Association Between Clonogenic Cell Growth and Clinical Risk Group in B-Cell Chronic Lymphocytic Leukemia


Chronic lymphocytic leukemia of B-cell origin (B-CLL) is a disease with a variable clinical course, despite the fact that the neoplastic cells in this disorder are homogeneous with respect to morphology, immunophenotype, and cell cycle stage. To further investigate the heterogeneity observed in the clinical behavior of B-CLL, we determined the phenotype and growth characteristics of clonogenic cells from 28 patients with B-CLL from low-, intermediate-, and high-risk groups as defined by the Rai staging system. Using methylcellulose as a semi-solid media with feeder cells and/or growth factors, colonies were observed with one or more of the culture conditions tested in 25 of 28 CLLs. Phenotypic analysis of colonies demonstrated that the clonogenic cells uniformly expressed la, CD19, CD20, CD5, and the identical light chain as the original CLL cell cultured. However, heterogeneity was observed in clonogenic B-CLL cell growth among the three different CLL risk groups. Clonogenic cells from patients with low-risk CLL required either irradiated unstimulated T cells, with or without conditioned media (CM) or irradiated activated T cells alone for colony formation. Both the number of colonies (227 ± 15) as well as the number of cells per colony (220 ± 82) were large, with a mean cloning efficiency of 0.33%. In contrast, clonogenic cells from patients with intermediate- and high-risk CLL required the combination of both irradiated activated T cells and CM. As compared with the low-risk CLLs, both the number and size of the colonies formed by the intermediate- (74 ± 17, 70 ± 39) and high- (83 ± 28, 40 ± 14) risk groups were significantly lower (P < .0001). Similarly, the mean cloning efficiency was significantly reduced to 0.15% and 0.14%, respectively. None of the recombinant cytokines (interleukin 1 [IL-1] to IL-7, tumor necrosis factor, α and γ-interferon, B-cell growth factor, and granulocyte macrophage colony-stimulating factor) alone or in combination with each other could entirely replace the stimulatory effect of the activated T cells. These data suggest that clinical progression of B-CLL is associated with a loss of clonogenic potential in the circulating pool of neoplastic cells, which require as yet undefined factors provided by activated T cells and CM.

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domly selected patients with low- (Rai stage 0), intermediate- (Rai stages I and II), and high- (Rai stages III and IV) risk disease. In the studies to be described below, we demonstrate that B-CLLs from low-risk patients can be more readily grown in vitro than cells isolated from patients with intermediate/high risk. The growth characteristics of each risk group was extensively studied with regard to specific growth conditions and phenotype of the clonogenic cell.

MATERIALS AND METHODS

Patients

Blood (Ph), bone marrow (BM), lymph node (LN), and/or spleen samples from 28 unselected patients with clinically, morphologically, and pathologically typical B-CLL were studied. Each patient's disease was assigned a Rai stage and corresponding risk group: low risk (stage 0), intermediate risk (stages I and II), and high risk (stages III and IV). The low-risk CLLs had lymphocytosis in Pb (>15,000/mm<sup>3</sup>) and BM (>40%) only. Intermediate-risk CLLs had lymphocytosis as well as enlarged LNs, and/or splenomegaly and/or hepatomegaly. The high-risk cases had lymphocytosis as well as anemia (hemoglobin [Hb] less than 11g%) and/or thrombocytopenia (platelets <100,000/mm<sup>3</sup>). Patients with autoimmune mechanisms of anemia (+ Coombs) and/or thrombocytopenia (anti-platelet antibodies) were excluded from analysis. Charts were reviewed retrospectively to assess other potential prognostic variables: cytogenetics (presence or absence of trisomy 12 and other cytogenetic abnormalities), pattern of BM involvement (nondiffuse or diffuse),<sup>23</sup> level of lymphocyte count, lymphocyte doubling time,<sup>24</sup> rapidity of disease progression to a new stage, prior number of chemotherapy regimens, and overall survival time from time of diagnosis.

Separation of Mononuclear Cells

Mononuclear cells from Pb and BM were isolated by Ficoll-Hypaque density sedimentation. Single-cell suspensions of lymph node and spleen tissue were prepared as previously described.<sup>25</sup> Monocyte depletion was performed by adherence to plastic tissue culture dishes. T cells were removed by E-rosetting.<sup>28</sup> Further enrichment of the B-CLL cells was performed by complement lysis using CD4, CD8, CD13, and CD14 monoclonal antibodies (MoAbs) as previously described.<sup>26</sup> The CLls were 99% CD19 and CD20 positive with less than 1% CD4, CD8, CD13, and CD14 positive cells by indirect immunofluorescence. Cells were either used fresh or cryopreserved in liquid nitrogen. Cells were recovered with a viability of 75% to 90%.

Preparation of Normal T Cells as Feeder Populations

Mononuclear cells obtained from normal individuals were depleted of monocytes by adherence and enriched for T cells by E-rosetting. The E-rosetted T cells were irradiated at 20 Gy and added to the culture mixture at a concentration of 2.5 × 10<sup>4</sup>/mL. To obtain activated T cells, 1 × 10<sup>6</sup>/mL E-rosetted peripheral blood lymphocytes (PBL) cells were stimulated for 48 hours with PHA (2.5 µg/mL), washed extensively to remove cell aggregates, and subsequently irradiated at 20 Gy and used at a concentration of 2.5 × 10<sup>4</sup>/mL.

Preparation of CM

Peripheral blood mononuclear cells obtained from normal individuals were cultured at a concentration of 1 × 10<sup>6</sup>/mL with 2 µg/mL of PHA and 10 ng/mL TPA in RPMI containing 10% fetal calf serum (FCS) at 37°C overnight. Cells were washed once and recultured in fresh RPMI/FCS for 3 days at 37°C. The supernatant was harvested and stored at −70°C (PBL-CM). CM was also obtained by incubating purified normal T cells with PHA for 3 days at 37°C. The supernatant of these cultures was filtered and used as a second source of CM (T-CM).

Cytokines and Mitogens

Colony assays were performed using various cytokines and/or mitogens either alone or in combination with each other. IL-1 to IL-7, γ-interferon (γ-IFN), and granulocyte macrophage colony-stimulating factor (GM-CSF) were generous gifts of the named organizations. The optimal concentration and source of each cytokine are listed. IL-1 (100 U/mL; Immunex Corp, Seattle, WA); IL-2 (25 U/mL; Biogen Corp, Boston, MA); IL-3 (1/500; Genetics Institute, Boston, MA); IL-4 (100 U/mL; Immunex); IL-5 (1/500; Genetics Institute); IL-6 (1/500; Genetics Institute); IL-7 (100 U/mL; Immunex); tumor necrosis factor (TNF) (100 U/mL; Asahi Incorporated, Japan); α-IFN (1,000 U/mL; Schering Corp, NJ); γ-IFN (1,000 U/mL; Biogen); B-cell growth factor (BCGF) (10%; Cellular Products, Buffalo, NY); GM-CSF (1/500; Genetics Institute); PHA (3.2 µg/mL; Sigma Chemical Co, St Louis, MO); and TPA (70 ng/mL; Sigma).

Phenotypic Analysis of CLls

The following MoAbs<sup>29</sup> were used at saturated binding concentrations to phenotype highly enriched CLL cells using indirect immunofluorescence staining as described previously:<sup>30</sup> slg (x and λ), IgM (IgG1), IgG (IgG1), IgD (IgG1), Ia, CD19 (B4, IgG2a), CD20 (B1, IgG2a), CD10 (J5, IgG2a), CD21 (B2, IgM), B5 (IgM), B7 (IgM), CD23 (Tac, IgG2a), CD23 (Blast-2, IgG1), CD71 (T9, IgG1), and CD5 (T1, IgG2a).

Cell Cycle Analysis of CLls

The position of the CLL cells in the cell cycle was assessed by acridine orange (AO) staining as previously described.<sup>31,32</sup>

 Colony-Forming Assay

The methylcellulose assay used in these experiments was a modification of the previously described technique.<sup>33</sup> T-cell and monocyte-depleted B-CLL cells (5 × 10<sup>4</sup>/mL) were cultured in Iscove's modified Dulbecco's medium (IMDM, GIBCO) supplemented with 20% FCS, 5 × 10<sup>-7</sup> mol/L 2-mercaptoethanol (2-ME), which was found to be essential for colony formation, and 0.9% methylcellulose. Each sample was cultured in the presence of media, irradiated unstimulated T cells (T)x at 2.5 × 10<sup>4</sup>/mL, irradiated activated T cells (Ta)x at 2.5 × 10<sup>4</sup>/mL, and/or 10% CM. Cells were plated in duplicates in Linbro tissue culture plates (Flow Lab, Inc, VA). The cultures were incubated at 37°C. The plates were frequently inspected for colony formation. Colonies were harvested on day 7 and phenotyped.

Assessment of CLL Colonies

A colony was defined as a distinct aggregate of greater than 20 cells. CLls that formed less than 30 colonies and small colonies of less than 20 cells were defined as unresponsive. Individual colonies were picked using micropipettes and assessed for morphology using Wright-Giemsa staining. Pooled colonies were washed and their phenotype was characterized by APAAP.<sup>31</sup> For each CLL sample the colony assay was repeated at least twice.

Self-renewal Assay

To examine the self-renewal ability of CLL clonogenic cells, pooled primary colonies were washed and replated in the same
cultural conditions as before. The cells were incubated for 7 more days, and secondary colonies were picked for phenotypic analysis.

**Inhibition of CLL Colony Formation**

CLL colony formation was inhibited using MoAbs and complement treatment. After incubation with either Ia, CD19, CD20, or CD5 MoAbs, cells were treated with rabbit complement (1/4 dilution) in Hanks buffered saline solution (HBSS) at 37°C for 45 minutes. The cells were washed and put into a colony assay as described above. The percentage inhibition of colony growth was determined on day 7. Corresponding controls were incubated in media and complement alone. In three CLL cases nonspecific binding by complement was examined by prior absorption of complement using 5 x 10^7/mL CLL cells.

**Statistical Analysis of Data**

*P* values were determined using Jonckheere’s test with appropriate adjustments for ties. This is a nonparametric (ie, no distributional assumptions are made about the data) test for trend, testing whether CLL colony formation was associated with increase in risk group. Briefly, the assumptions made were that the data value for each patient with the largest number of colonies was used as an estimate of optimal growth regardless of growth conditions. Estimated colony size was scored on a nonlinear scale. The midpoint of the range for any score was used as a multiplier to estimate the total cell count (no. colonies x colony size).

**RESULTS**

**Characteristics of B-CLL Patients**

Twenty-eight patients with classical B-CLL were characterized according to Rai stage and corresponding risk group, prior treatment, and cell surface phenotype. All B-CLLs demonstrated uniform morphology with no evidence of transformation at the time of study. All of the patients in the low-risk group (n = 8) were previously untreated, whereas 2 of 12 in the intermediate-risk group and 6 of 8 in the high-risk group had received prior therapy. Cell samples were from peripheral blood in 24 of the 28 cases.

Using highly enriched B-CLL cells from each of the above 28 patients, the cell surface phenotype was examined using flow cytometric analysis. After depletion of T cells and monocytes, CLL preparations were routinely greater than 95% CD20 and CD5 positive, and less than 5% of cells expressed CD2, CD4, CD8, CD13, CD14, or CD16. Highly enriched B-CLL cells from all 28 patients expressed the Ia antigen, CD19, CD20, CD21, CD5, and the B-cell activation antigen, B5. Within individual CLL specimens, virtually all tumor cells were positive for these antigens and the intensity of expression was similar from patient to patient. Monoclonal surface Ig could be detected on the tumor cell surface of only 13 of 28 CLls (7 expressed & and 6 expressed Ig heavy chain), and as previously reported the intensity was uniformly very weak. In contrast, no tumor cells expressed CD10 (CALLA).

**Growth Requirements for B-CLL Colony Formation**

To determine the growth requirements of clonalogenic cells in B-CLL, purified B cells were plated and grown in the presence of media, CM, irradiated unstimulated T cells (T)x, or irradiated activated T cells (Ta)x using the methylcellulose assay. Pilot experiments showed that methylcellulose provided the best colony growth in B-CLL as compared with the agar or fluid-agar techniques (data not shown); therefore, it was used in our studies. Twenty-five of the 28 cases grew with one or a combination of the above growth conditions. Colonies of 20 cells or greater were noted as early as 5 days and reached optimal colony number and size by day 7. Control cultures containing irradiated T cells alone did not result in colony formation. Both the number of colonies and colony size were graded for each CLL tested.

To confirm that the colonies were of B-cell origin, colonies were harvested on day 7 and the cells were found to be morphologically similar to CLls by Wright-Giemsa staining. APAAP analysis of pooled colonies demonstrated the expression of Ia, CD19, CD20, and CD5 antigens. For B-CLLs that expressed detectable Ia, colonies demonstrated the identical light chain isotype as the original CLL cells plated. Moreover, cells within these colonies were negative for T and monocyte/myeloid markers (CD4, CD8, CD13, and CD14) demonstrating less than 1% contamination of T cells or monocytes.

To demonstrate that the observed colonies were clonogenic, self-renewal properties were examined in four cases (CLls 1, 5, 7, and 11). To this end, individual colonies were plucked from the original cultures, pooled, and replated under the identical conditions as primary cultures. These cells were found to give rise to secondary colonies expressing the same light chain isotype as the original CLL cells.

**Low-risk CLls.** CLL 1 was the only CLL that demonstrated growth with media (200 [++] or CM alone (176 [++] ) (Table 1). Within the low-risk group, CLls 1 through 3 demonstrated colony growth in the presence of irradiated unstimulated T cells, and the addition of CM induced colony formation only in CLL 4. No colonies were observed for these CLls when cultured with irradiated activated T cells alone. In contrast, CLls 5 through 8 did not grow with irradiated unstimulated T cells, and the presence of CM had no effect on colony growth. In the presence of irradiated activated T cells these CLls all demonstrated significant colony growth. The addition of CM induced variable response with regard to numbers of colonies as well as size of colonies (no. of cells/colony):

- + < 20
- + + < 40
- + + + < 60
- + + + + < 200
- + + + + + < 1,000

Abbreviation: NA, no cells available.

*Irradiated unstimulated T cells.
†Irradiated activated T cells.
‡Refer to text.

<table>
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<td>209(++)</td>
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</table>

Size of colonies (no. of cells/colony):

- + < 20
- + + < 40
- + + + < 60
- + + + + < 200
- + + + + + < 1,000

Abbreviation: NA, no cells available.

*Irradiated unstimulated T cells.
†Irradiated activated T cells.
‡Refer to text.
CLONOGENIC CELL GROWTH IN B-CLL

as colony size. Of the eight CLLs in the low-risk group, all eight demonstrated colony numbers of 188 or greater (mean = 227 ± 15, median = 211) with one or more of the above growth conditions. Moreover, large colony size (mean = 220 ± 82) was observed in 6 of 8 CLLs. The mean cloning efficiency (number of colonies per well) for low-risk CLLs was 0.39%.

Intermediate-risk CLLs. All but one (CLL 20) of the 12 CLLs from the intermediate-risk group demonstrated colony growth with one of the growth conditions (Table 2). In comparison with the low-risk CLLs, very few grew with irradiated unstimulated T cells, whereas all others (excluding CLL 20) grew with irradiated activated T cells with or without CM. In fact, CLLs 18 and 19 grew only with the combination of irradiated activated T cells and CM. More importantly, the number of colonies observed was significantly reduced (mean = 74 ± 17, median = 54). The size of colonies was also reduced, with the overwhelming majority of colonies in the small size range (mean = 70 ± 39). The mean cloning efficiency also fell to 0.15%. Of interest is the observation that CLL 11 formed large colonies similar to those of the low-risk CLLs. This patient was placed in the intermediate-risk category only due to the presence of a single LN.

High-risk CLLs. Only CLL 21 grew with irradiated unstimulated T cells and CM (Table 3). Six of eight high-risk CLLs demonstrated colony formation with irradiated activated T cells with or without CM. As observed for intermediate-risk CLLs, CM induced variable effects. The number of colonies (mean = 83 ± 28, median = 68) was similar to that observed for intermediate-risk CLL. Again, like intermediate-risk CLLs, colony size was small (mean = 40 ± 14) and the mean cloning efficiency was 0.14%.

Effects of CM and Cytokines on B-CLL Colony Formation
The observation that activated T cells induced CLL colony formation in the majority of cases suggested that activated T cells might be producing a growth-promoting cytokine. Therefore, we attempted to replace this activity with CM and/or a panel of recombinant cytokines.

CM from two sources were evaluated for their ability to induce colony formation. CM produced from TPA-PHA activation of peripheral blood mononuclear cells (PBL-CM) induced CLL colony growth in 8 of 28 CLLs when added to either unstimulated or activated T cells (Tables 1 through 3). For the low-risk CLLs, the addition of CM to irradiated unstimulated T cells induced growth in only one case (CLL 4), whereas it induced growth with irradiated activated T cells in two cases (CLLs 1 and 4). The addition of CM had variable effects on both the number and size of CLL colonies (Table 1). For intermediate/high-risk CLLs, the addition of CM to irradiated unstimulated T cells induced colony growth in one intermediate- (CLL 13) and one high- (CLL 21) risk CLL, whereas addition of CM to irradiated activated T cells induced colony formation in 3 of 12 intermediate-risk CLLs and 1 in 8 high-risk CLLs (Tables 2 and 3). The number and/or size of colonies was increased in 4 of 13 intermediate- and 1 of 8 high-risk CLLs. A second source of CM produced by PHA activation of highly enriched peripheral blood T cells (T-CM) produced a very minimal effect when added to irradiated unstimulated and/or activated T cells (CLLs 3, 9, 11, and 14). To confirm the previous observation that the addition of IL-2 together with mitogens induced CLL colony growth, we examined the effects of PHA, TPA, and IL-2 alone and in combination. Whereas PHA or TPA alone had no effect, the simultaneous addition of PHA or TPA with recombinant IL-2 induced colony growth in 5 of 19 and 3 of 19 CLLs, respectively.

Considering that CM did not consistently replace the colony-stimulating growth induced by activated T cells, recombinant cytokines either alone or in combination were added to CLL cultures to assess their ability to induce colony formation. Cytokines including IL-1 to IL-7, TNF, α and γ-IFN, BCGF, and GM-CSF were added to B-CLL cells to determine if they could replace the growth-promoting effects observed with activated T cells alone. None of the above cytokines alone or in combination with each other induced significant (greater than 30) colony formation using our culture system. Furthermore, the combination of all cytokines did not induce growth. Some inconsistent growth was observed with IL-3, TNF, IL-3 + IL-5, TNF + IL-2, IL-2 + IL-4, IL-6 + IL-7, or α-IFN + IL-2.

In vivo, CLLs receive signals not only from hematopoietic cells but also from microenvironmental stromal cells. Therefore, we attempted to determine whether the cytokine IL-7, which induces pre-B cell growth and is produced by the stromal cells, has any effect on the CLL clonogenic cell growth when added to irradiated unstimulated or activated T cells. For the low-risk CLLs, no consistent effect was

### Table 2. Growth Requirements of the Intermediate-Risk CLLs

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Refer to Table 1 for explanation of symbols.

### Table 3. Growth Requirements of the High-Risk CLLs

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Refer to Table 1 for explanation of symbols.
observed. For intermediate/high-risk CLLs, 4 of 20 CLLs that did not grow with irradiated activated T cells demonstrated convincing colony growth. IL-7 in these cases induced both an increase in the number of colonies as well as an increase in colony size (e.g., CLL 19: no colonies with irradiated activated T cells alone and 329 colonies with the combination of irradiated activated T cells and IL-7).

**Phenotype of the Clonogenic CLL Cell**

In the context of the above determined growth requirements of the B-CLL clonogenic cell, we then attempted to characterize the phenotype of B-CLL cells capable of forming colonies in methylcellulose (Fig 1). The antigenic phenotype of the CLL clonogenic (progenitor) cell was examined in 14 CLLs (8 low-, 3 intermediate-, and 3 high-risk) by attempting to deplete this cell using MoAbs and complement. Removal of Ia and CD19 positive cells resulted in the abrogation of colony formation with a mean of 95% and 94%, respectively. Moreover, lysis of the CD20 and CD5 antigens inhibited colony formation by a mean of 82% and 93%, respectively. These data suggest that CLL clonogenic cells uniformly express Ia, CD19, CD20, and CD5 antigens on their surface.

**Correlation of B-CLL Colony Formation With Clinical and Biologic Parameters**

An attempt was made to correlate the ability of B-CLL cells to form colonies in vitro with clinical presentation, expression of cell surface antigens, and stage of the cell cycle (Table 4).

A high correlation, $r = .68$, $P = .0002$, was observed between the number of colonies and the colony size. A significant association was observed between low-risk versus intermediate-risk CLLs and maximum number of colonies formed, as well as colony size ($P < .0001$). No statistically significant difference was observed between the intermediate- and high-risk CLLs with regard to colony number and size. Stage is a perfect predictor of risk group and the $P$ values for the above measures were the same for stage. There is compelling evidence from these data that colony formation capability with the four growth conditions tested decreases with increasing CLL progression when measured by the number of colonies per well, the colony size, or the product of the two.

Several clinical parameters, including level of lymphocyte count, lymphocyte doubling time, paraproteinemia, hypogammaglobulinemia, BM histology, cytogenetic abnormalities, pace of disease progression, prior therapy, and survival from time of diagnosis were examined to determine if they correlated with CLL colony formation. No correlations could be identified between prior therapy, level of lymphocyte count (excluding stage 0), and site of disease or disease bulk. Moreover, with the data available on a limited number of patients, no correlation was observed between paraproteinemia, hypogammaglobulinemia, BM histology, or cytogenetic abnormalities with the ability to form CLL colonies in vitro.

Heterogeneity in cell surface antigen expression was observed for the B-cell activation antigens CD71, CD25, CD23, and B7. Eight cases expressed CD71, 11 expressed CD25, 17 expressed CD23, and 19 expressed B7. However, patterns of antigen expression did not correlate with the different growth requirements of the B-CLL clonogenic cell observed above or with the stage of the disease.

Cell cycle stage of 15 CLLs (5 low-, 6 intermediate-, and 4 high-risk patients) was examined before culture using acridine orange staining. Tumor cells isolated from seven cases had virtually all cells in Go whereas the remaining eight CLLs showed some variable percentage of cells in G1. Of the 8 cases that had tumor cells in both Go/G1, 2 were low-risk, 4 were intermediate-risk, and 2 in high-risk groups. The remainder of CLL cases demonstrated nearly all cells in Go. None of the CLLs demonstrated tumor cells in S or G2/M phase of the cell cycle. Similarly, the heterogeneity observed in the cell cycle did not correlate with growth or stage of CLL.

**DISCUSSION**

In this report, we have examined the in vitro clonogenic cell growth requirements of 28 randomly selected B-CLLs. These B-CLLs were homogeneous with respect to both morphology and coexpression of B-cell antigens CD19, CD20, CD21, as well as CD5. Twenty-five of 28 highly enriched B-CLLs formed self-renewable colonies of the identical cell surface phenotype as the original CLL cells plated. Seven of 28 B-CLLs demonstrated clonogenic cell growth when cultured with irradiated unstimulated T cells, whereas 23 of 28 grew with irradiated activated T cells. Clonogenic cell growth correlated best with CLL risk group. Low-risk B-CLLs demonstrated a mean of 227 ± 15 colonies, which were generally of large size (220 ± 82). In contrast, intermediate-risk and high-risk B-CLLs demonstrated a significantly lower mean cloning efficiency, with mean colony
Table 4. Clinical and Biological Characteristics of the CLLs

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Abbreviations: WBC, white blood cells; Rx, therapy; ND, not done.

* - = no LN.
+ = one LN site.
++ = two LN sites.
+++ = three LN sites.
† = no spleen.
+ = splenomegaly by CAT scan.
++ = palpable spleen tip.
+++ = palpable to mid-abdomen.
++++ = palpable to pelvic brim.

numbers of 74 ± 17 and 83 ± 28, respectively. Similarly, for intermediate- and high-risk CLLs, colony size tended to be much smaller (70 ± 39 and 40 ± 14). CM, individual cytokines, or combination of cytokines were not capable of inducing clonogenic cell growth comparable with that observed with irradiated activated T cells. The differences in clonogenic B-CLL growth observed between low-, intermediate-, and high-risk CLLs could not be explained by differences in the clonogenic cell phenotype, clinical presentation, numbers of cells in G1 phase of the cell cycle, and expression of cell surface Ig, CD71, CD25, CD23, and/or B7.

A number of investigators have previously studied the growth requirements of B-CLL clonogenic cells in vitro.\textsuperscript{14,21} This literature is confusing since each study was based on a variety of culture conditions as well as small numbers of patients whose clinical status was not always defined. The two major variables in these studies were matrix (soft agar or methylcellulose) and additives (mitogens, serum source, CM, and feeder cells). Considering these variables, we attempted to reproduce three of the most commonly used systems (soft agar, fluid-agar, and methylcellulose) to determine their advantages and disadvantages. The methylcellulose assay system was selected since colony growth was reproducibly observed and colonies could be easily isolated and readily phenotyped. Using either irradiated unstimulated or activated T cells as feeder cells, marked differences in colony growth were observed. These data demonstrate that the growth requirements of B-CLL clonogenic cells are heterogeneous.

Activated T cells are known to secrete a large number of cytokines that are not secreted by unstimulated T cells. These observations suggest that the signals required for CLL...
clonogenic cell growth for the majority of B-CLLs are produced by activated T cells. This hypothesis is further supported by the fact that clonogenic cell growth was not due to cell–cell adhesion since no cellular aggregation or even individual cells adhering to one another was observed visually immediately after plating. Although the CM was prepared by activating either whole mononuclear cell fractions (PBL-CM) or purified T cells (T-CM), these preparations did not replace the growth-promoting ability of irradiated activated T cells, and in some instances were even inhibitory. The inability of CM to replace activated T cells may be due to a short-lived, secreted growth factor or, alternatively, the presence of both stimulatory and inhibitory growth factors. The major difference between our system and those previously reported is that clonogenic CLL cells could be grown in a microenvironment provided by activated T cells without the requirement for mitogens.

Since we could not substitute CM for the growth-promoting stimuli provided by activated T cells, we reasoned that a single recombinant cytokine or combination of cytokines might provide the signal(s) necessary for B-CLL growth. It was surprising that no consistent colony growth could be induced by any of the cytokines tested. Moreover, attempts to combine cytokines were similarly disappointing. Our inability to induce CLL colony growth with cytokines might be due to one of several variables including dose, combinations, or source of cytokine. It is equally possible that the growth-promoting cytokine has yet to be discovered. Further work is needed to identify the growth factor required for CLL colony formation.

The most important finding of this study was that the ability to induce colony formation using the growth conditions tested correlated with CLL risk group. The number and size of the colonies as well as the cloning efficiency of the low-risk CLLs were much greater than those of intermediate- and high-risk groups. There was a significant correlation between low-risk versus intermediate/high-risk CLLs and maximum number of colonies formed, as well as colony size (P < .0001). No marked difference was observed between the intermediate- and high-risk CLLs with respect to colony number and size. Therefore, these data provide compelling evidence that the ability to form colonies decreases as the disease progresses from low- to high-risk CLL groups when measured by the number of colonies per well and the colony size. Although patient numbers are too small for statistical significance, it was of interest to note that the low-risk CLLs appeared to segregate into two subgroups. One subgroup formed colonies in the presence of irradiated unstimulated T cells while the other subgroup grew with activated T cells.

Therefore, these studies demonstrate that as the CLL advances and invades parenchymal organs, fewer colonies can be grown. This observation is in agreement with those of Perri and Kay,11 who concluded that CLL colony formation was reduced as the mass of CLL cells increased. The above findings suggest that the microenvironmental signal(s) required to grow the intermediate/high-risk CLLs are not sufficiently provided by activated T cells alone. They further suggest that additional signals provided by accessory or stromal cells may be required. Evidence for this hypothesis is derived from attempts to induce CLL colony formation by small pieces of CLL tissue isolated from infiltrated lymph nodes. In several preliminary experiments, large and numerous CLL colonies were only observed in the vicinity of the tissue specimen, suggesting that one or more soluble factors were produced.

It was assumed that the low-risk CLL clonogenic cell is different from that of the intermediate- and high-risk CLL, hence the reason for the different growth behavior. Therefore, the phenotype of the CLL clonogenic cell was analyzed. The clonogenic cell from each CLL risk group was found to be identical in coexpressing Ia, CD19, CD20, and CD5 antigens. These data are in accord with the previous findings of Perri et al.,17 who concluded that the CLL clonogenic cell expressed Ia, CD24, and CD5 antigens. Further work is needed to identify the exact level of maturity of the clonogenic cells using a greater selection of MoAbs. Heterogeneity observed in cell surface antigen expression and cell cycle did not correlate with colony formation.

In addition to Rai stage and corresponding risk group, several clinical parameters including level of lymphocyte count, lymphocyte doubling time, paraproteinemia, hypogammaglobulinemia, bone marrow histology, cytogenetic abnormalities, pace of disease progression, prior therapy, and survival from time of diagnosis were examined to determine if they correlated with CLL colony formation. No correlations could be identified between level of lymphocyte count (excluding stage 0), paraproteinemia, hypogammaglobulinemia, BM histology, cytogenetic abnormalities or prior therapy, and ability to form CLL colonies in vitro. In contrast, patients from the intermediate- and high-risk groups who demonstrated short lymphocyte doubling time, and/or a rapid progression in stage (CLLs 15 through 20 and CLL 25) formed very few CLL colonies in vitro. Colony growth was also poor in those patients exhibiting short survival times (CLL 20 and CLL 25). Previously treated patients did not react any differently to the different growth stimuli than untreated CLLs, suggesting that prior therapy has no effect on colony formation. This is in contrast to the findings of Ishiyama et al.,21 who reported that untreated CLLs irrespective of clinical stage grew better than treated CLLs. These investigators studied only a small number of CLL patients who were receiving treatment at the time of study. Thus, their observation of reduced growth could not be attributed to the clonogenic cell. In the few cases of Rai stages II and IV that had transformed to large cell lymphoma (Richter’s syndrome), there was a significant increase in the ability to form colonies with both irradiated unstimulated and activated T cells. This further suggests that the clonogenic cell at each stage of the disease has a different and specific growth requirement.

We conclude from our data that CLL clonogenic cell growth requirement correlates with CLL risk group and not with site of disease, phenotype, or cell cycle status which represents growth in vivo. The CLL clonogenic cell potential to grow in vitro decreases due to an unknown mechanism with organ infiltration. The growth factor(s) required for
colony formation by the low-risk and intermediate/high-risk groups is different; however, neither factor has yet been defined. Future studies involve the identification of the specific growth factor(s) required for colony growth of each CLL risk group using various sources of CM and combination of these with cytokines. A future use of this clonogenic assay would involve monitoring the efficacy of immunotoxin therapy in the killing of tumor cells.

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Association between clonogenic cell growth and clinical risk group in B- cell chronic lymphocytic leukemia

R Dadmarz, SN Rabinowe, SA Cannistra, JW Andersen, AS Freedman and LM Nadler

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