%CD5	ACD5	κ/λ ratio	PCR	Cat
7-JJ	89.1	79.2	2.7	2,138	96.6	76,500	0.7	554.4	99	75,740	λ	MC	1
7-KK	6.4	2	38.7	774	50.6	1,020	10.3	206	50.2	508	IND	MC	1
7-LL ^d	5.8	2.11	82	1,732	5.9	124	5.9	124.63	45	58	2.0	PC	4
7-MM	5.7	1.7	83.3	1,416	7.6	129	7.4	125.8	57.9	74	1.5	PC	4
7-NN	5.7	2.5	76	1,900	9.6	240	13.8	345	61	146	1.4	PC	4
7-OO	5.5	3	82	2,460	9.4	282	7.4	222	74	209	1.3	PC	4
8-PP	7.7	1.2	56.2	674	6.1	73	31.8	382	35	25	0.73	PC	2
8-QQ ^e	7.7	2.6	51.0	1,326	38.0	988	8.3	216	74	731	κ	MC ^f	3
8-RR	8.4	2.25	72.1	1,622	20.8	468	4.5	101	40	187	1.8 ^g	PC	4
8-SS	11.6	2.84	79.2	2,249	8.6	244	8.8	250	17	41	1.7 ^g	PC	2
8-TT	7.9	0.66	76.6	505	7.9	52	11.4	75	16	8	1.3 ^g	PC	2
8-UU ^e	26.1	21.1	13.7	2,891	81.5	17,197	4.3	907	100	17,197	κ	MC	1
9-W			1		97		1		97		0.01	NA	1
9-WW			85		9		3		78		3.3	NA	2
9-XX			78		15		4		87		14.0	MC ^f	3
9-YY	1.9	496	51	253	9	45	36	179	78	35	1.9	PC ^h	1
9-ZZ			79		12		5		50		1.2	PC	2

age, and MGUS in a certain percentage of individuals should progress or transform into BCLL or other related LPDs over time. Rawstrom et al. found exactly this age-dependent relation for BCML (46,47).

Thus, longitudinal studies of BCML in the setting of familial BCLL will be required to elucidate the importance of this precursor state and undoubtedly will provide insight into the mechanism of progression to BCLL. Given that BCLL kindred are a key resource in attempting to identify genetic susceptibility factors for this condition, further studies of individuals with BCML will be a high priority. Population and family studies are needed to identify the rate of progression to BCLL and to better understand the parallels between this clinical entity and BCLL. Due to an increasing aged population and an unexplained increase in the incidence of B-cell non-Hodgkin's lymphoma over the past decade, better understanding the determinants of BCML in families is a priority (55). This population is ideal for the study of early events in BCLL with the use of post genomic technologies (56–58). We believe these findings of familial CLL in the United States and the United Kingdom should be investigated in the setting of an international familial CLL consortium.

ACKNOWLEDGMENTS

We are grateful to Dr. Margaret Tucker for helpful discussions. This paper is dedicated to the memory of Glennelle C. Washington who died while this paper was being prepared.

LITERATURE CITED

- Weiss NS. Geographical variation in the incidence of the leukemia and lymphomas. *Natl Cancer Inst Monogr* 1979;53:139–142.
- Linet MS, Cartwright RA. Chronic lymphocytic leukemia: epidemiology and etiologic findings. *Nouv Rev Fr Hematol* 1988;30:353–357.
- Capalbo S, Trerotoli P, Ciancio A, Battista C, Serio G, Liso V. Increased risk of lymphoproliferative disorders in relatives of patients with B-cell chronic lymphocytic leukemia: relevance of the degree of familial linkage. *Eur J Haematol* 2000;65:114–117.
- Goldin LR, Sgambati M, Marti GE, Fontaine L, Ishibe N, Caporaso N. Anticipation in familial chronic lymphocytic leukemia. *Am J Hum Genet* 1999;65:265–269.
- Gunz FW, Dameshek W. Chronic lymphocytic leukemia in a family, including twin brothers and a son. *JAMA* 1957;164:1323–1325.
- Gunz FW, Gunz JP, Veale AM, Chapman CJ, Houston IB. Familial leukaemia: a study of 909 families. *Scand J Haematol* 1975;15:117–131.
- Horwitz M, Goode EL, Jarvik GP. Anticipation in familial leukemia. *Am J Hum Genet* 1996;59:990–998.
- Yuille MR, Houlston RS, Catovsky D. Anticipation in familial chronic lymphocytic leukaemia. *Leukemia* 1998;12:1696–1698.
- Yuille MR, Matutes E, Marossy A, Hilditch B, Catovsky D, Houlston RS. Familial chronic lymphocytic leukaemia: a survey and review of published studies. *Br J Haematol* 2000;109:794–799.
- Ardashnikov SN. Genetics of leukemia in man. *J Hyg* 1937;37:286–289.
- Conley CL, Misiti J, Laster AJ. Genetic factors predisposing to chronic lymphocytic leukemia and to autoimmune disease. *Medicine* 1980;59:323–334.
- Goldgar DE, Easton DF, Cannon-Albright LA, Skolnick MH. Systematic population based assessment of cancer risk in first-degree relatives of cancer probands. *J Natl Cancer Inst* 1994;86:1600–1608.
- Ishibe N, Sgambati MT, Fontaine L, Goldin LR, Jain N, Weissman N, Marti G, Caporaso NE. Clinical characteristics of familial B-CLL in the National Cancer Institute Familial Registry. *Leuk Lymphoma* 2001;42:99–108.
- Linet MS, Van Natta ML, Brookmeyer R, Khoury MJ, McCaffrey LD, Humphrey RL, Szklo M. Familial cancer history and chronic lymphocytic leukemia: a case-control study. *Am J Epidemiol* 1989;130:655–664.
- Wiernik PH, Ashwin M, Hu XP, Paietta E, Brown K. Anticipation in familial chronic lymphocytic leukaemia. *Br J Haematol* 2001;113:407–414.
- Dohner H, Stilgenbauer S, Benne A, Leupol E, Kröbe A, Bulling L, Döhne K, Bent M, Lichter P. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2001;343:1910–1911.
- Kyle RA, Rajkumar SV. Monoclonal gammopathies of undetermined significance. *Hematol Oncol Clin North Am* 1999;13:1181–1202.
- Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF, Melton LJ III. A long-term study of prognosis in monoclonal gammopathy of undetermined significance. *N Engl J Med* 2002;346:564–569.
- Jack A, Richards S, Evans P, Wilks C. Population screening for B-cell monoclonal lymphocytosis using PCR and flow cytometry to determine immunophenotypes. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p 93–96.
- Maiese RL, Braylan RC. Detection of low levels of B-cell lymphoproliferative disorders. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell

- chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p 61-68.
21. Marti GE, Muller J, Stetler-Stevenson M, Caporaso N. B-cell monoclonal lymphocytosis in three individuals living near a hazardous waste site. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p 37-50.
 22. Sarasua SM, Vogt RF, Middleton DC, et al. 'CLL-like' B-cell phenotypes detected in superfund studies: epidemiologic methods and findings. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p 7-18.
 23. Vogt RF, Meredith MNK, Powell J, et al. Results in eleven individuals with B-CLL-like phenotypes detected in environmental health studies. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p 19-35.
 24. Cartwright RA. Summary of epidemiology and immunobiology. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p 215-222.
 25. Slade BA. Follow-up investigation of B-cell abnormalities identified in previous ATSDR health studies. Publication PB99-138331. Bethesda: US Department of Health and Human Services, Public Health Service; 1999.
 26. Caporaso N, Fontaine L, Whitehouse J, Marti GE. Familial B-CLL: review of literature and the NCI Familial B-CLL Registry. In: Marti GE, Vogt RF, Zenger VE, editors. Proceedings of a USPHS workshop on laboratory approaches to determining the role of environmental exposures as risk factors for B-cell chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Atlanta, GA; 1997. p.173-180.
 27. Caporaso NE, Whitehouse J, Bertin P, Amos C, Papadopoulos N, Muller J, Whang-Peng J, Tucker MA, Fleisher TA, Marti GE. A 20 year clinical and laboratory study of familial B-chronic lymphocytic leukemia in a single kindred. *Leuk Lymphoma* 1991;3:331-342.
 28. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood* 1996;87:4990-4997.
 29. Zwiebel JA, Cheson BD. Chronic lymphocytic leukemia: staging and prognostic factors. *Semin Oncol* 1998;25:42-59.
 30. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick H, Sultan C. Proposals for the classification of chronic (mature) B and T lymphoid leukemias. *J Clin Pathol* 1989;42:567-584.
 31. Carter PH, Resto-Ruiz S, Washington GC, Ethridge S, Palini A, Vogt R, Waxdal M, Fleisher T, Noguchi P, Marti GE. Whole blood lysis: a flow cytometric analysis of three anticoagulants and five cell preparations. *Cytometry* 1992;13:68-74.
 32. Duque RE, Andreeff M, Braylan RC, Diamond LW, Peiper SC. Consensus review of the clinical utility of DNA flow cytometry in neoplastic hematopathology. *Cytometry* 1993;14:492-496.
 33. Fleisher TA, Marti GE. Detection of unseparated human lymphocytes by flow cytometry. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. Volume 1. New York: John Wiley & Sons; 1991. p 7.9.1-7.9.7.
 34. Kamihira S, Matutes E, Marco JG, Hoda S, Morilla R, Owusu-Ankomah K, Crawford A, Ellis J, Catovsky D. Flow cytometry detection of minimal DNA aneuploidy in mature lymphoid leukemias: Comparison with metaphase and interphase cytogenetics. *Int J Oncol* 1994;5:211-214.
 35. Zenger VE, Vogt R, Mandy F, Schwartz A, Marti GE. Quantitative Flow Cytometry: Inter-Laboratory Variation. *Cytometry* 1998;33:138-145.
 36. Nicholson JKA, Rao PE, Calvelli T, Stetler-Stevenson M, Browning SW, Marti GE. Artifacts staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. *Cytometry* 1994;18:140-146.
 37. Shanky TV, Rabinovitch PS, Bagwell B, Bower KD, Duque RE, Hedley DW, Myall BH, Wheelless L, Cox C. Guidelines for implementation of clinical DNA cytometry, International Society for Analytical Cytology. *Cytometry* 1993;14:472-477.
 38. Vindelov LL. Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. A new method for rapid isolation and straining of nuclei. *Virchows Arch B Cell Pathol* 1977;24:227-242.
 39. McCarthy KP, Sloan JP, Wiedemann LM. Rapid method for distinguishing clonal from polyclonal B-cell populations in surgical biopsy specimens. *J Clin Pathol* 1990;43:429-432.
 40. Griesser H. Applied molecular genetics in the diagnosis of malignant non-Hodgkins lymphoma. *Diagn Mol Pathol* 1993;2:177-191.
 41. Melo JV, Brito-Babapulle V, Foroni L, Robinson DS, Luzzatto L, Catovsky D. Two new cell lines from B-prolymphocytic leukaemia: characterization by morphology, immunological markers, karyotype and Ig gene rearrangement. *Int J Cancer* 1986;38:531-538.
 42. Marti GE. Mechanisms of a B cell neoplasia. In: Potter M, Melchers F, editors. Mechanisms of B cell neoplasia. Basel, Switzerland: Editiones Roche; 1993. p 129-136.
 43. Marti GE, Faguet G, Bertin P, Agee J, Washington G, Ruiz S, Carter P, Zenger V, Vogt R, Noguchi P. CD20 and CD5 expression in B chronic lymphocytic leukemia (B-CLL). *Proc N Y Acad Sci* 1992;651:480-483.
 44. Marti GE, Faguet GB, Stewart C, Branham P, Carter PH, Washington GC, Bertin P, Muller J, Zenger V, Caporaso N, Whitehouse J, Amos CI, Fleisher TA, Vogt R. Evolution of leukemic heterogeneity of human B-CLL lymphocytes between and within patients. *Curr Topics Microbiol Immunol* 1992;182:303-311.
 45. Rachel JM, Zucker ML, Plapp FV, Fox CM, Marti GE, Abassi F, Menitove JE. B cell monoclonal lymphocytosis in blood donors (abstract). *Blood* 2002;100:590a.
 46. Rawstron AC, Green MJ, Kuzmicki A, Kennedy B, Fenton JAL, Evans PAS, O'Connor SJM, Richards SJ, Morgan GJ, Jack AS, Hillmen P. Monoclonal B lymphocytes with the characteristics of "indolent" chronic lymphocytic leukemia are present in 3.5% of adults with normal blood counts. *Blood* 2002;100:635-639.
 47. Rawstron AC, Yuille MR, Fuller J, Cullen M, Kennedy B, Richards SJ, Jack AS, Matutes E, Catovsky D, Hillmen P, Houlston RS. Inherited predisposition to CLL is detectable as sub-clinical monoclonal B-lymphocyte expansion. *Blood*. 2002;100:2289-2291.
 48. Faguet GB, Agee JF, Marti GE. Clone emergence and evolution in chronic lymphocytic leukemia: characterization of clinical, laboratory, and immunophenotypic profiles of 25 patients. *Leuk Lymphoma* 1992;6:345-356.
 49. Bassan R, Buzzetti M, Marini B, Rambaldi A, Allavena P, Barbui T. Investigation of chronic lymphocytosis in adults. *Am J Clin Pathol* 1988;89:783-787.
 50. Han T, Ozer H, Gavigan M, Gajera R, Minowada J, Bloom ML, Sadamori N, Sandberg AA, Gomez GA, Henderson ES. Benign monoclonal B cell lymphocytosis—a benign variant of CLL: clinical, immunologic, phenotypic, and cytogenetic studies in 20 patients. *Blood* 1984;64:244-252.
 51. Mandelli F, De Rossi G, Mancini P, Alberti A, Cajozzo A, Grignani F, Leoni P, Liso V, Martelli M, Neri A. Progression in chronic lymphocytic leukemia: a retrospective multicentric study from the GIMEMA group. *J Clin Oncol* 1987;5:398-404.
 52. Wang C, Amato D, Fernandes B. CD5-negative phenotype of monoclonal B-lymphocytosis of undetermined significance (MLUS). *Am J Hematol* 2002;69:147-149.
 53. Yakhnina EI, Nikitin EA, Astsaturov IA, Varlamova EY, Kobzev YN, Kovaleva LG, Kremenetskaya AM, Melikyan AL, Pivnik AV, Samoilova RS, Vorobyev AI. A benign form of chronic lymphoid leukemia. *Ter Arkh* 1997;69:11-17.
 54. Byrd J. Clonal lymphocytosis of uncertain significance (CLUS): what clues will CLUS yield? *Blood* 2002;100:2277.
 55. Levine PH, Hoover RN. The emerging epidemic of non-Hodgkin's lymphoma: current knowledge regarding etiologic factors. *Cancer Res* 1992;52:5425S-5574S.
 56. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, Yang L, Pickeral OK, Rassenti LZ, Powell J, Botstein D, Byrd JC, Grever MR, Cheson BD, Chiarazzi N, Wilson WH, Kipps TJ, Brown PO, Staudt LM. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. *J Exp Med* 2001;194:1639-1647.
 57. Stratowa C, Loffler G, Lichter P, Stilgenbauer S, Haberl P, Schweifer N, Döhner H, Wilgenbus KK. CDNA microarray gene expression analysis of B-cell chronic lymphocytic leukemia proposes potential new prognostic markers involved in lymphocyte trafficking. *Int J Cancer* 2001;91:474-480.
 58. Voss T, Ahorn H, Haberl P, Döhner H, Wilgenbus K. Correlation of clinical data with proteomics profiles in 24 patients with B-cell chronic lymphocytic leukemia. *Int J Cancer* 2001;91:180-186.
 59. Fukushima PI, Nguyen PK, O'Grady P, Stetler-Stevenson M. Flow cytometric analysis of kappa and lambda light chain expression. *Cytometry* 1996;26:243-252.