Proliferation, Differentiation, and Cytogenetics of Chronic Leukemic B Lymphocytes Cultured With Mitomycin-Treated Normal Cells

By P. Nowell, T. V. Shankey, J. Finan, D. Guerry, and E. Besa

Lymphocytes from 6 patients with chronic lymphocytic leukemia of the B-cell variety (B-CLL) were cultured with equal numbers of mitomycin-treated mononuclear cells from normal blood. When stimulated with pokeweed mitogen (PWM), phytohemagglutinin (PHA), or the tumor-promoting agent, phorbol tetradecanoyl-acetate (TPA), the CLI cells proliferated actively by day 3 or 4 of culture, and in four cases, differentiated to significant numbers of immunoglobulin-containing cells. Chromosome studies on the proliferating lymphocytes demonstrated a cytogenetically abnormal clone in three patients, including two with a 14q- marker chromosome and two with a translocation involving the short arm of chromosome 9. One patient had a translocation from 22q to 14q, producing a Philadelphia chromosome as well as the 14q- marker. The results indicate that the neoplastic lymphocytes of B-CLL may proliferate and differentiate when appropriately stimulated in vitro, and that chromosomally abnormal clones are not uncommon. With several techniques now available for successful short-term culture of B-CLL lymphocytes, there is opportunity for better understanding of the cellular alterations in this disease.

UNTIL very recently, it has been difficult to stimulate the neoplastic B cells of chronic lymphocytic leukemia (CLL) to proliferate and differentiate in culture. This has led to the view that these cells are functionally incompetent, contributing to the hypogammaglobulinemia and immunologic deficiency common in this disorder.1,2 It has also prevented chromosome studies in most cases, because dividing neoplastic cells for karyotype analysis were usually not obtainable.3

Several laboratories have now reported successful stimulation of CLL-B cells in vitro, using various culture techniques,4,5 and limited cytogenetic data on this disease are appearing.3,7,11

We have extended to CLL a technique developed for the study of normal human B lymphocytes.12 It incorporates certain aspects of methods used successfully by others, and it has permitted us to investigate proliferation, differentiation, and cytogenetics in six patients with typical B-CLL.

MATERIALS AND METHODS

Patients

Six patients with chronic lymphocytic leukemia were studied. Clinical and hematologic findings, including marker data on circulating lymphocytes, are given in Table I. Three patients (219, 271, 273) had moderate anemia and thrombocytopenia. None had a monoclonal serum immunoglobulin, and surface immunoglobulin (slg) on circulating lymphocytes was sufficient for evaluation in only one patient (271). Two-thirds of his cells were positive for slg.

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14q- marker chromosome and two with a translocation involving the short arm of chromosome 9. One patient had a translocation from 22q to 14q, producing a Philadelphia chromosome as well as the 14q- marker. The results indicate that the neoplastic lymphocytes of B-CLL may proliferate and differentiate when appropriately stimulated in vitro, and that chromosomally abnormal clones are not uncommon. With several techniques now available for successful short-term culture of B-CLL lymphocytes, there is opportunity for better understanding of the cellular alterations in this disease.

Methods

Lymphocyte Separation and Membrane Markers

Blood from CLL patients or from normal donors was enriched for lymphocytes by Ficoll-Hypaque separation. Normal mononuclear cells (PBL) obtained in this way, to be used as "filler" cells in the CLI cultures, were treated with mitomycin-C (Sigma, St. Louis, Mo.). The cells were incubated for 45 min at 37°C with 25 μg mitomycin/10^7 cells in 1 ml Hank’s balanced salt solution (HBSS) supplemented with 10% human serum. After three washes with HBBS, the cells were greater than 90% viable by trypan blue exclusion.

Surface membrane markers on the CLI cells (E rosettes, EAC rosettes, slg) were determined as previously described.12 Depletion of T cells from the CLI lymphocyte preparations of some patients was accomplished using neuraminidase-treated sheep erythrocytes (SRBC). The lymphocytes (10^7/ml) were incubated for 5 min with an equal volume of neuraminidase-treated SRBC (10^7/ml) at 20°C, centrifuged for 10 min at 400 g, and incubated for 60 min at 20°C. Cells were resuspended, underlayered with Ficoll-Hypaque, and centrifuged at 20°C for 45 min at 400 g. The B-enriched CLI fraction was removed from the interface, washed with HBSS, and cultured as described below.

Culture Conditions

The CLI cultures consisted of 0.5 x 10^6 CLI cells mixed with 0.5 x 10^6 filler cells (mitomycin-treated PBL, allogeneic, opposite sex) in 13 x 100 mm round-bottom glass tubes. These were cultured at 37°C in 1 ml RPMI-1640 containing 10% pooled heat-inactivated human serum, 25mM Hepes buffer, 5 mM NaHCO₃, 2 mM l-glutamine, and antibiotics (neomycin, kanamycin). Controls included mitomycin-treated filler cells cultured alone and CLI cultures without filler cells or without mitogen. The mitogens used included phytohemagglutinin (PHA-M, Difco, 50 μg/ml), pokeweed mitogen (PMW, Difco, 25 μg/ml), concanavalin A (Con-A, Cal Biochem, 50 μg/ml), tetradecanoyl-0-phorbol-13-acetate (TPA, 50 ng/ml, Chemical Carcinogenesis, Minneapolis), lipopoly-
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Table 1. Clinical and Hematologic Data on Six Patients With B-CLL

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Sex</th>
<th>Duration of Disease (mo)</th>
<th>Previous Chemotherapy</th>
<th>Lymphadenopathy</th>
<th>Splenomegaly</th>
<th>WBC (x 10^9/liter)</th>
<th>Lymphocytes (%)</th>
<th>E Rosettes* (%)</th>
<th>EAC Rosettes* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>249</td>
<td>67</td>
<td>F</td>
<td>100</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>32</td>
<td>85</td>
<td>4</td>
<td>69</td>
</tr>
<tr>
<td>271</td>
<td>65</td>
<td>M</td>
<td>15</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>62</td>
<td>89</td>
<td>32</td>
<td>70</td>
</tr>
<tr>
<td>219</td>
<td>66</td>
<td>F</td>
<td>66</td>
<td>Chlorambucil</td>
<td>Yes</td>
<td>Yes</td>
<td>268</td>
<td>99</td>
<td>7</td>
<td>62</td>
</tr>
<tr>
<td>246</td>
<td>65</td>
<td>M</td>
<td>84</td>
<td>Chlorambucil</td>
<td>Yes</td>
<td>Yes</td>
<td>104</td>
<td>97</td>
<td>4</td>
<td>76</td>
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<tr>
<td>248</td>
<td>43</td>
<td>F</td>
<td>144</td>
<td>Chlorambucil</td>
<td>Yes</td>
<td>Yes</td>
<td>35</td>
<td>89</td>
<td>9</td>
<td>68</td>
</tr>
<tr>
<td>273</td>
<td>80</td>
<td>F</td>
<td>76</td>
<td>None</td>
<td>Yes</td>
<td>Yes</td>
<td>371</td>
<td>98</td>
<td>2</td>
<td>64</td>
</tr>
</tbody>
</table>

*Percent of cells in separated lymphocyte suspension that formed rosettes.

RESULTS

Proliferation

Although considerable individual variation was observed, the lymphocytes of all six CLL patients, when cultured with mitomycin-treated normal PBL, gave proliferative responses to PWM, TPA, and PHA with a time course generally similar to that illustrated in Fig. 1. Proliferation in the range of 30,000–70,000 counts per minute (cpm) typically occurred on day 3 or 4, and the response to TPA was usually the highest (Table 2). The maximum proliferation with PWM occurred later than with the other mitogens in some patients. Combining TPA with PHA or PWM did not enhance the response.

LPS and dextran sulfate produced a very low, delayed proliferative response in this CLL culture system, similar to that occurring in cultures without mitogen. The latter usually reached levels of 5000–10,000 cpm by day 6 or 7, was consistently below 2000 cpm earlier (Fig. 1), and probably reflected, at least in part, a mixed lymphocyte response of the patient’s T cells to the allogeneic feeder cells. In none of the cultures did the mitomycin-treated filler cells proliferate.

When the lymphocytes of these six CLL patients were cultured without the filler cells, they gave low delayed responses to all mitogens, similar to those typically recorded in CLL, and presumably reflecting continued proliferation of the small number of nonneoplastic T cells in the CLL inoculum.

In three patients (249, 271, 273), filler cultures were established with CLL cells from which the T lymphocytes had been partially depleted. The results were variable. In patient 249, reduction of E-rosetting cells from 4% to less than 1% produced a decreased response to all mitogens as well as reduced proliferation in control cultures without mitogen; but in patient 273, similar T-cell depletion, from 2% to <1%, had no effect. Patient 271 initially had a high frequency of

Fig. 1. Proliferation and differentiation of lymphocytes from CLL patient 273 in cultures containing mitomycin-treated normal PBL, and stimulated with different mitogens: (O—O), PWM; (■—■), TPA; (○—○), PHA; (□—□), no mitogen. Points represent mean of triplicate cultures. Standard deviation was less than 10% of the mean. Cells positive for clg were enumerated on day 7 of culture (see text for details).
Table 2. Proliferation and Differentiation of CLL Lymphocytes in Culture

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Date of Study</th>
<th>Maximum Proliferation (cpm)*</th>
<th>Mitogen</th>
<th>Maximum Differentiation (No. clg Cells)†</th>
<th>Mitogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>249</td>
<td>March 1980</td>
<td>32,000</td>
<td>PWM</td>
<td>&lt;3,000</td>
<td></td>
</tr>
<tr>
<td>271</td>
<td>May 1980</td>
<td>68,000</td>
<td>TPA</td>
<td>155,000</td>
<td>PWM</td>
</tr>
<tr>
<td>219</td>
<td>April 1980</td>
<td>52,000</td>
<td>TPA</td>
<td>&lt;3,000</td>
<td>†</td>
</tr>
<tr>
<td>246</td>
<td>April 1980</td>
<td>40,000</td>
<td>TPA</td>
<td>90,000</td>
<td>PWM</td>
</tr>
<tr>
<td>248</td>
<td>April 1980</td>
<td>58,000</td>
<td>TPA</td>
<td>184,000</td>
<td>PWM</td>
</tr>
<tr>
<td>273</td>
<td>May 1980</td>
<td>65,000</td>
<td>PWM</td>
<td>50,000</td>
<td>PWM</td>
</tr>
</tbody>
</table>

*Uptake of ³HThdR on day 3 or 4 of culture. Mean of triplicate cultures.
†Percent clg⁺ cells x no. surviving cells/culture on day 7 of culture. Mean of triplicate cultures.
‡Poor response to all mitogens.

E-rosetting cells (32%), and reduction to 12% resulted in a poor response to PHA and PWM, but a continued good response to TPA. The possible significance of these observations in connection with the cytogenetic findings will be considered below.

Differentiation

As an indication of differentiation within the neoplastic B-cell population, mitogen-stimulated cultures, identical to those used for the proliferation studies, were terminated on day 7, and cells containing cytoplasmic immunoglobulin were enumerated. The results are summarized in Table 2 and illustrated in Fig. 1. In four patients (271, 246, 248, 273), significant numbers of clg⁺ cells were observed in cultures stimulated with various mitogens (PWM, TPA, PHA). The maximum number ranged from 0.5 to 1.84 × 10⁶ clg⁺ cells, all in cultures stimulated with PWM (Table 2), as compared to a range of 1.5–5.6 × 10⁵ clg⁺ cells in PWM-stimulated cultures of normal B cells under identical conditions. Except for patient 271, the intensity of fluorescence in the CLL cells was generally less than that observed in comparable preparations of normal lymphocytes.

As noted, maximum differentiation in these four patients occurred in cultures stimulated with PWM, despite the fact that in three instances TPA was the most potent mitogen (Table 2). PHA consistently produced the fewest clg⁺ cells (0.06–0.8 × 10⁶), although the very low frequency in patient 273 (Fig. 1) was atypical. Depletion of T cells from the inoculum in two patients (271, 273) had little effect except to reduce the number of clg⁺ cells in those cultures that proliferated poorly. In general, differentiation was not observed in any cultures from these four patients (271, 246, 248, 273), which did not give a good proliferative response. This was true whether the failure to proliferate resulted from depletion of T cells, use of an ineffective mitogen (LPS, dextran sulfate), or absence of mitogen or filler cells in control cultures.

In two patients (219, 249), differentiation to clg⁺ cells did not occur even in cultures that proliferated well (Table 2).

Cytogenetics

The frequency of chromosomally abnormal cells in cultures from the six CLL patients stimulated with different mitogens is summarized in Table 3. Gaps in the data resulted from inadequate proliferation or metaphases of poor technical quality. Where a cytogenetic alteration was present, it was the same in all abnormal metaphases, and the karyotype of this clone is also given in Table 3.

Patient 249 was studied twice. In the first study (March 1980), 10%–20% of the metaphases in PWM cultures contained two translo-
Fig. 2
Representative G-banded karyotype from abnormal clone in patient 249 illustrating translocation from chromosome 22 to chromosome 14, producing a Philadelphia chromosome and a 14q⁻ marker. Karyotype was interpreted as: 46,XX,t(14;22)(q24–32;q11).
cation from the long arm of chromosome 22 (band q11) to the long arm of chromosome 14 (band q24–32), producing a Philadelphia (Ph) chromosome and a 14q’ marker (Fig. 2). In the second study of this patient (May 1980), cultures were established with the CLL lymphocyte suspension both before and after T-cell depletion. In TPA-stimulated cultures, this procedure increased the frequency of metaphases with the 14;22 translocation from 30% to 90%. No chromosomally abnormal metaphases were found in the PHA and PWM cultures before T-cell removal, and the reduced proliferation with these mitogens after T-depletion (see above) provided too few metaphases for study (Table 3). This patient had had asymptomatic CLL for 9 yr, without treatment.

Patient 271 also had a pseudodiploid clone with a 14q’ marker. Approximately one-third of the metaphases from PWM and TPA cultures showed a translocation of the long arm of chromosome 11 (band q13) to the long arm of chromosome 14 (band q32). There was also loss of material from the short arm of chromosome 9 (band p22), perhaps translocated to number 11 (Fig. 3). Thus, the abnormal karyotype was interpreted as most probably: 46,XY, t(9;11;14) (p22;q13;q32). In this patient also, partial depletion of T cells from the inoculum resulted in a somewhat higher frequency of cytogenetically abnormal mitoses, as well as reduced proliferation in PWM and PHA cultures. This patient had a history of relatively mild disease, with no treatment, for 15 mo.

In patient 219, there was a pseudodiploid clone, which was observed only in cultures stimulated with TPA (10 of 40 metaphases). The abnormality was a translocation of most of chromosome 13 (band q12) to the short arm of chromosome 9 (q22–24) (Fig. 4). The remaining centric portion of chromosome 13 resembled a Ph chromosome in unbanded material, and inspection of the few mitoses obtained in earlier attempts to grow this patient’s neoplastic lymphocytes in standard cultures indicated that it was probably present as early as December 1975, when she was first studied. This patient received radiation therapy to cervical nodes and chemotherapy with chlorambucil shortly after diagnosis of CLL in January 1975 and no treatment thereafter.

Although adequate numbers of metaphases for study were obtained by day 3 or 4 from one or more sets of mitogen-stimulated cultures in the remaining three patients (246, 248, 273), no chromosome abnormalities were detected. This included T-depleted cultures on patient 273. Two of these three individuals had been previously treated with chlorambucil.

**DISCUSSION**

The present findings confirm and extend indications from other laboratories that when appropriately stimulated by various mitogens in the presence of adequate “help” from normal PBL, the neoplastic B lymphocytes of CLL will proliferate and, in some instances, differentiate in culture. Until very recently, attempts to stimulate CLL cells with mitogens in vitro typically produced a very low delayed response, apparently reflecting cell division only of the nonneoplastic T cells in the inoculum. Several groups of investigators have now reported evidence of proliferation and/or differentiation of the neoplastic B cells in some cases of B-CLL using various mitogens, with or without added normal cells. Optimum conditions have yet to be defined, and variable results have been obtained with different mitogens, different sera, and different sources of helper T cells. In general, insufficient data on the kinetics of proliferation and differen-
tiation have been reported to permit detailed comparisons between laboratories and between patients. Gahrton et al.,11 for instance, obtained enough mitoses after 5 days of culture to permit chromosome studies, using either LPS or Epstein-Barr virus (EBV) as mitogens, without allogeneic T-cell help, but proliferation with these agents after shorter culture periods was low. Where differentiation to Ig-producing cells has been observed, this has usually involved those relatively rare CLL patients whose neoplastic cells are apparently also producing Ig in vivo, as indicated by the presence of a monoclonal serum immunoglobulin.17,18

Using our culture system, which includes PWM, TPA, or PHA as mitogen, plus mitomycin-treated normal PBL for T-cell “help,” we have obtained good proliferation by 3–4 days in vitro in 6 consecutive patients with typical B-CLL. In all cases, the CLL lymphocytes failed to proliferate in the absence of the allogeneic “filler” cells.

In four of these six patients, differentiation to Ig-containing cells was observed in the filler cultures by day 7, and PWM was the most effective stimulant in this regard. The relatively large numbers of clg- cells in these cultures argues against a major contribution from the small proportion of normal B cells present in the culture inoculum. Differentiation did not occur in cultures that failed to proliferate, and it is unclear from published reports whether prior cell division is always required for CLL-B cells to differentiate in vitro.17,20

In two patients, the neoplastic B cells did not undergo differentiation to clg- cells despite a good proliferative response. This could indicate a defect in the leukemic lymphocytes or differences in the “helper” capacities of the T cells from different normal individuals that were used in the filler cultures.19

The present findings, and the other recent observations cited above, suggest that although the immune defects in patients with B-CLL may result from the “crowding out” of normal B cells and T cells, it does not necessarily indicate an inherent inability of the neoplastic B cells to proliferate and differentiate when appropriately stimulated. In some cases there may be a qualitative or quantitative defect in their capacity to synthesize or secrete immunoglobulin;19,21 in other instances, the neoplastic B cells may be competent, but because of their clonal nature they may be capable of responding only to a very few antigens, and so are of little value to the host.

These studies of CLL-B cells in culture do not shed light directly on the question of T-cell function in these patients, a matter that has been debated.21,23 The failure of the patient’s own T lymphocytes to provide sufficient “help” for proliferation and differentiation of the neoplastic B cells in our cultures that did not contain added normal cells could simply indicate excessive dilution of the patient’s T lymphocytes and monocytes in the inoculum, rather than any fundamental deficiency in their helper capacities; and a similar phenomenon could occur in vivo.

The chromosome findings in the present study also extend recent reports by others,7,11 which suggest that cytogenetic abnormalities in B-CLL are not rare. Earlier conclusions that the karyotype is usually normal in CLL24 again probably reflect the proliferation only of nonneoplastic T cells in cultures used for such studies. Several laboratories, employing the newer culture methods, have reported chromosome data in small numbers of patients with B-CLL and have observed clones of cells with cytogenetic alterations in a proportion of the individuals studied.7,11

As in our series, there have been some cases with no demonstrable abnormalities and also varying numbers of normal metaphases in most individuals with chromosome aberrations. One cannot be certain whether a cytogenetically normal cell in these cultures represents a diploid tumor cell or a dividing nonneoplastic B or T lymphocyte.18,11 A good proliferative response at 72–96 hr in cultures where the inoculum presumably contains very few nonneoplastic cells, as in CLL patients with a high total lymphocyte count, should indicate that most mitoses represent the leukemic cells. However, our cytogenetic data from T-depleted CLL suspensions (Table 3) suggest that even a small initial fraction of T cells may, in some patients, contribute significantly to the early mitotic activity in culture. More studies are needed to determine whether the T-depletion procedure, which apparently may also adversely affect the B-cell response, should be used routinely to reduce the frequency of nonneoplastic mitoses.

It also appears, from our work and that of others,8,11 that there may be significant variation among normal T and B lymphocytes and neoplastic CLL-B cells in their responses to different mitogens in different patients, as has also been observed in T-CLL.25 The cells with chromosome abnormalities in our three patients were observed in cultures stimulated with TPA or with PWM, but not with PHA, although the time course and magnitude of the proliferative responses to all three mitogens were sufficiently similar to indicate that leukemic cells were dividing in each instance. We also did not obtain adequate cell division in our cultures stimulated with either LPS or dextran sulfate to permit chromosome studies, although these are considered B-cell mitogens and have been reported by others to be effective in CLL, as have EBV, protein A from Staphylococcus, and the
calcium ionophore, A23187. There have been no previous reports of the use of TPA in CLL, although its ability to stimulate normal human T and B cells has been recognized. More experimentation with various mitogens is needed, and the use of more than one in the cytogenetic study of any given patient may increase the chances of success.

The specific chromosome abnormalities observed in our three patients add to the relatively limited information available on this subject. All three had pseudodiploid clones, with apparently balanced translocations. In two instances, these rearrangements involved the terminal portion of the long arm of chromosome 14, producing a 14q- chromosome, an abnormality frequently associated in a nonrandom fashion with a variety of lymphoproliferative disorders, including CLL. In patient 271, there appeared to be a complex three-way translocation including chromosomes 9, 11 and 14. The involvement of 11q as a “donor” in the formation of the 14q- marker, as in this case, also appears to occur nonrandomly in lymphoid neoplasms. Interestingly, this patient had surface immunoglobulin, characterized as IgM,K, on his leukemic B cells, and sIg of the same constitution was observed by Gahrton et al. only in the one patient in their series of 11 cases of CLL who had an 11;14 translocation.

In patient 249, the translocation was from chromosome 22 to chromosome 14, producing a Philadelphia chromosome in addition to the 14q- marker. The frequent involvement of the same segment of chromosome 14 in many B-cell disorders, as well as in occasional chronic T-cell neoplasms, has suggested that this is the site of a gene locus important in neoplastic lymphoid proliferation. It may be significant that a gene for immunoglobulin (Ig) heavy chain synthesis is located on this chromosome arm and that a similar correlation has been observed in murine myelomas. As Ig on the cell surface is the receptor that “triggers” lymphocyte proliferation, perhaps structural alteration in an Ig gene could produce an abnormal response to environmental stimuli, leading to neoplasia.

The significance of the Philadelphia (Ph) chromosome in our CLL patient is less clear. It is typically associated with chronic granulocytic leukemia (CGL) and usually involves a translocation from chromosome 22 to the long arm of chromosome 9. This alteration apparently occurs in a marrow stem cell, and so the Ph chromosome may occasionally appear in cells differentiating along other pathways, including lymphoid. However, it has not been observed previously in CLL, and the present rearrangement involving translocation from chromosome 22 to the long arm of chromosome 14 is extremely rare in CGL. Alteration in chromosome 22 obviously can confer a selective advantage when it occurs in a marrow stem cell, but it is not certain to what extent it is contributing to the proliferative capacity of the neoplastic clone in B-CLL, which seems to arise from a lymphoid precursor committed to the B-cell lineage.

Despite these two nonrandom cytogenetic alterations in her leukemic cells, both apparently involving genes important in the development of human neoplasia, the disease in this patient was unusually benign, with no progression of a very mild disorder over 9 yr.

Two other aspects of our chromosome findings warrant comment. We did not observe trisomy of chromosome 12, although this appears also to be a nonrandom abnormality associated with B-CLL. Two of our three patients had alterations of the short arm of chromosome 9, perhaps involving the same band (p22-24). This aberration has not been previously reported in B-CLL, and more information is needed to determine if this chromosomal segment is involved with greater than random frequency.

Further investigation is also needed to demonstrate the relationship, if any, between the nature and frequency of chromosome abnormalities in B-CLL and the clinical course of the disease. Our findings, and other reports, do not indicate any clear association between an aberrant karyotype and the duration or stage of disease or previous exposure to mutagenic therapy (chemotherapy, radiation). It remains to be determined by follow-up studies whether CLL patients with chromosome alterations have a less favorable prognosis, and whether cytogenetic evolution is associated with clinical progression, as has been shown in CGL.

A number of techniques are now available for the successful short-term culture of leukemic lymphocytes from B-CLL. The neoplastic cells of individual patients may respond differently in vitro, and varying numbers of nonneoplastic B cells and T cells in different patients also represent a complication. Nevertheless, these methods should yield important new data on the characteristics of CLL lymphocytes and correlation with their cytogenetic patterns. In addition to providing further insights into the nature of the disease, such studies may also prove of practical value in diagnosis and prognosis.

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REFERENCES

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