T Cells in B-Cell Chronic Lymphocytic Leukemia: Quantitative Assessment of Cytotoxic and Interleukin-2–Producing Lymphocyte Precursors by Limiting Dilution Analysis

By Ottmar Janssen, Christoph Nerl, and Dieter Kabelitz

Controversy exists as to the functional capacity of T lymphocytes in patients with B-cell chronic lymphocytic leukemia (CLL). We have used a limiting dilution (LD) culture approach to quantitatively assess frequencies of proliferating lymphocyte precursors (PLP), cytotoxic lymphocyte precursors (CLP), and interleukin-2 (IL-2)–producing helper lymphocyte precursors (HLP). Unseparated mononuclear cells (MNC) or purified T cells (E⁺) and leukemic B cells (E⁻) were cocultured under LD conditions with irradiated OKT3 hybridoma cells in the absence (determination of HLP) or presence of recombinant IL-2 (determination of PLP and CLP). Under these conditions, low frequencies of PLP, HLP, and CLP (f = 1/65 to 1/4600) were measured in unseparated MNC of CLL patients. In contrast, purified T cells (50% to 92% CD3⁺) contained frequencies of PIP, HIP, and CIP (frequency of HIP) or presence of recombinant IL-2 (depletion) cells can be readily identified in CLL patients and, in fact, absolute numbers of circulating T cells are generally raised. Numerous studies have emphasized a disturbed balance of T helper and T suppressor cells as well as functional defects of T cells in B-CLL patients. In vitro production of interleukin-2 (IL-2) is reduced in CLL, as is the level of natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC). It is not entirely clear, however, whether these defects are due to a “dilution” of T helper function of the clonally expanded, irrespective of their individual antigenic specificity. Functional capacities such as lymphokine production and cytotoxic effector function of the clonally proliferating T cells can be explored; based on the quantitative distribution of responsive and nonresponsive microcultures plated under LD conditions, frequencies (f) of proliferating lymphocyte precursors (PLP), IL-2–producing helper lymphocyte precursors (HLP), and cytotoxic lymphocyte precursors (CLP) can be determined. Our present results indicate that frequencies of PLP, HLP, and CLP detected in CLL patients are similar to those measured in healthy controls when corrected for the percentage of CD3⁺ T cells. The data are discussed with regard to controversial reports on T cell function in B-CLL.

CHRONIC LYMPHOCYTIC leukemia (CLL) is usually characterized by an accumulation of monoclonal “resting” B cells. Although the vast majority of peripheral blood mononuclear cells (MNC) from patients with high WBC counts are leukemic B cells, a residual T cell population can be readily identified in CLL patients and, in fact, absolute numbers of circulating T cells are generally raised. Numerous studies have emphasized a disturbed balance of T helper and T suppressor cells as well as functional defects of T cells in B-CLL patients. In vitro production of interleukin-2 (IL-2) is reduced in CLL, as is the level of natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC). It is not entirely clear, however, whether these defects are due to a “dilution” of T helper function of the clonally expanded, irrespective of their individual antigenic specificity. Functional capacities such as lymphokine production and cytotoxic effector function of the clonally proliferating T cells can be explored; based on the quantitative distribution of responsive and nonresponsive microcultures plated under LD conditions, frequencies (f) of proliferating lymphocyte precursors (PLP), IL-2–producing helper lymphocyte precursors (HLP), and cytotoxic lymphocyte precursors (CLP) can be determined. Our present results indicate that frequencies of PLP, HLP, and CLP detected in CLL patients are similar to those measured in healthy controls when corrected for the percentage of CD3⁺ T cells. The data are discussed with regard to controversial reports on T cell function in B-CLL.

MATERIALS AND METHODS

Patients. Eight patients (seven women, one man) with B-CLL were included in this study. WBC counts ranged from 25 to 300 × 10⁹/L. The clinical stage was assessed according to Rai et al., and is listed in Table 1. None of the patients was on treatment within the last 6 months before the study. Heparinized blood was obtained from CLL patients and healthy control donors after informed consent. Isolation of lymphocyte subpopulations. MNC were isolated from peripheral blood by centrifugation on Ficoll-Hypaque density gradients. MNC were further separated into T (E⁺) and non-T (E⁻) cells by several cycles of rosetting with neuraminidase-treated sheep erythrocytes (Eα). Briefly, MNC (20 to 100 × 10⁶) were mixed with 1 mL of a 10% Eα suspension and were immediately centrifuged on Ficoll-Hypaque gradients. Cells recovered from the interphase were depleted of residual E-rosette-forming T cells by a second cycle of Eα rosette separation. Nonrosetting cells obtained after the second cycle are referred to as E⁻ cells. These cells consisted of <0.5% CD3⁺ (Leu4⁺) T cells and 92.0% ± 4.1% CD20⁺ (Leu16⁺) B cells. Rosette-forming cells pelleting after the first Ficoll-Hypaque gradient were gently resuspended and centrifuged again on Ficoll-Hypaque. This step was repeated once more. Sheep erythrocytes were lysed in a buffered NH₄Cl solution. Rosette-forming cells obtained after three cycles of density separation are referred to as E⁺ or T cells. The cell fraction consisted of 71% ± 14.1% CD3⁺ (Leu4⁺) T cells (mean ± SD of seven separations not including patient LAN where only low T cell purity [14.6% CD3⁺] was achieved). Purified CLL E⁻ cells consisted of 48.8% ± 16% CD4⁺...
Graded numbers of responder cells were cocultured with irradiated OKT3 hybridoma cells (15,000 per well) in a total volume of 150 µL in wells of V-shaped microtiter plates (Nunc). The culture medium was the same as described above with the exception that no exogenous IL-2 was added; it was further supplemented with phorbol ester TPA (1 ng/mL; Sigma Chemicals, St Louis) and ionomycin (250 ng/mL; Calbiochem, San Diego). After three or four days, 75 µL of supernatant were transferred to flat-bottom microtiter plates, and IL-2 content was measured on IL-2-dependent murine CTLL cells by a colorimetric method as described in detail elsewhere.14 The CTLL cell line specifically detects IL-2; it is not reactive to human IL-1, IL-3, or IL-4.14

Statistical analysis. To determine frequencies of PLP, HLP, and CLP, all wells were considered positive in which 3H-thymidine uptake, IL-2 release, or specific cytotoxicity exceeded the median spontaneous values in 16 to 24 control wells (no responder cells added) by at least 3 SD. Frequencies (f) of PLP, HLP, and CLP were calculated according to the Poisson distribution of negative and positive wells with the aid of a computer program based on statistical methods of Taswell15 and kindly provided by Dr K. Heeg, ULM University. The statistical analysis included determination of P values for single hit kinetics and 95% confidence limits of frequency estimates. In all experiments described in this report, P was greater than 0.2, indicating a high probability of single hit kinetics.16

RESULTS

Frequency of PLP and CLP in B-CLL patients. We have recently described an experimental protocol in which CD3+ T cells are clonally activated by irradiated anti-CD3 producing OKT3 hybridoma cells.13 In combination with LD culture conditions, this method can be efficiently used to quantitatively assess the frequency of growth-inducible CD3+ T cells in a given lymphoid cell population, independent of their antigen specificity. Here we have applied this approach to measure frequencies of PLP in T-enriched and T-depleted lymphocyte subpopulations from B-CLL patients. As detailed in the Materials and Methods section, leukemic B cells purified by two consecutive cycles of E-rosette depletion were virtually free (<0.5%) of contaminating CD3+ T cells. T cells purified by repeated cycles of E-rosette formation consisted of 30% to 92% (mean, 70% ± 14.1%) CD3+ T cells with the exception of one patient (LAN) where CD3+ T cells could not be enriched to greater than 14%. In contrast to published data,33 E-rosette-purified T cells from the majority of this series of CLL patients did not have decreased CD4/CD8 ratios.

As expected from the low percentage of CD3+ T cells in unseparated MNC (0.5% to 13.9%, mean, 4.1% ± 4.3%), we measured only low frequencies of growth-inducible PLP when using CLL MNC as responder cells. As reported in Table 2, from one of 65 to one of 2,800 CLL MNC developed into a proliferating cell clone when activated under LD conditions by OKT3 hybridoma cells in the presence of exogenous IL-2. Purified leukemic B cells did not contain measurable numbers of CD3+ PLP that could be activated in the present LD system; frequency estimates of PLP within CLL B cells were less than 1/50,000 in all cases (not shown), which substantiates in functional terms the non-T nature of CLL E- cells. In contrast, E-rosette-purified CLL T cells were highly enriched for PLP that could be clonally activated under our LD culture conditions. When frequency estimates

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Stage of the disease was determined according to Rai et al.19
were corrected for the actual percentage of CD3+ (Leu4+)-positive T cells in a given CLL E+ responder cell population, we measured PLP frequencies of 1/4 to 1/10. This range of PLP frequency estimates is similar to control experiments with healthy donors where one of two to nine E-rosette-purified T cells developed into a proliferating clone when activated by OKT3 hybridoma cells (Table 2). These data demonstrate that CD3+ T cells within E-rosette–purified B-CLL T cells are as efficiently activated by OKT3 hybridoma cells as are normal T cells.

To determine the functional capacity of clonally developing CD3+ T cells in CLL, we measured the cytotoxic activity of individual microcultures in a lectin-facilitated 51Cr-release test. This assay detects cytotoxic effector cells independent of their antigen specificity and thus, in combination with OKT3 hybridoma-stimulated LD cultures, allows a frequency estimation of CD3+ CLP. As reported in Table 2, frequencies of CLP within CLL MNC responder cells were in the same order of magnitude as those of PLP (1/170 to 1/4,600), indicating that a large fraction of clonally activated PLP acquired cytotoxic effector function. As for PLP, no measurable cytotoxic lymphocyte precursors (f < 1/50,000) were present in T-depleted leukemic B cell populations (not shown). In contrast, CLP were highly enriched in purified CLL T cell fractions (see Table 2). Frequency estimates of CLP, corrected for the actual percentage of CD3+ cells, were as high as 1/5 to 1/12. Similar CLP frequencies (1/3 to 1/12) were measured in five independent experiments when E+ T cells from healthy control donors were clonally activated by OKT3 hybridoma cells (see Table 2). As shown in Fig 1, straight lines were obtained when the number of responder cells (MNC, E+, E−) seeded per well was plotted on the X axis v the logarithm of the fraction of negative (ie, nonresponding) cultures (Y axis). P values were calculated according to Taswell.16

Frequency of IL-2–producing HLP in B-CLL patients. To determine frequencies of IL-2–producing HLP,
the above LD protocol was slightly modified. Exogenous IL-2 was omitted from the culture medium; instead, cultures were supplemented with phorbol ester TPA (1 ng/mL) and ionomycin (250 ng/mL). After three days, supernatants of individual microcultures were screened for IL-2 content, and frequencies of IL-2 producing HLP were determined as described in Materials and Methods. Under these conditions, one of four to one of 11 E-rosette-purified T cells from five normal control donors produced IL-2 (Table 2). Unseparated MNC from B-CLL patients contained IL-2-producing HLP in low frequency (f = 1/250 to 1/2,070). HLP were greatly enriched in CLL T-cell populations (f = 1/8 to 1/24 of CD3+ T cells) where they were almost as frequent as in E+ T cells from healthy control donors. As for PLP and CLP, purified leukemic B cells were devoid of HLP (f < 1/50,000) in all but one patient. As shown in a frequency plot in Fig 2, highly purified leukemic B cells from patient LAN contained on repetitive testing a significant fraction of HLP (f = 1/1,700, P = .24) in the absence of detectable PLP and CLP (f < 1/50,000). It has been shown that certain normal and transformed B cells can produce IL-2.18,19 Further studies at the level of mRNA expression are required, however, to substantiate the hypothesis that patient LAN’s B cells are capable of producing and secreting IL-2.

**DISCUSSION**

Our study is a first attempt to quantitatively measure functional T lymphocytes in patients with B-CLL. There are numerous reports dealing with the distribution and with functional aspects of T lymphocyte subsets in B-CLL. These studies have emphasized a decreased ratio of helper to suppressor cell activity20 and colony formation in soft agar are within normal ranges.21

We have used a quantitative approach to measure frequencies of growth-inducible as well as cytotoxic and IL-2-producing helper lymphocyte precursors in CLL. The rationale of this approach is based on the fact that a large fraction of all CD3+ T cells can be clonally activated (independent of individual antigenic specificity) when T cells are triggered via anti-CD3 MoAbs. When combined with LD culture conditions, it is thus possible to determine frequencies of lymphocyte precursors that clonally develop into effector cells.21,22 This approach would be expected to reveal any major defect in the number of circulating PLP, CLP, and HLP in patients as compared with healthy control donors.

As expected from the low percentage of T cells, unseparated MNC from all CLL patients studied here contained few precursors that gave rise to proliferating, cytotoxic, or IL-2-producing T cells (f = 1/65 to 1/4,600). Leukemic B cell fractions rigorously depleted of residual T cells did not contain measurable numbers of PLP, CLP, or HLP (f < 1/50,000), in line with the surface marker analysis. In contrast, E-rosette-purified T cells were highly enriched for PLP, CLP, and HLP; in fact, when corrected for the percentage of CD3+ cells, frequencies of PLP, CLP, and HLP were as high in CLL E+ cells as they were in control E+ cells. These data strongly suggest that cell populations exerting two basic T cell-associated functions, ie, cytotoxicity and IL-2 production, are numerically preserved in B-CLL patients including those with extremely high (>100 x 10^9/L) WBC counts.

In conclusion, we have successfully applied LD culture technology to the quantitative analysis of functional T cell subsets in B-CLL. These studies did not reveal major defects in the number of circulating cytotoxic or IL-2-producing
lymphocyte precursors. It should be stressed, however, that our experimental protocol activates CD3+ T cells independent of their antigenic specificity; any defect in antigen-specific T cell activation pathways would not be detected in the experimental system used here.

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REFERENCES

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