In vivo Dynamics of Stable Chronic Lymphocytic Leukemia Inversely Correlate with Somatic Hypermutation Levels and Suggest No Major Leukemic Turnover in Bone Marrow

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Abstract

Although accumulating evidence indicates that chronic lymphocytic leukemia (CLL) is a disease with appreciable cell dynamics, it remains uncertain whether this also applies to patients with stable disease. In this study, ^2H_2O was administered to a clinically homogeneous cohort of nine stable, untreated CLL patients. CLL dynamics in blood and bone marrow were determined and compared with normal B-cell dynamics in blood from five healthy individuals who underwent a similar ^2H_2O labeling protocol. Average CLL turnover rates (0.08–0.35% of the clone per day) were ~2-fold lower than average B-cell turnover rates from healthy individuals (0.34–0.89%), whereas the rate at which labeled CLL cells in blood disappeared (0.00–0.39% of B cells per day) was ~10-fold lower compared with labeled B cells from healthy individuals (1.57–4.24% per day). Leukemic cell turnover variables inversely correlated with the level of somatic hypermutation of the CLL clone (IgVH mutations). Although CLL cells in bone marrow had a higher level of label enrichment than CLL cells in blood, no difference between proliferation rates and proapoptotic and antiapoptotic profiles of CLL cells from these compartments was observed. These data suggest that, in stable disease, there is a biological relationship between the degree of somatic hypermutation of the CLL clone and its dynamics *in vivo*. Furthermore, in contrast to lymph nodes, the bone marrow does not seem to be a major CLL proliferation site. [Cancer Res 2008;68(24):10137–44]

Introduction

For several decades, chronic lymphocytic leukemia (CLL) has been regarded as a homogeneous accumulative disease in which intrinsic apoptotic defects of clonal $CD5^+CD19^+$ B cells account for the etiology and progression of the disease. However, in recent years, evidence has been gathered that challenged this paradigm. Although the balance between proapoptotic and antiapoptotic regulators in CLL is shifted toward an antiapoptotic profile compared with normal B cells (1), an intrinsic defect in apoptosis has not been found. In addition, it has become clear that the disease is rather heterogeneous, both in its clinical behavior and in its biology.

Based on IgVH gene mutations (2–4), specific chromosomal abnormalities (5, 6), and expression of the surface marker CD38 (4, 7, 8) and the intracellular protein ZAP-70 (9–13), prognostic subgroups can be defined in which cases with unmutated IgVH genes, deletion of the short arm of chromosome 17 or the long arm of chromosome 11, and expression of CD38 and/or ZAP-70 have an inferior clinical outcome (4–8).

CLL cells receive signals from the microenvironment, which provide them with the means to enter the cell cycle and to circumvent apoptosis, resulting in accumulation of the total tumor clone. These survival signals are hypothesized to be induced by cell-cell interactions with stromal cell–derived factor (SDF)-producing nurse-like cells (14, 15), bone marrow stromal cells (16, 17), CD40L-expressing $CD4^+$ T cells (18, 19), and stimuli from chemokines or growth factors, such as vascular endothelial growth factor (20), SDF-1 (15), BAFF (21), and APRIL (21). The interaction with these microenvironmental stimuli is thought to occur predominantly in specific tissues such as lymph nodes, spleen, and bone marrow (22). Indeed, in a comparative study between CLL cells derived from lymph nodes and peripheral blood, we recently confirmed not only increased expression of the cell cycle markers Ki67 and survivin but also a strong overexpression of antiapoptotic regulators in lymph node–derived CLL cells (1, 17). These observations imply that CLL may not simply result from an accumulation of leukemic cells but may be a dynamic disease where cells proliferate and die with various rates in different compartments.

"Heavy water" labeling experiments can be used to study cell dynamics *in vivo*. Administration of deuterated water (^2H_2O) is a safe and reliable method for labeling of dividing cells *in vivo* for long time spans and has resolved the issues of label toxicity that have hampered previous labeling studies (23, 24). During intake of ^2H_2O, deuterium (^2H) is incorporated into the deoxyribose moiety of newly synthesized DNA and thereby labels dividing cells. The presence of deuterium can be detected by gas chromatography and mass spectrometry (25). Up-labeling and down-labeling profiles of cells can be analyzed mathematically, providing cell-specific turnover characteristics for each individual.

Recently, Messmer and colleagues applied ^2H_2O labeling to a clinically heterogeneous CLL patient cohort. They showed that there is substantial leukemic cell turnover (production) in CLL, with proliferation rates ranging from 0.1% to 1.0% of the entire clone per day (26), which strongly supports the concept of CLL as a

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-08-2325

dynamic disease. Nevertheless, several issues remain unresolved. First, it remains to be elucidated whether the observed dynamic state also applies to patients with untreated clinically stable disease and whether the dynamic state is related to the level of IgVH gene mutations as has been suggested by *in vitro* telomere length analyses (27). Second, it is unclear whether recently divided leukemic cells tend to contribute to the maintenance of the total CLL clone or tend to be lost rapidly. Third, most leukemic cells do not reside in the blood but mainly in lymph nodes and bone marrow. These sites may provide CLL cells with several microenvironmental stimuli. However, it still has to be determined whether leukemic cells preferentially proliferate at these sites *in vivo*.

The aim of the present study was to determine *in vivo* leukemic cell dynamics in stable CLL patients by measuring not only CLL turnover in both blood and bone marrow but also disappearance rates of recently divided cells during an extended down-labeling period of approximately 1 year. In this cohort of clinically stable CLL patients, an inverse correlation between the level of mutations in the IgVH gene and both the average CLL turnover rate and the disappearance rate of recently proliferated CLL cells was found. Furthermore, based on labeling data of leukemic cells in blood and bone marrow in concert with the proapoptotic and antiapoptotic profile at the RNA and protein level, we show that the bone marrow does not seem to be a major site of CLL proliferation.

Materials and Methods

Patients and *in vivo* ^2H_2O labeling protocol. One female and nine male participants, who were diagnosed with CLL by established criteria (28) and who had stable disease (stable leukemic cell counts) for at least 1 y without any need for treatment, were enrolled in the study after having provided written informed consent (Table 1). All patients were admitted for 1 d at the Academic Medical Center (Amsterdam, the Netherlands) where they received an initial bolus of 10 mL ^2H_2O (99.8% enriched) per kg body water in small portions. Blood was withdrawn before the first portion and urine was collected after the last portion. Body water was estimated to be 60% of body weight for males and 50% of body weight for females. As a maintenance dose, the subjects drank 12.5% of the initial dose at home daily during a 9-wk labeling period. Blood and urine were collected five or six times at regular intervals during the labeling period and eight times during the down-labeling period of about 1 y. From CLL04, no data were obtained as this participant dropped out of the study at day 14 because of personal reasons. Six participants (CLL01, CLL02, CLL07, CLL08, CLL09, and CLL10) underwent a bone marrow puncture at the end of the labeling period. To minimize contamination of the harvested bone marrow with peripheral blood, only the first syringe of aspiration was used. Microscopic analysis of the aspirates confirmed high-quality bone marrow–derived cells as indicated by the presence of stromal cells, nucleated precursor red cells, and megakaryocytes in all cases. In addition, three patients (CLL01, CLL03, and CLL08) underwent a bone marrow biopsy.

Next to the CLL participants, five healthy participants [for individual details, see Vrisekoop and colleagues (29)] were enrolled in this study. They underwent a similar ^2H_2O labeling protocol as described above, with the exception that the down-labeling period consisted of 150 d in which blood and urine were collected seven times. This study was conducted in accordance with the ethical standards of our institutional medical committee on human experimentation as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

Flow cytometry and cell sorting. Peripheral blood mononuclear cells (PBMC) or bone marrow mononuclear cells were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and were stained with a combination of monoclonal antibodies [mAb; CD5-PE, CD19-PerCP, and CD38-APC (Becton Dickinson)]. Proliferation of $CD5^+CD19^+$ CLL cells in blood and bone marrow was studied by measuring expression of Ki67 nuclear antigen. Briefly, cells were fixed (FACS Lysing Solution, Becton Dickinson), permeabilized (FACS Permeabilizing Solution 2, Becton Dickinson), and stained intracellularly with Ki67-FITC (Monosan). Cells were analyzed on a FACSCalibur (Becton Dickinson) with CellQuest software.

Bone marrow mononuclear cells from CLL patients were stained with CD5-PE and CD19-APC mAb (Becton Dickinson). After washing, $CD5^+CD19^+$ cells were isolated by cell sorting on a FACSAria (Becton Dickinson) to determine label incorporation of bone marrow leukemic cells. Purity of sorted cells was at least 97%.

MACS cell separation. Granulocytes were obtained following lysis of erythrocytes from the cell pellet after Ficoll-Paque density gradient centrifugation. Because the granulocyte fractions of CLL patients were often contaminated with leukemic cells, these cells were purified by cell separation over MACS Separation Columns (Miltenyi Biotec GmbH) using CD16-conjugated magnetic beads according to the manufacturer's instructions. $CD19^+$ cells from healthy participants and from CLL patients were purified from PBMC in a similar fashion. Purity of all MACS-sorted cell populations was >90%. $CD19^+$ cells from CLL patients mainly consisted

Table 1. Characteristics of CLL patients

Patient	Age at day 0 (y)	Gender	Rai stage	Time since diagnosis (y)	IgVH mutation (%)	No. lymphocytes (10^9 per mL)		CD38* (%)	Cytogenetic abnormalities †
						Start of study	End of study		
CLL01	75.3	M	2	5	2.8	55.7	61.1	71.4	11q- (21%)
CLL02	77.1	M	0	6	11.2	42.2	42.9	0.9	None
CLL03	58.6	M	2	6	4.0	57.8	47.0	85.0	Trisomy 12 (58%)
CLL05	72.5	M	0	2	3.0	44.4	39.0	0.3	13q- (75%)
CLL06	71.1	M	0	2	6.6	5.8	6.3	0.7	None
CLL07	71.0	F	0	3	1.3	14.0	18.9	0.7	13q- (48%)
CLL08	50.9	M	0	1	0.4	57.6	64.0	1.0	Trisomy 12 (52%)
CLL09	66.1	M	4	15	3.0	121.3	113.0	0.1	13q- (56%)
CLL10	41.7	M	0	1	8.9	37.1	46.2	0.4	13q- (17%)

Abbreviations: M, male; F, female.
*Expressed as the median CD38 expression level on $CD5^+CD19^+$ cells during study follow-up.
†As was detected by fluorescence *in situ* hybridization analysis.

of CLL cells, as the percentage of $CD19^+$ cells expressing CD5 was 93.6%. From CLL03, no pure $CD16^+$ fractions could be obtained. Genomic DNA was isolated from each cell population using the Blood Quick Pure kit (Bioké Nucleospin, Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions.

Measurement of ^2H_2O enrichment in body water and DNA. Deuterium enrichment in urine was measured by a method adopted from Previs and colleagues (30). The isotopic enrichment of DNA isolated from granulocytes and $CD19^+$ cells was measured according to the method described by Neese and colleagues (25) with minor modifications (29).

Mathematical modeling. The label enrichment in urine, granulocytes, and $CD19^+$ cells was fitted to a steady-state kinetic heterogeneity model, which was described previously (29, 31). This model takes into account the fact that the kinetics of cells that have recently divided (and hence picked up label) may be intrinsically different from those that have not. For example, the labeled fraction may be biased toward cells with relatively rapid turnover. The model thereby allows the average rate of cell production (determined during up-labeling) to differ from the rate of disappearance of labeled cells after label cessation (due to death, maturation, or migration) even under steady-state conditions.

Labeling data were fitted according to the method we described previously (29). In brief, we first fitted the urine enrichment data, as a measure of body water enrichment (Supplementary Fig. S1), to determine the fraction f of ^2H_2O in the drinking water, the baseline urine enrichment β attained after the boost of label by the end of day 0, and the turnover rate δ of body water, for each individual (Supplementary Table S1). These variables were incorporated when fitting the enrichment in granulocytes (Supplementary Fig. S1), CLL cells (Fig. 1*A*), and B cells from healthy individuals (Fig. 1*B*). Assuming that granulocytes undergo complete turnover, the enrichment of granulocytes of each individual was analyzed mathematically to determine the maximal level of label enrichment cf that cells could possibly attain (Supplementary Table S2). This maximum is determined not only by the fraction f of ^2H_2O in the drinking water but also by an amplification factor c because the derivative that was analyzed contains multiple hydrogen atoms that can be replaced by deuterium. At each time point, the percentage of labeled DNA of CLL cells in blood and bone marrow and of labeled DNA of normal B cells in blood was normalized by dividing by this maximum cf for each individual (29).

RNA isolation and reverse transcription-multiplex ligation-dependent probe amplification assay. Total RNA was isolated using the RNeasy Mini kit (Qiagen). A reverse transcription–multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously (32, 33) using 100 ng total RNA as input. Results were analyzed using the programs Genescan analysis and Genotyper (Applied Biosystems) and further analyzed with Excel spreadsheet software (Microsoft). Data were normalized by setting the sum of all signals at 100% and expressing individual peaks relative to the 100% value.

***In vitro* CD40 stimulation and Western blotting.** *In vitro* CD40 stimulation of CLL cells followed by Western blotting of lysates was performed as described (1, 33). Blots were probed with the following antisera: polyclonal Mcl-1 (Pharmingen, BD Biosciences), monoclonal anti-Noxa (clone 114C307.1; Imgenex), polyclonal anti-Bcl-XL (Pharmingen, BD Biosciences), polyclonal anti-Bcl2 (Alexis), and antiserum to β-actin (clone I-19; Santa Cruz Biotechnology).

Statistical analyses. Correlations were performed using the nonparametric Spearman's rank correlation coefficient. Differences in Ki67 expression and ^2H enrichment data between blood and bone marrow CLL cells were analyzed by the nonparametric Wilcoxon signed rank test for paired data. Differences in mRNA expression of proapoptotic and antiapoptotic molecules were determined using the Student's t test.

Results

Patient follow-up. To assess leukemic cell dynamics in stable disease, 10 untreated CLL patients with stable disease activity were enrolled in the ^2H_2O labeling study. One patient (CLL04) dropped out at day 14. Data of the remaining nine patients were analyzed. All patients had stable lymphocyte numbers during follow-up (Table 1). Participants did not receive any disease-related treatment before and during the study, except CLL09 who had received two cycles of chlorambucil treatment 10 years before the start of the study. This participant died after 205 days of follow-up due to uncontrolled sepsis following pneumonia. CLL01 was monitored until day 136 because of cardiac surgery.

From each participant, the ^2H_2O enrichment in urine (Supplementary Fig. S1) and ^2H enrichment in the DNA of granulocytes (Supplementary Fig. S1) were fitted to the mathematical model (Materials and Methods), as well as ^2H enrichment in the DNA of CLL cells (Fig. 1*A*) or B cells from healthy individuals (Fig. 1*B*). The strength of this model is that it allows for discrimination between the average turnover rate (p) of the total leukemic or normal B-cell compartment in blood and the rate (d) at which recently divided cells (which have taken up label) disappear from the population by death, maturation, or migration to another body compartment (31).

Because granulocytes presumably undergo complete turnover, label enrichment of these cells was assessed to estimate the maximum level of label intake that cells could possibly attain (Supplementary Fig. S1; refs. 25, 34).

Low leukemic cell turnover in blood of stable CLL patients. Labeling data were fitted to the mathematical model (Materials and Methods) to determine kinetic variables of leukemic cells (Fig. 1*A*) or B cells from healthy individuals (Fig. 1*B*) in blood. The average turnover rate of the total leukemic population (p) and the disappearance rate (d) of labeled leukemic cells in the blood for each participant are given in Table 2. For patients CLL01 and CLL09, we only report p, and not d, because only a limited number of down-labeling data could be collected from these patients. As purification of granulocytes from CLL03 proved unsuccessful, the kinetic variables of the leukemic clone of this patient were calculated using the median of all granulocyte enrichment maxima from the other CLL participants.

Average CLL turnover rates varied between 0.08% and 0.35% of the CLL clone per day. In a subset of patients (CLL03, CLL06, CLL07, and CLL08), these turnover rates were balanced by similar disappearance rates of labeled leukemic cells (0.24–0.39% per day), whereas in others (CLL02, CLL05, and CLL10) hardly any or no labeled leukemic cells disappeared (0.00–0.07% per day) during the down-labeling period. The latter group of patients also had the lowest average CLL turnover rates (0.08–0.15% per day). Turnover rates of CLL cells were ~2-fold lower compared with turnover rates of B cells from healthy individuals (0.34–0.89% of the B-cell population per day). The largest difference between the dynamics of CLL cells and B cells from healthy individuals was the disappearance rate of recently proliferated cells: labeled B cells from healthy individuals were lost at ~10-fold higher rates (1.57–4.24% of the B-cell population per day) compared with CLL cells in patients (0.00–0.39% of the CLL clone per day).

The IgVH mutation level correlates negatively with leukemic cell turnover. As expected in a cohort of patients selected for stable disease activity, the majority of patients (seven of nine) had leukemic cells with mutated IgVH genes (≥2% mutations compared with germ-line sequence) with a range of 0.4% to 11.2% (Table 1). Because prior studies found a robust correlation between the level of mutations and telomere lengths (used as surrogate variable for cell turnover; ref. 27), we assessed whether the level of IgVH mutations correlated with the dynamics of the CLL clone. We found a significant inverse correlation between the

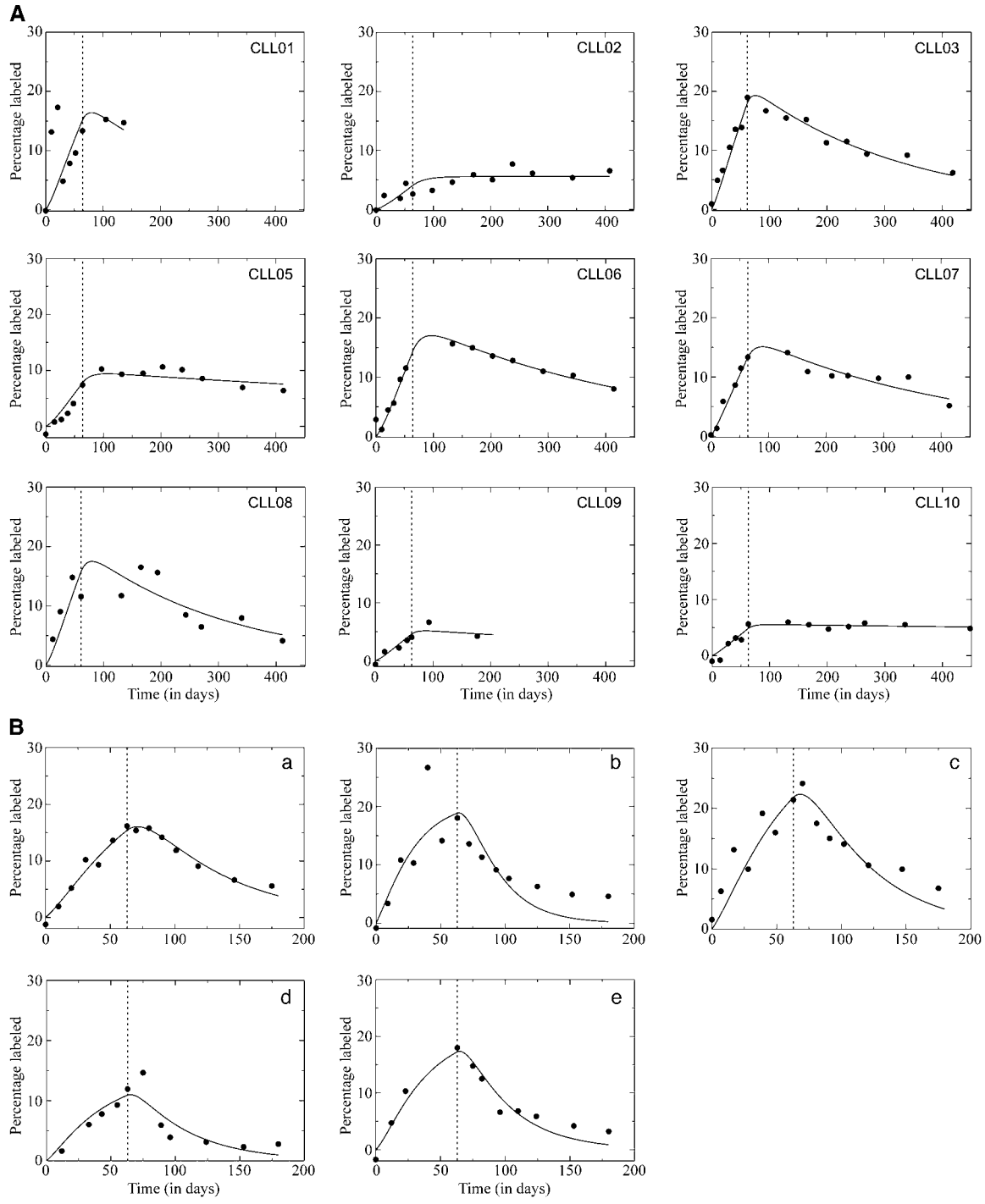

Figure 1. *In vivo* cell dynamics. ^{2}H enrichment of deoxyadenosines in the DNA of peripheral blood (*A*) CLL cells and (*B*) B cells from healthy individuals. Label enrichment was scaled between 0% and 100% by normalizing for the maximum percentage of label obtained in granulocytes. The dotted line represents the time point of label cessation. Labeling data were fitted as described in Materials and Methods.

level of IgVH mutations and (*a*) the level of average leukemic cell turnover p (Fig. 2*A*; $P = 0.017$; $r = -0.80$) and (*b*) the disappearance rate of labeled cells d (Fig. 2*B*; $P = 0.023$; $r = -0.82$). In addition, we found a strong correlation between the average leukemic cell turnover rate p and expression of the proliferation marker Ki67 (which is expressed in late G_1 phase of the cell cycle) in leukemic cells residing in blood (Fig. 2*C*; $P = 0.010$; $r = 0.83$). Collectively, these data suggest that in this stable CLL patient cohort, there is a biological link between the level of somatic hypermutation and leukemic cell dynamics.

No increased proliferation of leukemic cells in the bone marrow compared with blood. We have previously shown (17)

Table 2. Average turnover rates and disappearance rates of labeled cells in blood

CLL patient	CLL kinetics according to the model: Turnover rate, p (% per day)	Disappearance rate, d (% per day)
CLL01	0.30	unr.
CLL02	0.08	0.00
CLL03*	0.35	0.37
CLL05	0.15	0.07
CLL06	0.29	0.24
CLL07	0.25	0.28
CLL08	0.33	0.39
CLL09	0.08	unr.
CLL10	0.08	0.02
Median	0.25	0.24
Healthy participant	**Normal B-cell kinetics according to the model: Turnover rate, p (% per day)**	**Disappearance rate, d (% per day)**
A	0.43	1.57
B	0.89	4.24
C	0.65	1.97
D	0.34	2.28
E	0.61	2.81
Median	0.61	2.28

Abbreviation: unr., unreliable because of too few data points.
*The average turnover rate (p) was calculated by using the median of the granulocyte enrichment maxima of the other CLL participants.

that lymph node CLL cells are characterized by a strong expression of the proliferation markers Ki67 and *survivin* (a molecule that is only expressed during the G_2-M phase in proliferating cells; ref. 35) compared with blood CLL cells. These findings suggest that the level of ^{2}H incorporation in the DNA of CLL cells in the blood mainly reflects proliferation at other sites in the body. To assess the contribution of leukemic cell turnover in the bone marrow, six patients underwent a bone marrow puncture at the end of the labeling period. The level of ^{2}H incorporation in the DNA of bone marrow CLL cells was compared with that in blood CLL cells (Fig. 3*A*). Bone marrow CLL cells were found to have significantly higher levels of ^{2}H incorporation than CLL cells in blood (P = 0.046). When we performed a paired analysis between the average level of Ki67 expression of CLL cells in blood and bone marrow, however, no statistical difference was observed (P = 0.574; Fig. 3*B*). Proliferation levels were also assessed by mRNA expression levels of *survivin* (measured by MLPA). *Survivin* was hardly expressed both in blood and bone marrow CLL cells (Fig. 3*C*). *Survivin* levels measured by MLPA in lymph node samples of CLL patients in the aforementioned study (17) were approximately 10-fold higher [relative expression in lymph nodes: 0.60 $\pm$ 0.35 (n = 9); bone marrow: 0.06 $\pm$ 0.09; Fig. 3*C*]. Although only the first syringe of bone marrow aspirate was used for further analysis, it cannot be excluded that the aspirates were slightly contaminated with peripheral blood CLL cells, which could result in an underestimation of the actual number of proliferating cells in the bone marrow. Therefore, we also analyzed bone marrow biopsies from three patients (CLL01, CLL03, and CLL08). High-power field microscopy and immunohistochemical staining showed an average of 65% bone marrow infiltration with small CD5$^+$CD20$^+$ cells. Staining of these samples with Ki67 revealed only very sparse (≤1%) Ki67$^+$ cells within leukemic cell fields without the presence of clear proliferation centers (data not shown). Collectively, these data suggest that the higher level of ^{2}H incorporation in the DNA of CLL cells from the bone marrow is due to migration of CLL cells that have proliferated elsewhere into the bone marrow.

Similar apoptotic profile of leukemic cells in blood and bone marrow. The expression levels of apoptosis-regulating genes of lymph node CLL cells have also been shown to differ from those in the blood (higher expression of the antiapoptotic proteins Bcl-XL and Mcl-1 and lower expression of the proapoptotic protein Noxa; ref. 17). We examined whether the apoptotic profile of circulating CLL cells in the blood differed from CLL cells in the bone marrow. A paired comparison by MLPA was performed to

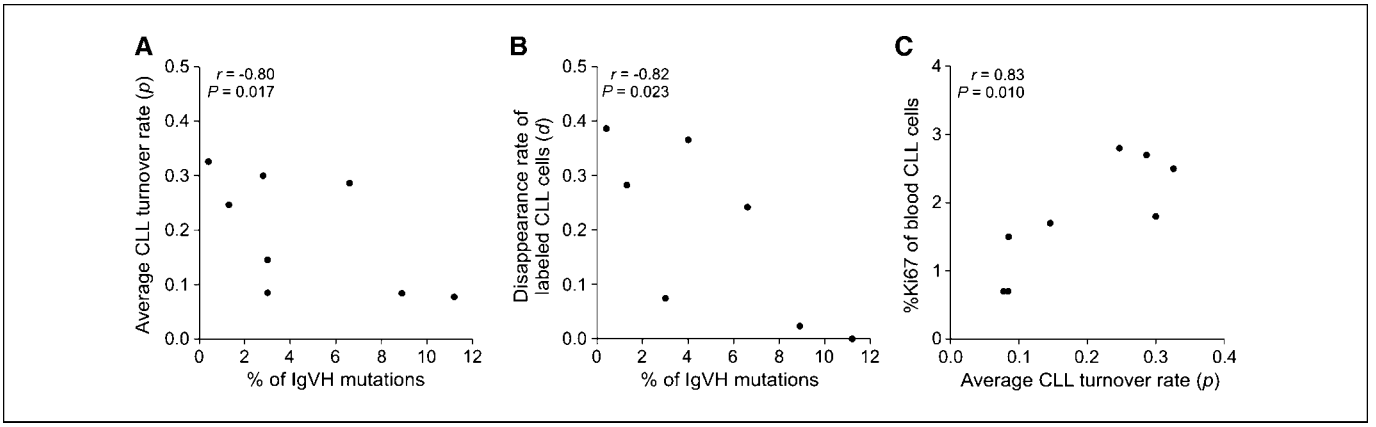

Figure 2. Relation of the level of IgVH mutations with the calculated variables. *A,* inverse correlation between the average CLL turnover rate in blood and the percentage of IgVH mutations. *B,* the disappearance rate of labeled cells from the blood is inversely correlated with the percentage of IgVH mutations. *C,* average Ki67 expression of blood CLL cells is positively correlated with the average CLL turnover rate in blood. Statistical significance (P < 0.05) was determined by the nonparametric Spearman's rank correlation coefficient. The turnover rate of CLL03 was not included in the analyses of *A* and *C* as this value was calculated using the median of the maximum granulocyte enrichment of the other patients.

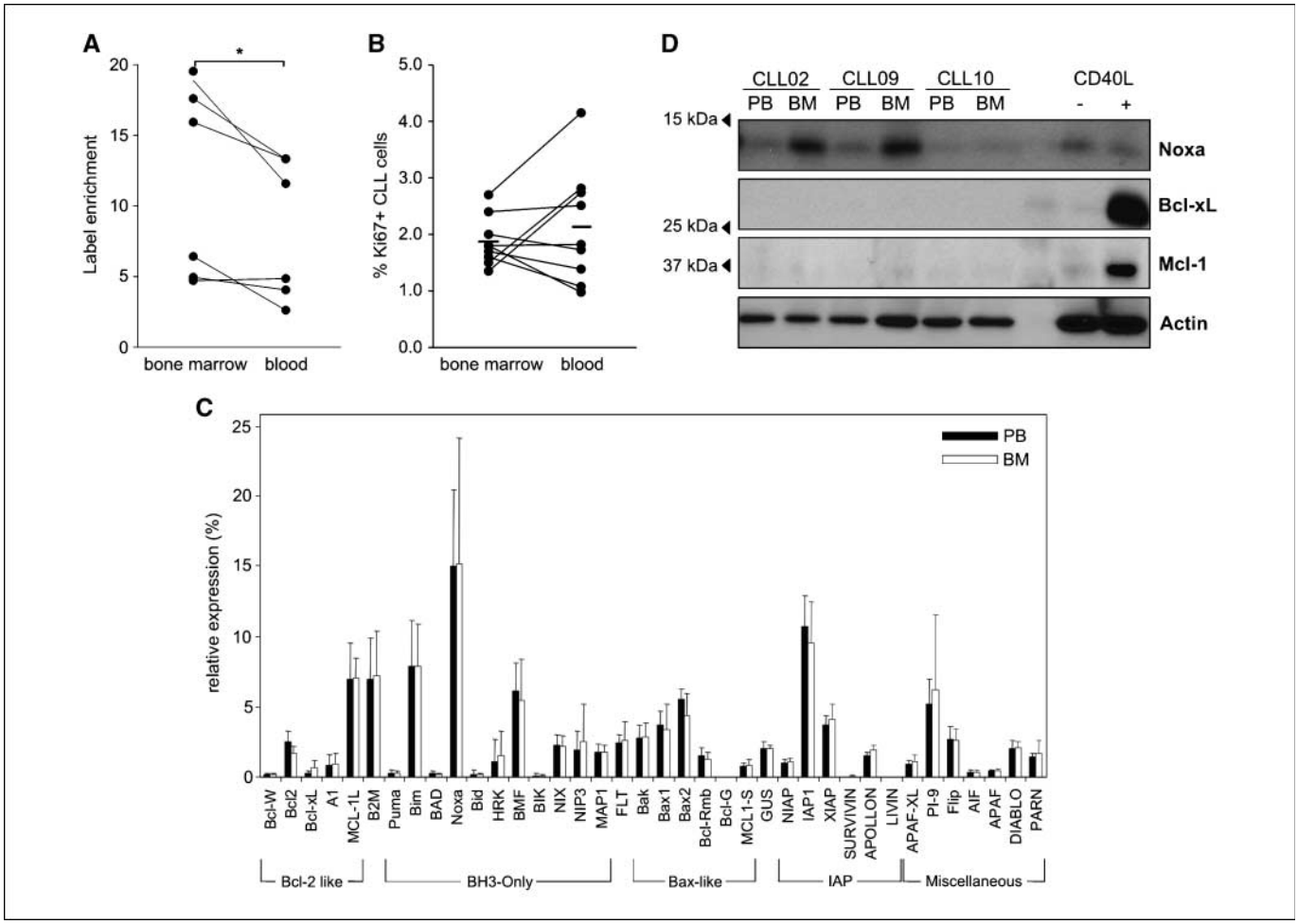

Figure 3. Comparison of proliferation and survival of blood and bone marrow CLL cells. *A,* ^{2}H enrichment in DNA of bone marrow and blood CLL cells at the end of the labeling period. *B,* the average percentage of Ki67 expression of blood CLL cells compared with the percentage of Ki67 expression of bone marrow CLL cells. Statistical significance ($P < 0.05$) was determined by the nonparametric Wilcoxon signed rank test for paired data and is denoted by an asterisk. *C,* relative expression of 34 apoptosis regulators was investigated of six paired blood (*black columns*) and bone marrow (*white columns*) samples. Results of individual apoptosis regulatory genes are shown as the median expression of the samples relative to the total signal in the samples, with SD. Nonapoptosis genes included as housekeeping genes are *β2-microglobulin* (*B2M*), *ferritin light chain* (*FLT*), *β-glucoronidase* (*GUS*), and *polyadenylate-specific RNase* (*PARN*). Statistical significance ($P < 0.05$) was determined using the Student's *t* test. *D,* protein lysates of three paired blood and bone marrow samples were subjected to Western blot analyses. Blots were stained with antibodies directed against Noxa, Mcl-1, or Bcl-XL and reprobed with an antibody against β-actin as a loading control. As a control, CLL cells were stimulated for 3 d with irradiated untransfected or CD40L-transfected fibroblasts, mimicking the expression pattern of lymph node CLL cells.

determine mRNA levels of a large panel of apoptosis regulators in CLL cells in these two compartments. A shift in the balance between survival and apoptosis genes could not be observed (Fig. 3*C*). A subset of these are posttranscriptionally regulated, and therefore, protein levels of molecules with known altered expression in lymph node CLL cells (17) were measured in paired samples from patients CLL02, CLL09, and CLL10 by Western blot analyses (Fig. 3*D*). Because prolonged CD40 activation of CLL cells results in expression profiles of apoptosis-regulatory genes quite similar to lymph node CLL cells, CD40-stimulated peripheral blood CLL cells were used as a control, and these showed the expected induction of Bcl-XL and Mcl-1 and decreased expression of Noxa (Fig. 3*D*). In sharp contrast to lymph node CLL cells and CD40-stimulated blood CLL cells, no expression of Bcl-XL and Mcl-1 could be detected in either blood or bone marrow CLL cells. In two of the three patients, increased expression of Noxa was found in bone marrow leukemic cells compared with CLL cells from peripheral blood. Hence, besides similar expression of proliferation markers of CLL cells in blood and bone marrow, these data suggest that in bone marrow prosurvival microenvironmental stimuli, like CD40 activation (18, 19), are not a dominant feature.

Discussion

The dogma that CLL is a static disease of a leukemic clone that is slowly accumulating because of impaired apoptosis is gradually replaced by the view that proliferation and death within the leukemic clone is taking place at substantial levels. In a prior study by Messmer and colleagues (26), considerable production rates of leukemic clones were observed in a heterogeneous group of CLL patients. We extend their findings and add new insights at three levels. First, we assessed leukemic cell dynamics in a homogeneous patient group with stable disease activity and determined whether the level of IgVH mutations correlated with the observed dynamics. Second, with a substantial down-labeling period and the use of our mathematical model, we were able to evaluate the fate of labeled cells, which thus provided the means to identify whether cells that

had recently proliferated contributed to the maintenance of the leukemic compartment or had a smaller survival chance than undivided leukemic cells. Finally, we not only studied CLL dynamics in blood but also evaluated the role of the bone marrow as a potential site for leukemic cell production.

In the ^2H_2O labeling study performed by Messmer and colleagues (26), it was found that patients with average CLL turnover rates higher than 0.35% of the CLL clone per day were more likely to exhibit or develop active disease. These findings fit very well with the fact that daily average turnover rates of CLL cells in our small cohort of patients with stable disease activity were at maximum 0.35% of the CLL clone per day. We showed that the *in vivo* CLL dynamics differed to a large extent from B-cell dynamics in healthy individuals: average turnover rates and disappearance rates of recently labeled cells were, respectively, 2- and 10-fold lower in CLL patients compared with healthy individuals. Two factors complicate this comparison. First, normal B-cell dynamics were assessed in individuals who were younger than the CLL patients. A comparison of these two groups nevertheless seems legitimate because in a 6,6-$^2H_2$-glucose labeling study hardly any differences in B-cell dynamics were observed between individuals younger than 35 and older than 65 years of age (36). Second, it is not straightforward to which cell population the dynamics of $CD5^+CD19^+$ CLL cells should be compared. Based on microarray data, it has been suggested that CLL cells bare most resemblance with memory B cells (37), suggesting that the most fair comparison would be to compare CLL dynamics with memory B-cell dynamics in healthy individuals. 6,6-$^2H_2$-glucose labeling studies have pointed out that the turnover rate of $CD27^+$ memory B cells is higher than the average turnover rate of the overall B-cell compartment (36). If we had compared CLL dynamics with healthy memory B-cell dynamics, the low turnover rates and, in particular, the low disappearance rates of CLL cells would thus most likely only have become more apparent.

Examination of CLL dynamics between individuals revealed that differences exist between patients about average turnover rates and the loss of label after label cessation. In CLL03, CLL06, CLL07, and CLL08, labeled cells were lost with similar rates as cells acquired label during the labeling phase. In contrast, in CLL02, CLL05, and CLL10, average turnover rates were lower and hardly any labeled cells were lost during the down-labeling phase. Yet, the average number of circulating tumor cells remained stable in all patients. Although we measured only a few patients, these data suggest that CLL dynamics as measured in blood can differ between patients. Furthermore, the CLL clone within an individual patient may consist either of a single population or of multiple populations each having different dynamics. What causes these differences in CLL dynamics between and in individual patients is not known but may involve differential expression of site-specific homing markers or variations in retention and release of CLL cells from tissues such as bone marrow, spleen, and lymph nodes. Moreover, whether the labeling patterns we observed in our patient cohort are similar to those in CLL patients with aggressive disease is not known but could be of interest and should be studied in a larger cohort of patients.

CLL dynamics inversely correlated with the level of mutations in the *IgVH* gene. This association suggests that *in vivo* a biological link exists between the level of somatic hypermutation and proliferation and death of the CLL clone. It has been shown that the prognosis is poor in the subset of patients with <2% of mutations (unmutated CLL), but also within the group with >2% of mutations (mutated CLL), the prognosis differs with the best prognosis for the subset of patients with the highest degree of somatic hypermutation (38). In addition, it was shown that within a group of patients with mutated CLL, telomere length of the leukemic cells correlated with overall survival (39). The well-established difference in prognosis based on the division between <2% and ≥2% IgVH mutations led to the hypothesis that CLL comprises two different disease entities: one in which the oncogenic hit occurred early in a germinal center reaction and one in which the oncogenic hit took place after a germinal center reaction (40). *In vitro* studies have shown that unmutated CLL cells become activated by B-cell receptor (BCR) triggering, which possibly results in increased proliferation and more aggressive behavior of the CLL clone, whereas the majority of cases of mutated CLL are more or less anergic to BCR signaling (41). Damle and colleagues (27) found a positive correlation between telomere length and the percentage of IgVH mutations and an inverse correlation between the level of IgVH mutations and telomerase activity. In agreement, in preliminary observations, we found an inverse correlation between the expression of Ki67 in freshly isolated blood CLL cells and the level of IgVH mutations ($P = 0.01$) in 18 randomly selected CLL patients.[7] Collectively, these findings suggest that the level of mutations, rather than the mutational status, is indicative for the proliferative capacity of the leukemic clone. A biological rationale for this concept was provided in a recent study, which showed that the normal B-cell response is a highly dynamic process based on the (re-) entrance of memory B-cell clones in multiple germinal centers, resulting in increasing percentages of mutations following each germinal center round (42). Our data that CLL dynamics inversely correlated with the percentage of mutations in the IgVH gene hint at a model in which susceptibility of CLL cells to BCR triggering does not depend on the mutation status per se but rather on the number of germinal center rounds the cells went through before the (final) oncogenic hit.

The turnover of CLL cells has been hypothesized to occur primarily in lymph nodes and in bone marrow (43), but the assumed influence of microenvironmental stimuli on the maintenance of the leukemic clone within these compartments is largely based on *in vitro* studies (16, 18, 19, 44–46). Recently, examination of lymph node CLL cells provided additional support to this hypothesis as these cells were shown to have an antiapoptotic expression profile with up-regulation of Mcl-1, Bcl-XL, and survivin and concomitant down-regulation of Noxa when compared with CLL cells in blood (17). Insights in proliferation centers in bone marrow are limited as these are almost exclusively seen in cases with extensive marrow involvement (47). We showed that in our patient cohort with stable disease, the large majority of bone marrow CLL cells did not proliferate and, in general, did not possess an altered apoptotic regulator profile compared with peripheral blood CLL cells. The higher level of labeled CLL cells in bone marrow compared with blood may be explained by the possible migration of leukemic cells that have divided at other sites in the body into the bone marrow. Taken together, our data suggest that, at least in stable disease and in contrast to the findings for lymph nodes, the bone marrow is not a major CLL proliferation site.

In conclusion, we have shown that also in stable CLL, the leukemic population is maintained by a balance of ongoing proliferation and cell death. A better understanding of cell kinetics of the

[7] D.P. Luijks et al., unpublished data.

leukemic clone will hopefully result in a further tailoring of therapeutic approaches for the different subtypes of this still incurable disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/25/2008; revised 9/18/2008; accepted 9/22/2008.

Grant support: Landsteiner Foundation of Blood Tranfusion Research grant 0319. A.P. Kater is personally supported by a "Veni" grant from The Netherlands Organization for Health Research and Development. J.A.M. Borghans was financially supported by the Netherlands Organization for Scientific Research (VICI grant 016.048.603 to Rob J. de Boer).

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We thank the participants for their collaboration in this study, Mariëtte Weterman and colleagues of the Hematology outpatient clinic of the Academic Medical Center (Amsterdam, the Netherlands) for performing the blood withdrawal of the participants, and Dr. C.J. van Noesel (Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands) for reviewing the bone marrow biopsy specimens.

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