Chronic Lymphocytic Leukemia

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Evolving View of the *In-Vivo* Kinetics of Chronic Lymphocytic Leukemia B Cells

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B-cell chronic lymphocytic leukemia (B-CLL) has long been considered a disease of "accumulation," due to a presumed defect in programmed cell death. Recent data, however, suggest that B-CLL cells are born at a normal to an accelerated rate, with the rate of proliferation varying among patients. In addition, differences in birth rates, activation state, and inducibility appear to exist among subpopulations of cells within individual leukemic clones. The extent to which such dissimilarities influence clinical course and outcome is still unclear. This review examines the evidence supporting the existence of a proliferative compartment in B-CLL and the role that proliferating cells might play in the progression and evolution of this disease.

I. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is a liquid tumor consisting of a clonal $CD19^+/CD5^+$ B lymphocyte population. The disease is diagnosed primarily in individuals in the fifth and sixth decades of life with some patients having an indolent clinical course. For four decades, B-CLL has been considered a disease of "accumulation," due to a presumed defect in programmed cell death, with "immunologically incompetent" B-CLL cells building up over time to produce disease.[1]

However, the new perspective emerging from recent investigations suggests first that B-CLL cells derive from competent B lymphocytes selected for clonal expansion and eventual transformation by multiple encounters and responses to (auto)antigen(s). The transformed B cells, particularly from patients with the worst prognostic markers, appear to retain their ability to respond to signals delivered through several cell surface receptors, indicating they have at least partial immune competence. Second, and perhaps more surprisingly, rather than being sluggish, B-CLL cells are born at a normal or even accelerated rate, with the rate of proliferation varying among patients and even among subpopulations of cells within the leukemic clones of individual patients. The extent to which such dissimilarities

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influence clinical course and outcome is still unclear. In this review we examine primarily the contribution of proliferation to the progression and evolution of B-CLL; the matter of immune competence has been addressed recently.[2]

II. The Controversy

To what extent do accumulation and proliferation contribute to the pathobiology of B-CLL?

A. Original view: B-CLL is a disease primarily resulting from the accumulation of clonal B lymphocytes that do not die

A number of clinical and laboratory observations have led to this view. First, some patients with the disease live for many years after the diagnosis is first made, never requiring therapy and dying with leukemia rather than because of it.[3] Second, by light microscopy circulating leukemic cells resemble small, resting lymphocytes with a high nuclear to cytoplasmic ratio. The nuclear chromatin of these cells is usually condensed, suggesting minimal metabolic activity.[4] Finally, even using sensitive flow cytometric techniques, B-CLL cells progressing through the cell cycle are rarely detected in the blood.[5]

B. Evolving view: B-CLL is a disease of clonal B lymphocytes that replicate at a normal to higher than normal rate and do not appear to have an inherent apoptotic defect

Even though most B-CLL cells have a small resting appearance when evaluated by light microscopy, somewhat larger cells with more cytoplasm and less condensed chromatin are seen in some patients.[6] The numbers of these prolymphocytes and atypical lymphocytes often are initially small, but in some patients they increase with time and presage a less favorable clinical outcome. Richter's transformation, the conversion to a more aggressive, rapidly proliferating lymphoma, is seen more frequently in patients with increasing numbers of prolymphocytes.[7] Both findings establish a link between cell proliferation and clinical outcome.

Regarding clinical course, two subgroups of B-CLL exist which differ in IgVH gene mutation status[8] (mutated B-CLL [M-CLL] and unmutated B-CLL [U-CLL]), CD38[9] and ZAP-70[10] levels, and gene expression profiles,[10,11] and these subgroups have different clinical outcomes.[9,12] The cases with leukemic clones containing minimal or no mutations and elevated numbers of $CD38^+$ and ZAP-70^+ cells are at a much higher risk for clinical decompensation than those cases with clones with significant mutations and few $CD38^+$ or ZAP-70^+ cells. Irrespective of the subgroup to which a patient belongs, the leukemic cells always display surface activation markers, albeit with some differences. U-CLL cases resemble B cells that have been recently induced (e.g., increased proportion of $CD69^+$ cells and levels of HLA-DR), whereas M-CLL cells exhibit markers that emerge later after an activation stimulus.[13] In addition, cells from U-CLL more frequently express the unfavorable prognostic markers CD38[9] and ZAP-70.[14,15] Gene expression profiles suggest that cells from both B-CLL subgroups most closely resemble antigen-experienced, memory B cells.[10,11]

In addition, since telomere length can be an index of the number of times a cell has replicated, the finding that B-CLL cells have shorter telomeres than normal age-matched B cells[16] not only suggests that the leukemic cells divide, but that they have done so more frequently than the stem cells giving rise to normal B-cell counterparts. The leukemic cells from poor outcome U-CLL patients have even shorter telomeres than those of patients in the good outcome M-CLL subgroup, implying that the rate of cycling of the leukemic cells from U-CLL patients or their progenitors is more rapid than that of M-CLL patients.

Finally, although there is a virtual absence of cycling cells in the blood of B-CLL patients, ill-defined areas of apparent proliferation are seen in the bone marrow and affected lymph nodes. These "proliferation centers" contain larger cells with less condensed nuclear chromatin that display the cell cycle marker Ki-67.[17] These may be the primary sites of clonal growth.[4]

C. Evidence that B-CLL cells require exogenous signals to survive

Several observations suggest that B-CLL cells are not immortal, but require signals delivered through cell surface receptors to maintain viability. The most obvious is the inability of B-CLL cells to remain viable *in vitro*.[18] Unless B-CLL cells are cultured in the presence of other cell types[19] or soluble cytokines and chemokines[20] or other molecules that engage important cell surface receptors such as the BCR,[21] in particular the IgD form of the receptor,[22] CD38,[23] and CD40,[24] they undergo apoptosis more rapidly than normal B lymphocytes. This is in keeping with the lack of a defined apoptotic defect inherent to the clone. The extent to which these survival mechanisms affect the entire clonal population is unclear as only a subset of clonal members will have the opportunity to receive and respond to trophic, survival signals due to anatomic locations of survival signals, trafficking differences among B-CLL cells, etc. Therefore, it would not be surprising if many members of the B-CLL clone could not survive for long periods to remain a part of the "accumulating" pool.

III. Direct Measurements of the Proliferative Capacities of B-CLL Cells *in Vivo*

Since the observations mentioned above fail to unequivocally resolve the relative contributions of cellular accumulation and proliferation, *in vivo* measurements of the proliferative potential of B-CLL cells are essential. Although this need has been acknowledged for decades, *in vivo* kinetic studies have been hampered for many years by the inability to safely, accurately, and conveniently mark and quantify proliferating cells in healthy and ill subjects.

A. Direct measurement of DNA synthesis and cell proliferation of B-CLL cells in vivo

A1. Approach: In the last decade, Hellerstein and colleagues introduced isotopic labeling techniques that use deuterium (^2H) to label DNA of dividing cells as a measure of cell birth.[25] By one embodiment of this approach, ^2H, taken orally as heavy water (^2H_2O), is metabolically incorporated into the covalent C-H bonds of the deoxyribose moiety of replicating DNA but not into stable, non-replicating DNA. Once inserted, the presence of ^2H in DNA of cells is quantified by mass spectrometry.[26] This approach is safe for humans because deuterium is not radioactive and has no known toxicities at the levels achieved in these studies. Furthermore, the number of cells needed for these assays (10^4-10^5) and the accuracy of the measurements obtained using mass spectrometry make the ^2H-labeling approach very easy to exploit in practice, provided that the leukemic cells are purified in order to exclude labeled DNA from other proliferating cells. In B-CLL, purification is readily achieved because the leukemic cells express surface membrane CD5 and CD19, markers found on only a minor fraction of adult human B cells.

Messmer et al used ^2H-labeling of replicating DNA to directly measure the birth rates of B-CLL cells *in vivo*.[27] In this pulse/chase approach, patients drank ~60 mL of ^2H_2O daily for 12 weeks to achieve a level of ~1-2% of ^2H_2O in total body water. At regular intervals (~1-2 weeks), $CD5^+$/$CD19^+$ B-CLL cells were purified from circulating mononuclear cells, and genomic DNA was isolated from these cells to measure ^2H enrichment.

Serum samples were also collected at the same time intervals to quantify the enrichment of ^2H_2O in blood. Equilibrium levels were usually reached by 14 days, maintained throughout the 12-week labeling period, and decreased after ceasing ^2H_2O intake. These values were used to calculate cellular birth rates as well as to assure that sufficient levels of ^2H_2O were achieved for adequate cellular labeling and that patients were compliant with the protocol.

Table 1. Birth, growth and death rates of B-cell chronic lymphocytic leukemia (B-CLL) cells *in vivo.*

Patient No.	Birth Rate (%/day)	Growth Rate (Δ WBC %/day)	Death Rate* (%/day)
400	1.76	0.712	1.05
408	1.09	–1.052	2.14
189	0.81	0.656	0.15
472	0.54	–.053	0.59
169	0.49	–0.001	0.49
360	0.48	–0.311	0.79
355	0.45	0.302	0.15
336	0.41	–0.289	0.70
403	0.39	0.241	0.15
282	0.29	0.654	*–0.26*
321	0.28	0.035	0.26
418	0.24	–0.141	—
331	0.24	0.033	0.21
332	0.23	0.567	–0.33
107	0.22	0.006	0.22
280	0.18	0.087	*0.13*
394	0.11	0.045	0.14
109	0.29	–0.125	*0.24*

* Values in italics represent data from poor model fits.

A2. Kinetics of B-CLL cell birth rates: Using three mathematical methods to analyze the data (see reference [27] for details), normal to above normal proliferation of B-CLL cells was identified in every patient. Depending on the case, from 0.11% to 1.76% of the entire clone divided each day (**Table 1**). **Figure 1** (see Color Figures, page 512) illustrates kinetic patterns of patients with faster and slower birth rates. If one assumes the leukemic cell burden in a typical patient to range between $1 \times 10^{12} - 1 \times 10^{14}$, then *at a minimum* between 1×10^9 to 1×10^{11} B-CLL cells were produced daily. Thus, cellular proliferation occured in each B-CLL patient, at levels that equal or exceed those of B cells from four normal subjects studied.

A3. Estimation of clonal growth and death rates. Clonal growth rates were calculated by fitting an exponential curve to the WBC counts obtained at each blood drawing, from the beginning to the end of the study period. The growth rates for the cohort of patients ranged from –1.052 to + 0.712% per day[27] (**Table 1**). In patients whose WBC levels increased or remained virtually unchanged, positive or essentially zero growth rates were computed; for several patients, WBC counts declined during the 6-month study period, yielding a negative growth rate.

Of note, birth rates and growth rates did not correlate in all patients. For example, in one subject, CLL 408, with an especially high birth rate (1.09% of the clone per day), WBC number fell during the course of the study; for others, birth rates exceeded growth rates. Both of these discrepancies strongly suggested that concomitant leukemic cell death occurred.

Therefore, B-CLL cell death rates were estimated by subtracting growth rates from birth rates (**Table 1**). Accordingly, from –0.33% to +2.14% of the clonal cells were eliminated from the blood daily.[27] For these calculations it was assumed that, in the absence of identifiable changes in the size of measurable peripheral lymphoid tissues, elimination from the blood represented cell death, a reasonable assumption given the impaired recirculation of the majority of leukemic cells in B-CLL.[28] Furthermore, labeling over a period of months likely resulted in equilibrium between tissue and blood compartments in most subjects.[27]

Cumulatively, these data indicate that the observed blood lymphocyte counts in all B-CLL patients represent a

dynamic interplay between ongoing birth and death within the clones, not simply a linear, monotonous accumulation of inert leukemic cells.

A4. Relationship between *in vivo* B-CLL cell kinetics and disease activity and progression. Although the cohort studied was not large ($n = 19$), Messmer et al found a correlation between higher birth rates (≥ 0.35% of the clone being born each day) and disease progression and/or requirement for therapy, preceding, during, or following the study period[27] (**Table 2**). Since birth and growth rates did not necessarily move coordinately, the proliferative rate of the clone appeared more important in determining clinical course than changes in WBC or absolute lymphocyte counts.

This concept was supported by analysis of de-labeling curves for 4 patients whose birth rates were measured again 1-2 years after ^2H_2O intake ended.[27] In these patients, the kinetic estimates were on target over the prolonged interval, implying stable disease and an unchanged clinical course, which in fact was the case for these patients. One could envision that if the level of cellular proliferation determined at a follow-up blood drawing changed significantly, either higher or lower than predicted by extrapolating from the value measured at the initial study, this might raise concern about changes in birth or death within the clone and presage a change in clinical course. A national trial is currently being conducted to analyze the kinetics of a large cohort of new-onset B-CLL cases and to correlate these with various prognostic parameters and disease course, to determine the clinical utility of the ^2H-labeling technique in B-CLL. In this regard, there was a trend in the initial study for higher birth rates being associated with U-CLL; this did not reach statistical significance.[27]

IV. Questions Arising from the Finding that Proliferation Can Be Considerable in B-CLL

Although the preceding data indicate that the proliferative compartment at a point in time in B-CLL is relatively small, it is crucial to understand the extent to which all cells of the clone can amplify, the relative rates of division that fractions of the clone can undergo (if not all cells do so at once), and the extent to which this proliferation is spontaneous or induced.

A. Are all or only a fraction of cells in a B-CLL clone dividing and can those cells that have divided be identified phenotypically?

These are important pathophysiologic as well as therapeutic considerations that we are trying to answer by sub-fractionating B-CLL cells from patients who have ingested ^2H_2O, using combinations of cell surface markers and isolation by fluorescence-activated cell sorting. Because CD38 is known to be relevant to disease course[9] and possibly pathogenesis,[29] in preliminary studies we have isolated $CD19^+/CD5^+/CD38^+$ and $CD19^+/CD5^+/CD38^-$ cells from 9 patients at week 8, well into the pulse/maintenance phase of the study (C Calissano et al, 2006 submitted).

When DNA from these fractions was analyzed, mean ^2H enrichment in the $CD38^+$ population was approximately twofold higher than that in the $CD38^-$ fraction. Enhanced ^2H uptake into the $CD38^+$ population was detected in every patient, with $CD38^+/CD38^-$ labeling ratios ranging from slight (1.15) to high (5.58). In 4 patients, this ratio was well above 2.0. These *in-vivo* findings indicate that within an individual B-CLL patient, the leukemic subpopulation marked by surface membrane CD38 contains more cells that have recently undergone cell division than the $CD38^-$ fraction.

These kinetic analyses are complemented by the finding that more $CD38^+$ cells co-express markers of cellular activation and proliferation than the $CD38^-$ cells from an individual patient (CD69, CD62L, ZAP-70, and Ki-67; R

Table 2. *In vivo* birth rates of B-CLL cells correlate with disease progression.

Patient No.	Birth Rate (%/day)	Active/ Progressive Disease[1]	Treatment[2]	Mutation Status (% difference)[3]
400	1.76	Yes	Pre and Post	0.0
408	1.09	No	No	0.3
189	0.81	Yes	Pre and Post	0.3, 6.8[4]
472	0.54	Yes	No	0.0
169	0.49	Yes	Post	5.1
360	0.48	No	No	0.3
355	0.45	Yes	Post	0.0
336	0.41	Yes	Post	2.0
403	0.39	Yes	Post	0.0
282	0.39	Yes	Pre and Post	2.4
321	0.29	No	No	0.3
418	0.28	No	No	4.0
331	0.24	No	No	3.7
332	0.24	Yes	Post	1.0
107	0.23	No	No	6.9
280	0.22	No	No	—
394	0.18	No	No	0.0
109	0.11	No	No	7.1

1. Based on National Cancer Institute criteria (BD Cheson *et al.* Blood 1996;87:4990-4997)
2. Timed in relation to the heavy water protocol
3. Percent difference in nucleotide sequence between the expressed IgVH gene of the B-CLL patient and the most similar germline counterpart.
4. This leukemic clone expressed two VHDJH rearrangements.

Damle et al, 2006 submitted). Furthermore, CD38$^+$ B-CLL cells are more responsive to BCR crosslinking than CD38$^-$ cells from the same patient (G Cutrona et al, 2006 submitted). Thus, kinetic, phenotypic, and functional analyses indicate that the CD38$^+$ fraction is more active and inducible than the CD38$^-$ counterparts.

B. Is the in vivo *proliferation of B-CLL cells spontaneous or induced?*

B cells can proliferate because of antigen-induced and antigen-independent mechanisms. Antigen-induced proliferation is a selective process whereby responding B cells are culled for expansion from a large available pool by antigen binding. Antigen-independent proliferation, often referred to as homeostatic proliferation, replenishes B cells from the same available pool, although this replacement is not directly mediated by antigen.[30] Such replacement can be indirectly influenced by antigen stimulation, if the need for B-cell replenishment is due to clonal activation, maturation, or elimination initiated by an encounter with an immunogenic molecule. Although these two mechanisms differ in their need for direct antigen mediation, they both require an intact BCR for initiation.[30,31] Similarly, both types of proliferation can be T-cell independent, although normal B-cell activation and differentiation is often T-cell mediated.

B1. Do B-CLL cells proliferate in vivo in response to antigen encounter? Leukemic cells from both M-CLL and U-CLL probably derive from clonally expanded, antigen-experienced B cells, although the type of antigen each has encountered (i.e., T-cell dependent vs. T-cell independent) and the maturation stage at which they were frozen (before vs. after classical germinal center reactions) remains controversial.[2,20,32] Regardless, clonal selection by antigen has skewed the IgVH gene repertoires of both M-CLL and U-CLL compared to normal B cells and to each other.[2,20,32]

If antigen-BCR interactions stimulate full-fledged B-CLL cells *in vivo*, as *in vitro* studies suggest,[33,34] then at least part of the cellular proliferation in B-CLL could be antigen initiated. Since B-CLL clones, particularly from unmutated cases, produce polyspecific (natural) antibodies, engagement of these BCRs by foreign and autoantigens could lead to cellular activation. The outcome of the stimulation by (auto)antigens could be cell survival/proliferation or apoptosis depending upon the molecular form of the stimulating antigen (soluble vs. tissue bound), its anatomic location (dispersed vs. localized), the types of ancillary cells involved, and other concomitant stimuli such as those delivered through CD40, Toll-like receptors or by cytokines. These different outcomes could affect the dynamic birth and death rates of leukemic cells. Other receptor-ligand interactions, could also be important for clonal replenishment and expansion or apoptosis.

B2. Do B-CLL cells also proliferate in an antigen-independent manner? However, not all cellular proliferation may occur because members of the leukemic clone engage antigen via the BCR or other ligands via additional receptors, especially if the relevant antigens are localized to discrete anatomic compartments and if many of the cells of the clone are impaired in their recirculation capacity, thereby inhibiting antigen encounter.

Homeostatic proliferation is designed to maintain an adequately sized and diverse cellular pool. There are currently no direct data documenting the level of homeostatic proliferation in B-CLL. However, since this process is normally limited and inhibited by mature, follicular B lymphocytes,[30] the immune-deficient state that most B-CLL patients develop could promote the emergence of an antigen-independent proliferating clonal pool. Since in the normal setting these cells emerge from stem cells, a similar process could occur in B-CLL via leukemic stem cells. Because these stem cells would have the IgH and L chain variable region rearrangements encoding the BCR of the leukemic cell, the stem cell progeny would be susceptible to antigen stimulation and the physiologic effects mentioned above. Indeed, both M-CLL and U-CLL could be replenished by an antigen-independent mechanism, but only the U-CLL cases might receive an antigen-dependent drive because of their enhanced poly/autoreactivity[35] and retained ability to be stimulated through the BCR[34]; in contrast, M-CLL might not, either because of a lack of antigen reactivity[35] and/or an inability to respond to such signals[32] (**Figure 2**; see Color Figures, page 512).

V. Potential Clinical Implications

Whether cell turnover *in vivo* is facilitated by external signals or not, the existence of a sizable proliferating pool of leukemic cells in B-CLL has clinical relevance. This rate of cell division may promote mutation, resulting in the creation of cells with new genetic lesions that bestow a growth advantage for the leukemic clone and impart deleterious consequences for the patient. Such clonal evolution with the appearance and outgrowth of variants with ominous chromosomal abnormalities (e.g., deletions at 11q and 17p) is well known in B-CLL. Since the CD38$^+$ cells represent the major component of the proliferative compartment, these cells in particular might be more susceptible to developing such lesions, and indeed cases with high numbers of CD38$^+$ cells are enriched in chromosomal aberrations[36,37] and *p53* dysfunction.[38] The concept that these dividing cells represent a feeder population for clonal maintenance, growth, and diversification is consistent with the findings that active progressive disease correlates with higher birth rates.[27] Thus, this reservoir of dividing leukemic cells could be a prime therapeutic target to limit clonal burden and prevent dangerous clonal evolution.

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