Response Assessment in Chronic Lymphocytic Leukemia After Fludarabine Plus Prednisone: Clinical, Pathologic, Immunophenotypic, and Molecular Analysis

By L.E. Robertson, Yang O. Huh, James J. Butler, William C. Pugh, Cheryl Hirsch-Ginsberg, Sanford Stass, Hagop Kantarjian, and Michael J. Keating

The goals of this study were to evaluate the response to treatment in chronic lymphocytic leukemia (CLL) according to clinical, pathologic, immunophenotypic, and molecular features, as well as to address the clinical significance of each finding. One hundred fifty-nine CLL patients with either advanced Rai stage III or IV (81 patients) or progressive Rai stage 0 to II (78 patients) were treated with fludarabine [30 mg/m²/d intravenously every day for 5 days] plus prednisone [30 mg/m²/d orally daily for 5 days]. Thirty-six patients were previously untreated. The response rates were 12% complete response (CR), 30% nodular complete response (nCR), and 18% partial response (PR). In all patients who achieved a complete response (both CR and nCR) less than 30% of nucleated cells were lymphocytes on marrow aspirate differential analysis; however, nCR patients had residual nodular and/or interstitial lymphocyte involvement on marrow biopsy examination. There was no evidence of leukemic infiltration on marrow biopsy examination in CR patients. With a median follow-up of 35 months, comparison of time to progression in the CR and nCR groups at 2 years showed a projected 87% versus 55% progression-free survival (P < .001). Residual disease assessment by flow cytometry using simultaneous dual-color staining on blood and marrow lymphocytes was also performed on each patient. Residual disease was determined by the expression of CDS on B lymphocytes and the monoclonality of surface light-chain expression. After six courses of fludarabine plus prednisone, no residual disease was detected by flow cytometry in 89% of the CRs, 51% of the nCRs, and 19% of the PRs. Clinical residual disease in PR patients with no residual disease detectable by flow cytometry was limited to lymphadenopathy. Time to progression at 2 years was longer in CR and nCR patients having no residual disease detected by flow cytometry (84% v 39% 2-year progression-free survival, P < .001). Posttreatment Ig gene rearrangement analysis using JH, Jκ, and Cλ probes demonstrated no rearranged bands and a return to the germline configuration in five of seven CRs and two of eight nCRs studied. The molecular studies were concordant with the dual-parameter immunophenotype results and none of the patients who reverted to a germline DNA pattern after treatment have experienced relapse. The absence of detectable minimal residual disease by bone marrow biopsy, dual-color flow cytometry, and Ig gene rearrangement analysis is achievable in CLL with fludarabine and is predictive of the response duration.

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THE NATURAL history of chronic lymphocytic leukemia (CLL) has been carefully described by a number of investigators. Over the years, the often indolent nature of this disease has been emphasized. This feature, combined with the frequent advanced age of CLL patients, the palliative effect of available therapy, and concerns of further immunosuppression from treatment, initially resulted in a relatively low level of interest in the development of new and innovative treatment strategies. Recently, with the identification of several active agents and regimens, interest in clinical research in CLL has been revitalized. Consequently, both the National Cancer Institute-Sponsored Working Group and The International Workshop on CLL have recommended objective guidelines for patient eligibility and response criteria to facilitate clinical trial comparison. Both working groups stress the need to pursue minimal residual disease assessment and note the persistence of residual lymphoid marrow nodules as the only clinical evidence of disease in some responding patients.

Herein, we summarize the results of residual disease evaluation in CLL by clinical parameters, bone marrow histopathology, flow cytometry using dual-parameter immunofluorescence staining, and Ig gene rearrangement analysis in a large number of patients treated with fludarabine plus prednisone. The clinical relevance and implications of these findings are addressed.

PATIENTS AND METHODS

Patients. One hundred sixty-one patients with progressive or symptomatic CLL entered into a clinical trial between August 1988 and December 1989. The median age was 62 years (range 34 to 82 years). One hundred sixteen (73%) were male and 123 (77%) had received prior cytotoxic therapy. The median number of prior therapies was two. The distribution according to Rai stage was Rai 0, 6%; Rai I, 19%; Rai II, 24%; Rai III, 24%; and Rai IV, 27%. The median follow-up was 36 months.

Diagnostic criteria. All patients fulfilled the National Cancer Institute-Sponsored Working Group diagnostic criteria for CLL. Pretreatment evaluation included a medical history, physical examination, complete blood cell count, differential analysis, platelet count, chemical survey, bone marrow examination, Ig quantitation, lymphocyte immunophenotype evaluation, and Ig gene rearrangement analysis.

Treatment. Fludarabine (30 mg/m² intravenously [IV] daily for 5 days) plus prednisone (30 mg/m² orally daily for 5 days) were administered approximately every 4 weeks for a total of six courses. Patients were thoroughly reevaluated after every three and six courses of therapy. The majority of patients received six courses of treatment. There was no statistically significant difference in the number of treatments in any of the subgroups.

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Response criteria. The sites considered for clinical response included the peripheral blood, bone marrow, lymph nodes, spleen, and liver. The criteria for response are shown in Table 1. These criteria are similar to the National Cancer Institute-Sponsored Working Group recommendations with only minor differences: bone marrow examinations were performed at the end of the last treatment; a 50% decrease in marrow lymphoid infiltration was required for a partial response (PR); and a normocellular marrow for age was not required (however, adequate values for hemoglobin concentration, absolute neutrophil count, and platelet count as defined in Table 1 were required). In addition, complete response (CR) patients were stratified based on the presence or absence of residual marrow lymphoid findings into CR and nodular CR (nCR) groups. We defined clinical relapse as an increase in the absolute lymphocyte count above 10,000/μL, more than 50% lymphocytes on marrow differential analysis, more than a 50% increase in the sum of the products of at least two lymph nodes, the appearance of new lymph nodes, more than 50% increase in the liver/spleen span below the costal margin, the new appearance of palpable hepatosplenomegaly, or the development of an aggressive lymphoma.

Pathologic review. All responding patients underwent posterior iliac crest marrow biopsies at both treatment initiation and response evaluation. Marrow biopsy specimens from CR patients (both CR and nCR) were evaluated by two pathologists (J.J.B. and W.C.P.) for the presence of residual lymphoid findings. Two patients were excluded from analysis after pathologic review and reclassified as leukemic-phase small-cleaved cell lymphoma based on paratrabeicular involvement and lack of CD5 expression on B lymphocytes. Marrow biopsy specimens were evaluated for cellularity, percentage lymphocytes, and pattern of bone marrow infiltration. Five different patterns were recognized: (1) normal, no evidence of lymphoproliferation in the bone marrow; (2) nodular pattern, nodules of small mature lymphocytes that lack clear germinal centers; (3) interstitial pattern, replacement of normal hematopoietic tissue by small mature lymphocytes infiltrating between fat without distortion of marrow architecture; (4) mixed pattern, both nodular and interstitial involvement; and (5) diffuse pattern, eradication of marrow architecture by small mature lymphocytes.

Immunophenotyping. Immunophenotyping was performed on peripheral blood and bone marrow samples collected in EDTA by flow cytometry using a simultaneous dual-color staining technique, before treatment and after three courses and after six courses of fludarabine. The following combinations of phycoerythrin (PE)-conjugated and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MoAbs) were used: IgG2-PE/IgG1-FITC (control), CD14-PE/CD45-FITC, HLA-DR-PE/CD2-FITC, CD4-PE/CD8-FITC, CD20-PE/CD5-FITC, CD5-PE/IgM+D-FITC, CD5-PE/κ-FITC, and CD5-PE/λ-FITC. Ten microliters of each MoAb was added to 0.5 × 10^6 to 1.0 × 10^6 cells and incubated at 2°C to 8°C for 15 minutes in the dark. The red blood cells in the sample were lysed using ammonium chloride for 10 minutes followed by a washing step. (For staining IgM + IgD, κ and λ, the last two steps are reversed in sequence). The cells were resuspended and fixed with 1% paraformaldehyde. The analysis was performed by FACScan (Becton-Dickinson, San Jose, CA) using a Consort 30 program. Residual disease was determined by coexpression of CD5 on B lymphocytes in conjunction with monoclonality of surface light-chain expression on CD5 positive B cells (Fig 1). The presence of more than 10% of the total lymphocyte population coexpressing CD20 and CD5 with monocytic light-chain expression was considered positive for residual disease. A κ/λ or λ/κ ratio exceeding 3:1 was considered as monocytic light-chain expression (Fig 1).

Ig gene rearrangement detection. Ig gene rearrangement detection was performed on marrow aspirates from all patients before therapy. Approximately 20% of the responding patients had repeat gene rearrangement study after six courses of fludarabine. DNA from the marrow aspirate cell pellets was purified by standard phenol/chloroform extractions and ethanol precipitation after proteinase K digestion. Ten-microgram samples of DNA were digested individually to completion with the following restriction endonucleases: EcoRI, HindIII, and BamHI. The samples were electrophoresed on a Probe Tech I (Oncor, Gaithersburg, MD) using a 0.7% agarose gel and transferred to a nylon filter (Oncor), in accordance with the manufacturer's instructions. Hybridization studies were performed with the following oligolabeled DNA probes: JH, Ig heavy-chain J region (Oncor); JK, Ig λ light chain J region (Oncor); CA, Ig A light chain C region (courtesy of Dr P. Ledet, Harvard Medical School, by licensing arrangement); and TCB, T-cell receptor β-chain gene constant regions C1 and C2 (Oncor). After hybridization the Southern blots were exposed to Kodak X-OMAT AR x-ray film (Eastman Kodak, Rochester, NY) for 3 days at −70°C. For cases lacking sufficient DNA to set up the required enzyme digests for all the lineage probes simultaneously, previously probed membranes were stripped of the radioactive probe and rehybridized with additional probes in accordance with the manufacturer's instructions.

Statistical analysis. For comparison of distinct variables between two groups, either the two-sample t-test or the χ² test was used to determine statistical significance. Actuarial survival and time to progression were measured from the date of treatment initiation. The endpoint for time to progression was clinical relapse, as previously defined under response. Survival and time of progression were estimated by the method of Kaplan and Meier. The log-rank test was used to assess statistical significance between subgroups.

RESULTS

All 159 patients were assessed for response. The response rates after six courses of fludarabine plus prednimisone are shown in Table 2. A CR or an nCR was obtained in 12% and 30% of patients, respectively. The PR rate was 18% for an overall response rate of 60%. The overall response rate was 81% in early Rai stage (0 to II) patients versus 36% in advanced Rai stage (III and IV) patients. The failure rate was 40% with 48 of 64 patients having refractory disease and 16 of 64 dying while on study. Five

### Table 1. Criteria for Response in CLL

<table>
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<tr>
<th>Response</th>
<th>Blood*</th>
<th>Marrow</th>
<th>Liver/Spleen</th>
<th>Nodes</th>
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<tr>
<td>CR</td>
<td>≤ 4,000 Lymphocytes/μL</td>
<td>&lt;30% Lymphocytes on aspiration differential</td>
<td>Impalpable</td>
<td>No pathologic nodes</td>
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<tr>
<td>PR</td>
<td>≥ 50% Decrease lymphocytes/μL</td>
<td>≥ 50% Decrease in infiltrate</td>
<td>≥ 50% Decrease in span below costal margin</td>
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*CR and PR patients must have a neutrophil count ≥ 1,500/μL, platelet count ≥ 100,000 μL, and an untransfused hemoglobin ≥ 11.0 g/dL.

†CR Patients are further divided into CR and nCR groups based on the presence or absence of residual marrow lymphoid findings.

‡Infiltrate, lymphocytes × marrow cellularity.
MINIMAL RESIDUAL DISEASE IN CLL

patients achieved a response compatible with CR (one), nCR (three), or PR (one), but failed to have normalization of their peripheral counts. The overall median survival time is 120 weeks. An improved CR and nCR rate was noted for patients receiving fludarabine as frontline therapy ($P < .002$).

In the 66 patients meeting clinical criteria for a complete response, residual marrow lymphoid findings were identified in 47 patients. The bone marrow infiltration patterns observed included: nodular, 27 patients; interstitial, 14 patients, and mixed, 6 patients. By definition, all CR patients (both CR and nCR) had less than 30% lymphocytes on marrow aspirate differential analysis. Minimal variation was observed on the extent of involvement between groups on aspiration differential evaluation. However, evaluation of the extent of lymphoid involvement on marrow biopsy evaluation showed significant differences. Comparison of the lymphocyte marrow biopsy infiltrate (cellularity $\times$ percentage lymphocytes/lOO) showed patients with any nodular involvement to have significantly higher marrow lymphocyte infiltrate (mean $\pm$ SEM, 12.1 $\pm$ 12.0) than patients with pure interstitial findings (mean $\pm$ SEM, 7.85 $\pm$ 7.6) ($P < .01$).

To investigate if biopsy assessment of the marrow infiltration pattern would be of clinical utility in determining time of progression, the CR and nCR groups were compared. A statistical difference was observed with 87% of CR patients versus 55% of nCR patients progression-free at 2 years ($P < .03$) (Fig 2). Not shown is the time to progression in the PR patients, which was remarkably similar to that observed in patients with residual lymphoid marrow findings.

Further separation of the nCR group into patients with any residual lymphoid nodules, or those with solely interstitial involvement, also showed differences in time to progression. Patients with any nodular involvement had a longer progression-free interval than those with purely interstitial involvement at 2 years (63% v 39%, $P < .02$).

Analysis of overall survival showed no significant difference in complete responders subgrouped by the marrow findings (CR v nCR) ($P =$ not significant [NS]). However,
three of the four CR patients who died had no clinical evidence of relapse. In contrast, in the 10 deaths in the nCR group, six of eight evaluable patients had clinical evidence of disease recurrence. Patients who obtained a PR or for whom fludarabine failed had much shorter survival (Fig 3).

Surface immunophenotyping by flow cytometry using dual-color staining on the peripheral blood and bone marrow was performed on all of the 66 complete responders and showed no residual disease in 89% of patients obtaining a CR. In the nCR and PR groups, the percentage of patients demonstrating no residual disease on two-parameter flow cytometry was less (51% and 19%, respectively). Further subgrouping of the nCR patients showed that residual disease detection was more common in patients with interstitial involvement versus those with purely nodular involvement (74% v 42%). In the PR patients demonstrating no residual disease on two-color flow cytometry, persistent disease was clinically outside the peripheral blood and bone marrow compartments and limited to residual lymphadenopathy. In the failure group, residual disease was detected in all patients tested.

Comparison of flow cytometry taken simultaneously from the peripheral blood and bone marrow showed a high degree of concordance. However, in approximately 5% of cases residual disease was detected in the marrow aspirate and not detected in the peripheral blood.

Achievement of no residual disease status by dual-parameter immunofluorescence staining in complete responders (CR and nCR) was a highly significant predictor of time to progression. For complete responders having no residual disease on flow cytometry the 2-year progression-free survival rate was 84% versus 39% in patient having residual disease detected by two-color flow cytometry ($P < .001$) (Fig 4).

In the 12 relapses that occurred in patients demonstrating no residual disease on dual-parameter flow after six courses of fludarabine, eight had evidence of CLL in the blood detected by two-color flow cytometry at relapse. Detection of disease by flow cytometry preceded clinical
relapse in six patients from 3 to 14 months. In four patients, no interim analysis was obtained. In the three patients with negative flow cytometry at the time of relapse, the relapse was caused by isolated progressive lymphadenopathy including two patients who developed a more aggressive histology (Richter’s Syndrome).

Pretreatment and posttreatment Ig gene rearrangement detection using JH, Jκ, and CA probes was performed in a number of responding patients. One or more rearranged Ig heavy- and light-chain genes were observed before treatment initiation. In five of seven patients obtaining a CR, a return to the germline configuration was noted. In contrast, no rearranged bands were detected in only two of eight of the nCR patients. In all of the PR patients and nonresponders undergoing repeat testing, rearranged bands were demonstrated. There was no discordance between residual disease detection by dual-parameter flow cytometry and gene rearrangement study. None of the seven patients who demonstrated no detectable Ig gene rearrangement have yet relapsed.

The pretreatment characteristics and posttreatment findings of the 19 patients obtaining CR are shown in Table 3. These patients ranged in age from 35 to 76 years (median, 64 years). Eleven patients (58%) were men, and 11 (58%) had received prior treatment. The initial Rai stage ranged from 0 to IV (median, II). Diffuse marrow lymphocyte infiltration at presentation was observed in 58%. After treatment, no residual marrow lymphoid findings were observed. Seventeen of 19 (89%) had no detectable residual disease by two-parameter flow cytometry, and five of seven (71%) had no evidence of clonal Ig gene rearrangement. Time to last follow-up was 51 to 182 weeks. Five patients have relapsed and four died. Four of the relapse patients remain alive. Causes of death included small cell lung cancer in one patient and infection in three patients (listeriosis, pneumocystis, and bacterial pneumonia).

**DISCUSSION**

Comparison of treatments for CLL in many earlier studies is difficult because of marked variations in the criteria for response. Earlier clinical trials failed to use uniform response criteria, and bone marrow evaluation was often not included in the response assessment. Because of an increase in the number of clinical trials in CLL, the National Cancer Institute-Sponsored Working Group and the International Workshop on CLL have published criteria for eligibility and response. Both working groups note the frequent occurrence of residual lymphoid marrow nodules. The differentiation of benign lymphocytic nodules from small lymphocytic lymphoma/CLL can be difficult, even on repeat biopsy. Because of this difficulty in establishing a malignant nature, an equivocal diagnosis must be frequently made. As a result, they are generally considered compatible with a CR.

In contrast to the National Cancer Institute and the International Workshop guidelines, we further stratified complete responders based on the presence or absence of residual lymphoid findings. The response rates in this group of patients treated with fludarabine plus prednisone is similar to the response rates reported with fludarabine as a single agent. Paralleling the overall improvement in response, patients in the no prior therapy group were more likely to demonstrate no residual marrow findings than the prior therapy group. Comparison of the CR and nCR groups showed significantly improved progression-free survival in patients having no residual lymphoid marrow findings ($P < .03$). Among the nCR patients, the nodular pattern group had a longer progression-free interval than

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Abbreviations: PB, peripheral blood; BM, bone marrow.
the group with only interstitial involvement. This is similar to previous studies in lymphoma where a nodular growth pattern was a favorable predictive feature.\textsuperscript{14,15} Patients with nodularity also demonstrated less residual disease detectable by flow cytometry than patients with interstitial involvement. Whether this is because some of these nodules are truly benign lymphocytic nodules or because of greater difficulty in aspirating these nodules remains speculative.

The bone marrow infiltration pattern at diagnosis is a well-recognized prognostic feature based on several independent investigations.\textsuperscript{16-20} Based on multivariate analysis of 329 patients, Rozman et al\textsuperscript{20} report that the bone marrow histologic pattern is the best single prognostic parameter in CLL. Our data suggest that the marrow findings posttherapy are also important in assessing the response to therapy and remission duration.

To further define CR, dual-parameter immunofluorescence flow cytometry was used to detect minimal residual disease. B-cell CLL cells typically display low-density surface Ig. Traditionally, clonality in B-cell CLL has been defined by the expression of a single light chain, either $\kappa$ or $\lambda$.\textsuperscript{21} In addition, CLL cells usually express B-cell–associated antigens such as CD19 and CD20. A highly characteristic feature of B-cell CLL lymphocytes is the expression of the nominal T-cell–associated antigen, CD5,\textsuperscript{22,23} which has also been identified on a subpopulation of normal B cells in fetal cord blood, spleen, and lymph nodes and at the periphery of the germinal center in adult lymph nodes.\textsuperscript{24} This characteristic coexpression of CD5 with B-cell–associated antigens in CLL and monoclonality of surface light-chain expression can be easily and rapidly identified by dual-parameter flow cytometry. Using dual-parameter flow cytometry, a state of no residual disease was detected in a greater number of complete responders having no residual lymphoid findings, suggesting that the residual marrow lymphocytes in some of the patients represent CLL cells. Comparison of time to progression was consistent with this, as over 60% of the nCR patients relapsed in 2 years compared with only approximately 15% of the CR patients ($P < .03$).

The concordance rate between residual disease detected by flow cytometry simultaneously taken from the blood and marrow compartments was high. However, in 5% of patients having differing results residual disease was detected in the marrow and not the blood, suggesting the marrow may be a more sensitive site for residual disease detection.

In patients in the flow cytometry negative group who had a clinical relapse, a proportion of cases were predicted earlier by repeat flow cytometry. In three patients there was no evidence of residual disease detectable by two-color flow cytometry in the peripheral blood and bone marrow at the time of relapse. All three of these patients had recurrent disease limited to lymphadenopathy, and Richter’s syndrome was diagnosed in two of these patients. This supports the need for further close clinical follow-up in patients, despite continued negative posttreatment flow cytometry.

Ig gene rearrangement studies using JH, Jk, and Ca probes was performed in a proportion of CR patients. Despite the small number tested, there was a significant difference in the obtainment of germline rearrangement pattern in CR patients compared to patients with residual marrow abnormalities. This finding, in conjunction with the dual-parameter flow cytometry findings, lends further support to the notion that these residual lymphocytes may represent CLL. It is noteworthy that the dual-parameter surface immunophenotype results were concordant with the molecular studies and none of the patients who demonstrated no residual disease on gene rearrangement study have yet relapsed.

Brugiatelli et al\textsuperscript{25} studied CLL patients obtaining a complete clinical remission after treatment with chlorambucil. Single-parameter surface lymphocyte analysis by flow cytometry showed 16 of 28 patients had normalization of circulating B cells as measured by mouse rosette-forming cells, SmIg, and CD24 labeling. All but six patients had an abnormal $\kappa$-$\lambda$ ratio and only one of nine patients studied lacked evidence of clonal Ig gene rearrangement. A significant improvement in the remission duration was observed in patients having no detectable residual disease. Using dual-parameter flow cytometry, Vuillier et al\textsuperscript{26} evaluated 22 CLL patients with a complete clinical remission confirmed by marrow examination. Eight patients were in a phenotypic remission. Clinical correlation with relapse pattern was not made in this preliminary analysis. In addition to lymphocyte surface marker analysis, Ig gene rearrangement assessment appears to provide information. Sopper et al\textsuperscript{27} sequentially studied 12 CLL patients and found clinical status correlated with changes in the relative proportion of cells with germline versus rearranged Ig genes determined by densitometric quantification. Our analysis of nine patients returning to a germline pattern also suggests that this is also of value in determining freedom from progressive disease.

Other possible methods to assess the completeness of cytoreduction in CLL include cytogenetic analysis, fluorescence in situ hybridization (FISH), evaluation for residual monoclonal idiotype expression, and the polymerase chain reaction (PCR). Serial karyotypic analysis using an abnormal/normal metaphase\textsuperscript{28} ratio or FISH\textsuperscript{29} could further define the completeness of response. However, this requires a specific chromosomal abnormality, and approximately 50% of CLL patients are diploid.\textsuperscript{30,31} The use of anti-idiotype detection requires knowledge of the idiotypic determinants in each case, and the more sensitive technique of DNA amplification by the PCR requires prior knowledge of the unique Ig gene rearrangement. All of these methods are more labor intensive compared with marrow histopathology assessment and dual-color flow cytometry.

The observation that response to therapy in CLL correlates with a significant benefit in survival has been made in numerous clinical trials.\textsuperscript{32-36} Such observations do not necessarily imply that the efficacy of therapy resulted in improved survival, as responders must live long enough to achieve a response.\textsuperscript{37} Comparison of survival in two groups that have reached the clinical landmark of CR is subject to less bias. The striking findings of this study were the marked increase in progression-free survival in responding patients having no residual lymphoid marrow biopsy findings and no residual disease detectable by dual-parameter flow cytometry. The comparison of overall survival within complete
responders was not significant, perhaps because of the present duration of follow-up. In addition to predicting the duration of remission, the evaluation of the completeness of cyto reduction can be used to better select patients for bone marrow harvest and future autologous bone marrow transplant.

In the past, the attainment of CR was rare in CLL. With fludarabine, this goal is achievable. Our results have implications for the further definition of CR in future clinical trials in CLL. Morphologic assessment of the bone marrow biopsy and flow cytometry using dual-parameter staining easily and rapidly measure the quality of CR. These additional endpoints can be used for interim comparative analyses of clinical trials and appear to be of clinical value based on significant improvability in durability of remission and a trend toward increased survival in patients with minimal residual disease. Further study is required to demonstrate if more complete eradication of the malignant clone will be beneficial to overall survival and quality of life in CLL.

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Response assessment in chronic lymphocytic leukemia after fludarabine plus prednisone: clinical, pathologic, immunophenotypic, and molecular analysis [see comments]

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