Expression of NK-Lineage Markers on Peripheral Blood Lymphocytes with T-Helper (Leu3⁺/T4⁺) Phenotype in B Cell Chronic Lymphocytic Leukemia

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Heterogeneity within lymphocyte subsets expressing T-helper (T4⁺/Leu3⁺) or T-suppressor (T8⁺/Leu2⁺) markers was analyzed in 38 patients with B cell chronic lymphocytic leukemia (B-CLL) and in 11 age-matched controls. Co-expression of NK-lineage markers (M1, Leu7) on Leu2⁺ or Leu3⁺ cells was investigated by two-color immunofluorescence, and the proportion of granular lymphocytes within each subset was determined by cytochemical staining for acid phosphatase. B-CLL patients and normal controls had similar absolute numbers of cells per μl with T-suppressor phenotype. However, the proportion of Leu2⁺ cells co-expressing the Leu7 antigen was higher in the B-CLL patients than in the control subjects (54 ± 3% vs 27 ± 4%, P < .0001). The absolute number per μl of cells with T-helper phenotype was somewhat decreased in B-CLL patients compared with normal subjects (649 ± 104 vs 799 ± 33, P < .02), with a consequent decrease of the helper/suppressor ratio. Furthermore, co-expression of the Leu7 and, more strikingly, of the M1 markers was found in 27 ± 4% of the B-CLL patients compared with normal controls (11 ± 2% vs 2 ± 0.7%, P < .002 for Leu7 and 40 ± 5% vs 4 ± 1%, P < .00001 for M1). Cytochemical studies showed that a large proportion of Leu3⁺ cells from B-CLL patients were granular lymphocytes, as suggested by the co-expression of natural killer (NK) cell markers. The emergence of a population of Leu3⁺ granular lymphocytes with NK markers, which is barely detectable in normal subjects, may provide an explanation for the impairment of T cell functions repeatedly described in B-CLL.

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20% of the T4⁺/Leu3⁺ cells can be identified morphologically and cytochemically as granular lymphocytes. Cells with the T-helper phenotype co-expressing NK-lineage markers (Leu7, M1) and displaying granular lymphocyte morphology have been found to be increased in lymphoproliferative disorders. Similar phenotypes (Leu3⁺/Leu7⁺) have also been detected in germinal-center cells from normal and lymphomatous lymphoid tissues. Imbalances of the component cell types within the helper or suppressor subsets may be responsible for the impairment of T cell function found in several disease states. B cell chronic lymphocytic leukemia (B-CLL) contains a small fraction of this subset when Leu3⁺ cells are positively or negatively selected for the expression of certain other surface markers (5/9, TQ1, Leu8, HB 10, HB 11). Other functional studies have shown that suppressor and cytotoxic functions can also be generated from T4⁺/Leu3⁺ precursors. Furthermore, a small proportion of peripheral blood T4⁺/Leu3⁺ cells express the Leu7 antigen, and up to 20% of the T4⁺/Leu3⁺ cells can be identified morphologically and cytochemically as granular lymphocytes. Cells with the T-helper phenotype co-expressing NK-lineage markers (Leu7, M1) and displaying granular lymphocyte morphology have been found to be increased in lymphoproliferative disorders. Similar phenotypes (Leu3⁺/Leu7⁺) have also been detected in germinal-center cells from normal and lymphomatous lymphoid tissues.
offers a potential example. Numerous studies have analyzed the phenotypic and functional characteristics of T lymphocytes in B-CLL patients, with the aim of detecting abnormalities that might explain the accompanying this disorder. T cells, although decreased in absolute number,29 Moreover, imbalances in the T cell subsets were detected, with an increase of Tγ cells and an inverted helper/suppressor ratio.28 T cell proliferative responses to mitogens or alloantigens were reported to be delayed or decreased in some studies and to be normal in others. The same defect was also shown in isolated T cells with helper phenotypes. A defective helper function for B cell differentiation have also been demonstrated for T cells isolated from B-CLL patients. Conflicting data have been reported for the T cell suppressor functions, which were normal in some studies or increased in others. Antibody-dependent cellular cytotoxicity (ADCC) and NK function exerted by cells forming E rosettes were also found decreased in B-CLL. In this study we have analyzed the heterogeneity of T cell subsets in B-CLL patients by means of two-color immunofluorescence analyses and cytochemical methods capable of identifying granular lymphocytes. We show that, in B-CLL, increased proportions of cells with the T suppressor phenotype express NK-cell markers. Furthermore, we find that in B-CLL patients a large proportion of cells with the helper phenotype also express myelomonocytic and NK-cell antigens and display morphologic and cytochemical features of granular lymphocytes.

MATERIALS AND METHODS

Patients and Controls

Thirty-eight B-CLL patients were included in this study. The diagnosis was confirmed by the finding of a monoclonal proliferation of B lymphocytes in the peripheral blood. Eighteen patients were men and 20 were women. Age ranged from 41 years to 82 years. The white blood cell counts ranged from 8,000 to 132,000 cells per microliter. According to the Rai classification scheme, 15 patients were at stage 0, four at stage I, 13 at stage II, one at stage III, and five at stage IV. Six patients were newly diagnosed, 20 had been off treatment (prednisone or chlorambucil or both) for at least six months prior to the time of study, and 12 patients had received no treatment in the preceding two weeks. None was receiving treatment at the time of study. The six patients seen at presentation were also studied in the course of treatment and two months after cessation of therapy.

Eleven healthy age-matched subjects were used as controls. The same defect was also shown in isolated T cells with helper phenotypes. A defective helper function for B cell differentiation have also been demonstrated for T cells isolated from B-CLL patients. Conflicting data have been reported for the T cell suppressor functions, which were normal in some studies or increased in others. Antibody-dependent cellular cytotoxicity (ADCC) and NK function exerted by cells forming E rosettes were also found decreased in B-CLL. In this study we have analyzed the heterogeneity of T cell subsets in B-CLL patients by means of two-color immunofluorescence analyses and cytochemical methods capable of identifying granular lymphocytes. We show that, in B-CLL, increased proportions of cells with the T suppressor phenotype express NK-cell markers. Furthermore, we find that in B-CLL patients a large proportion of cells with the helper phenotype also express myelomonocytic and NK-cell antigens and display morphologic and cytochemical features of granular lymphocytes.

Preparation of Mononuclear Cells

Mononuclear cell fractions were separated from heparinized peripheral blood samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation.

Antibodies

FITC- or RITC-conjugated goat anti-human μ, δ, γ, and α heavy chains and κ and λ light chains, affinity-purified and tested for specificity as described previously, were generously provided by Dr W. Gathings and used at a concentration of 300 to 500 μg/ml. Anti-T cell and anti-T cell subset mouse monoclonal antibodies of the Leu series were provided by Becton Dickinson (Mountain View, Calif). Leu4, and IgG1 antibody recognizing a pan-T antigen, Leu3, an IgM monoclonal antibody that identifies a subset comprising helper/inducer T cells, and Leu2, an IgG1 antibody that is specific for a subset comprising suppressor/cytotoxic T cells, were used as FITC-conjugates at a concentration of 100 μg/ml. Leu7, an IgM monoclonal antibody that identifies NK cells, was kindly provided by Drs T. Abo and C. M. Balch and used as FITC- or RITC-conjugates at the concentrations of 10 or 20 μg/ml, respectively.

OKM1, and IgG2b monoclonal antibody that recognizes an antigen expressed by myelomonocytic and NK cells, was purchased from Ortho Pharmaceutical Corp (Raritan, NJ) and used at the concentration of 100 μg/ml. An RITC-conjugated goat antibody anti-mouse γ2b, absorbed extensively with human and murine immunoglobulin, was used as the developing reagent for the M1 monoclonal antibody at the concentration of 100 to 300 μg/ml. Leu11, an IgG1 monoclonal antibody reacting with a type of Fc receptor on granulocytes and NK cells, was obtained from Becton Dickinson and used as an FITC-conjugate at a concentration of 100 μg/ml. OK-1a, an IgG2a monoclonal reagent recognizing HLA-DR antigens, was purchased from Ortho Pharmaceuticals and developed with affinity-purified rhodamine-conjugated goat anti-mouse γ2a antibodies.

Immunofluorescence

Typing of the leukemic clones was performed by using RITC-conjugated goat antibodies to human κ, μ, and γ chains in combination with FITC-conjugated goat antibodies to λ, δ, and α chains, respectively. Analysis of the T cell subsets was performed by two-color immunofluorescence using FITC-conjugated Leu3 or Leu2 antibodies in combination with RITC-conjugated Leu7 or M1 (developed with an RITC-conjugated goat antibody anti-mouse γ2b), or OK-1a (developed with RITC-conjugated goat antibody anti-mouse γ2a). Leu3* cells were analyzed for co-expression of Leu4 or Leu11 markers using an avidin-conjugated Leu3 reagent developed with rhodamine-coupled biotin, followed by fluorescein-labeled Leu4 or Leu11. Staining with each antibody was followed by a washing step before the next antibody was added. Negative controls for the indirect staining with M1 or OK-1a were obtained by using, respectively, an IgG2b or an IgG2a mouse monoclonal antibody reactive with human determinants, followed by the appropriate RITC-conjugated goat antibody.

Co-expression of Leu2 or Leu3 antigens on Leu7-positive cells was determined in similar fashion to that described above. Co-expression of M1 on Leu7-positive cells was analyzed by staining mononuclear cells with FITC-conjugated Leu7, followed by M1 and RITC-conjugated goat anti-mouse γ2b antibodies.

Wet preparations of viable cells were examined with a Leitz Orthoplan microscope equipped with epi-illumination and filters for fluorescein or rhodamine. The absolute percentages of positive cells were determined by counting at least 300 viable mononuclear cells. Co-expression of two markers on the same cells was determined by scoring 100 cells positive for one marker and determining the percentage of those cells also expressing the other marker.

Data are expressed as absolute cell counts per microliter or as mean percentages ± SE. The level of significance for the differences
observed between normal and B-CLL mononuclear cells has been calculated with the two-sample rank test.35

**Isolation of Leu3+ cells**

Mononuclear cells from three healthy controls and from three B-CLL patients were stained with fluorescein-conjugated Leu3 monoclonal antibody. Positive cells were subsequently isolated with a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Sunnyvale, Calif) and processed for the cytochemical localization of acid phosphatase activity.

**Cytochemical Staining for Acid Phosphatase Activity**

Leu3+ cells obtained by fluorescence-activated cell sorting were fixed in suspension with 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.6, for 30 minutes at room temperature and washed with phosphate-buffered saline. Fixed cells were cyt centrifuged onto slides and incubated for one hour at 37°C in a substrate containing naphthol-AS-BI-phosphonic acid (Sigma Chemical Co, St Louis) and Fast Garnet GBC salt (Sigma), in acetate buffer, pH 5.2.56 Following incubation, slides were washed in deionized water and mounted with Fluoromount (Southern Biotechnology Assts, Birmingham, Ala). At least 200 cells were counted in each preparation. Cells displaying numerous positive granules scattered in the cytoplasm were scored as granular lymphocytes.

**RESULTS**

**Imbalance of Leu3+/Leu2+ Phenotypes**

Absolute numbers per µL of cells expressing T cell antigens (Leu4, Leu3, Leu2) and an NK-cell antigen (Leu7) are shown in Fig 1, where B-CLL and control mononuclear cell preparations are compared. A slight but statistically significant decrease of Leu3+ cells was found in B-CLL patients compared with normal controls (P < .02). Thus, the helper/suppressor (Leu3/Leu2) ratio appeared significantly decreased (P < .01) in B-CLL patients as compared with normal controls.

**Fig 1.** Absolute numbers per microliter of Leu4+, Leu3+, Leu2+, and Leu7+ cells in the peripheral blood of B-CLL patients (B-CLL) vs normal controls (NC). Horizontal bars indicate mean values. The level of significance for the differences, as determined by a two-sample rank test, is shown at the top of each column. The means ± SE for each marker in B-CLL vs NC were the following: Leu4+ (N = 38 v 11), 1,359 ± 58 v 1,277 ± 176; Leu3+ (N = 38 v 11), 649 ± 104 v 799 ± 33; Leu2+ (N = 38 v 11), 628 ± 88 v 620 ± 55; Leu7+ (N = 38 v 11), 589 ± 90 v 179 ± 24. The last column on the right shows the Leu3+/Leu2+ ratio, which corresponded to 1.0 ± 0.1 in B-CLL patients and 1.7 ± 0.2 in normal controls.

Furthermore, the number of Leu7+ (NK) cells was significantly higher in B-CLL than in controls (P < .02).

**NK-Lineage Markers on Leu3+ Cells**

Two-color immunofluorescence staining was performed to detect co-expression of other markers on cells with NK (Leu7), suppressor (Leu2), or helper (Leu3) phenotypes. As shown in Fig 2, no significant difference between normal controls and B-CLL patients was found for the expression of the Leu3, Leu2, or M1 markers on Leu7+ cells. When marker co-expression was analyzed on Leu2+ cells, it was shown that higher proportions of cells with the suppressor phenotype expressed the Leu7 marker in B-CLL in comparison with normal controls (P < .001), whereas no difference for the co-expression of the M1 antigen was noted (Fig 3). Highly significant differ-
ences between B-CLL patients and controls were found in the Leu3^+ population when marker co-expression was investigated. Both the Leu7 and, more strikingly, the M1 markers were increased on Leu3^+ cells from B-CLL patients (P < .002 and P < .00001, respectively) (Fig 4). In 24 of 28 patients, the percentage of Leu3^+ cells also expressing M1 was above two standard deviations (>16.1%) from the mean determined in normal controls. In three patients with high percentages of Leu3^-M1^- cells, we did not find co-expression of Leu2, Leu11, or Ia-like antigens on Leu3^+ cells. On the contrary, all of the Leu3^+ cells bore the T3/Leu4 antigen.

In the group of patients studied at presentation, during treatment, and after treatment, no significant changes in the number of circulating Leu3^-M1^- or Leu3^-Leu7^- cells were detected.

Granular Lymphocyte Morphology of Leu3^+ Cells With NK-lineage Markers

Since the Leu7 and the M1 markers are both expressed on granular lymphocytes, the nature of the Leu3^+ cells expressing these markers in B-CLL patients was further investigated by cytochemical analysis of Leu3^+ cells isolated by cell sorting. The acid phosphatase marker was chosen because, contrary to other acid hydrolases (eg, acid esterase), this enzyme activity is detected cytochemically in the large majority of granular lymphocytes. In three normal controls, the percentage of Leu3^+ cells displaying granular lymphocyte morphology and expressing acid phosphatase ranged from 14% to 16%. A much higher percentage of acid phosphatase-positive, granular lymphocytes was found in Leu3^+ cells from three B-CLL patients with a range from 50% to 75% (Fig 5). In the same patients, co-expression of the M1 marker on Leu3^+ cells ranged from 38% to 62%, as opposed to 0% to 1% found in the three normal controls.

DISCUSSION

Our study primarily addressed the analysis of phenotypic heterogeneity of cell subsets expressing T-suppressor or T-helper surface markers. The underlying hypothesis was that increased heterogeneity, or the emergence of new phenotypes, might explain the impairment of T cell functions described in several disease states. The choice of B-CLL was promoted by the voluminous literature suggesting that an intrinsic T cell defect might characterize this lymphoproliferative disorder. The degree of the heterogeneity within subsets of cells with T-suppressor or T-helper markers was determined through the co-expression of markers of the NK-lineage (Leu7, M1).

Analysis of T cells expressing pan-T, helper, or suppressor markers confirmed previous observations^28^ 34^ indicating that (1), the absolute number of T cells is not decreased in B-CLL patients compared with normal controls and (2), a slight but statistically significant decrease of cells with the T-helper phenotype occurs in B-CLL, leading to reduction or inversion of the T-helper/suppressor ratio.

Absolute numbers of cells with T-suppressor (T8/Leu2) phenotype were similar in B-CLL and controls (Fig 1). However, co-expression of the Leu7 antigen on these cells was newly doubled in B-CLL, without significant variation in the co-expression of the M1 marker. This increased proportion of Leu2^-Leu7^- cells in B-CLL may reflect an increase of immature NK-cells^1^ which, in turn, might explain the defective NK function shown in B-CLL patients.49^ 51^
marker was even more pronounced. Indeed, the most striking change among lymphocytes with T markers was the appearance of a cell with a novel phenotype, Leu3^-/M1^+, which is barely detectable in normal individuals but is prominent in 80% of the B-CLL patients. Leu3^- cells from these patients did not express Leu2, Ia-like antigens, or FcIgG receptors (as detected by the Leu11 monoclonal antibody), but consistently displayed the T3/Leu4 antigen. Since both the Leu7 and the M1 antigen are markers of granular lymphocytes, we studied the cytochemical characteristics of Leu3^- cells positively selected from the peripheral blood of B-CLL patients and found that the percentage of Leu3^- granular lymphocytes was increased significantly in B-CLL patients compared with normal controls. When these FACS-purified Leu3^- fractions were tested for their NK function, no cytotoxic activity against K562 cells was found (data not shown).

The emergence of Leu3^- cells with NK lineage markers and granular lymphocyte morphology did not appear to be related to the stage of clinical presentation or to therapy. In particular, when the total white blood cell count decreased in response to therapy, the absolute numbers of circulating Leu3^-/M1^+ cells did not show significant changes.

The increased proportion of M1-positive granular cells within the subset with T-helper phenotype may provide an alternative explanation for the multiple functional T cell defects described in B-CLL, such as decreased mitogen responsiveness, decreased helper activity, or excessive suppressor activity, which are commonly ascribed to an intrinsic T cell abnormality. In fact, the absolute number of helper T cells, presumably a portion of the Leu3^-/M1^+ subset, is even more significantly decreased in B-CLL compared with controls (P < .001) than the Leu3^- cells altogether (P < .02). The concept of a quantitative rather than a qualitative T cell defect in B-CLL is reinforced by our preliminary finding that Leu3^-/M1^+ cells show poor proliferative responses to T cell mitogens and do not support the PWM-driven B cell differentiation (data not shown).

In conclusion, our study shows that in B-CLL patients, cells with T-helper markers display a high degree of heterogeneity because of an increased proportion of cells with markers of the NK lineage. Finally, the emergence of cell subsets with the Leu3^-/M1^+ phenotype may also be detected in other neoplastic and nonneoplastic disorders that represent convenient models for functional studies of these cells.

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