Interphase cytogenetics by means of in situ hybridization with the chromosome 12-specific biotinylated α satellite DNA probe pSP 12-1 was used for the study of trisomy 12, the most common chromosomal abnormality in chronic lymphocytic leukemia. In situ hybridization was performed on methanol/acetic acid fixed cells of conventional cytogenetic preparations from eight patients and on morphologically and immunologically classified cells of cytospin preparations from seven patients. The results show that trisomy 12 is more common than assumed on the basis of karyotype analysis of metaphase chromosomes: 2 of 13 patients with a normal karyotype in G-banding analysis were shown to have trisomy 12 by interphase cytogenetics. Immunophenotyping of the cells of one patient showed that the trisomy was restricted to cells with Ig light chain clonality. For the evaluation of the prognostic, therapeutic, and biologic significance of trisomy 12, in situ hybridization should be used in parallel with karyotype analysis because it allows the study of all cell populations of both interphase and mitotic cells, whether neoplastic or normal.

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pokeweed mitogen (PWM; 100 pg/mL; GIBCO, New York, for 3-day cell cultures. One culture was set up in the presence of son).

IES (MoAbs) were used: Leu14 (anti-CD22; Becton Dickinson, Erembodegem, Belgium), anti-K and anti-A (Coulter Clone, Coulter Immunology, Hialeah, FL), and Leu4 (anti-CD3; Becton Dickinson).

Conventional cytogenetic preparations. Whole blood was used for 3-day cell cultures. One culture was set up in the presence of pokeweed mitogen (PWM; 100 μg/mL; GIBCO, New York, NY) and one in the presence of 12-O-tetradecanoylphorbol-13-acetate (TPA; 2 μg/mL; Sigma, St Louis, MO). To arrest the metaphases, the cultures were treated with 0.125 μg of deacetylmyelocycline (Colcemid; GIBCO). For chromosome preparations, the cells were subjected to hypotonic treatment with a 0.56% solution of potassium chloride for 10 minutes. The cells were fixed in methanol/acetic acid (3:1) for 40 minutes. After centrifugation, the cells were resuspended in fixative, dropped onto slides, and air-dried. The preparations were stained by a standard trypsin-Giemsa G-banding procedure (GTG). For patient nos. 4 through 9 and 12 through 15, 20 metaphases from PWM-stimulated and 20 metaphases from TPA-stimulated cultures were analyzed, whereas for patient nos. 1 through 3 and 10 through 11, 20 metaphases from TPA-stimulated cultures only were studied.

MAC preparations of immunologically classified cells. MAC preparations were made of noncultured cells. Thus, only interphase cells were analyzed. The MAC (morphology-antibody-chromosomes) technique has been described earlier. Briefly, mononucleated cells from the peripheral blood were isolated by one-step density-gradient centrifugation in Ficoll-Paque and washed twice with phosphate-buffered saline. For immunologic classification, 1 million cells were suspended in 1 mL of RPMI 1640 medium, to which 1 mL of hypotonic solution was added. The hypotonic solution consisted of 50 mmol/L of glycerol, 5 mmol/L of potassium chloride, 10 mmol/L of sodium chloride, 0.8 mmol/L of magnesium chloride, 1 mmol/L of calcium chloride, and 10 mmol/L of sucrose. After hypotonic treatment for 5 minutes, the cell suspension was divided into cytocentrifuge chambers and centrifuged at 400g for 10 minutes. After air-drying overnight, the cells were identified by immunoperoxidase or alkaline phosphatase antialkaline phosphatase antialkaline phosphatase technique using the following antibodies: Leu14 (anti-CD22), anti-κ, anti-λ, and Leu4 (anti-CD3).

In situ hybridization. The hybridization was performed with a biotin-labeled, chromosome 12-specific satellite DNA probe pSP12-1, containing a 340-bp EcoRI fragment. Previous experiments using fluorescence in situ hybridization have shown that this probe is highly specific for the centromeric region of chromosome 12 and displays essentially no cross-hybridization with other chromosomes at high stringency. The probe was labeled by nick-translation using biotin-11-dUTP (Bethesda Research Laboratories, Gaithersburg, MD) or biotin-16-dUTP (Boehringer Mannheim, GmbH, Germany) according to the instructions of the supplier. Hybridization was performed by a modification of methods described earlier.

Conventional cytogenetic preparations (patient nos. 1 to 8) were made from methanol/acetic acid-fixed cell suspensions (stored at +4°C for 1 to 6 months) derived from TPA-stimulated cultures. Fresh slides were incubated in sequential ethanol series (70%, 94%, 99%; 5 minutes each) and air-dried for a few hours. MAC preparations of immunologically classified cells (patient nos. 9 through 15) were destained by incubation in methanol/acetic acid fixative for 1 hour at room temperature and air-dried for 1 to 4 hours. The preparations were then, right away or after storage at −70°C for 0.5 to 2 months, incubated in 0.01 N hydrochloric acid containing 0.1% to 0.1 mg of pepsin per milliliter at 37°C for 4 to 10 minutes to remove the cytoplasm. To stop the reaction, the slides were washed in distilled water for 2 to 3 minutes, which was followed by sequential incubation in ethanol. The slides were allowed to dry at room temperature for 1 to 4 hours before the hybridization was performed.

Immediately before hybridization the slides were incubated in a solution consisting of 60% formamide, 2X standard saline citrate

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age (y)</th>
<th>Therapy Before Study</th>
<th>Leukocyte Count (10⁹/L)</th>
<th>% of B Cells</th>
<th>Light Chain Clonality</th>
<th>% of T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/66</td>
<td>None</td>
<td>26</td>
<td>89/10</td>
<td>NS</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>M/75</td>
<td>None</td>
<td>29</td>
<td>81/14</td>
<td>NS</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>F/66</td>
<td>None</td>
<td>44</td>
<td>66/18</td>
<td>NS</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>F/70</td>
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<td>35</td>
<td>89/3</td>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>M/67</td>
<td>None</td>
<td>44</td>
<td>80/8</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>F/75</td>
<td>Chlorambucil</td>
<td>8</td>
<td>48/64</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>F/61</td>
<td>Cyclophosphamide</td>
<td>63</td>
<td>75/20</td>
<td>NS</td>
<td>91</td>
</tr>
<tr>
<td>8</td>
<td>M/63</td>
<td>Cyclophosphamide</td>
<td>29</td>
<td>69/28</td>
<td>+</td>
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</tr>
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<td>+</td>
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<tr>
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<td>M/66</td>
<td>None</td>
<td>39</td>
<td>26/48</td>
<td>+</td>
<td>15</td>
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<td>11</td>
<td>M/68</td>
<td>Cyclophosphamide</td>
<td>49</td>
<td>82/11</td>
<td>+</td>
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</tr>
<tr>
<td>12</td>
<td>F/71</td>
<td>None</td>
<td>19</td>
<td>68/31</td>
<td>+</td>
<td>7</td>
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<td>50</td>
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<td>84/12</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>M/68</td>
<td>None</td>
<td>15</td>
<td>73/24</td>
<td>+</td>
<td>75</td>
</tr>
</tbody>
</table>

Abbreviations: L, % of lymphocytes; N, % of neutrophils; NS, not studied.
(2X SSC = 0.3 mol/L NaCl, 0.03 mol/L trisodium citrate, pH 7.0), and 0.05% polysorbate (Tween) 20 at room temperature for 15 minutes. The hybridization buffer consisted of 50% formamide, 10% (wt/vol) dextran sulphate, 2X SSC, and 0.5 mg/mL of herring sperm DNA. The slides were covered with 0.04 µg of the biotinylated chromosome 12-specific DNA probe in 10 µL of the hybridization buffer. Denaturation of the probe and cells was performed at 70°C for 3 minutes. The slides were incubated in a moist chamber at 42°C for 12 to 16 hours. After hybridization the slides were washed in three changes of phosphate-buffered saline containing 2X SSC biotinylated chromosome 12-specific DNA probe in 10% (wt/vol) dextran sulphate, 2X SSC, and 0.5 mg/mL of herring sperm DNA. The slides were covered with 0.04 µg of the probe and then with peroxidase-conjugated rabbit antimalouse serum (Dakopatts). The color was developed by incubating the slides in diaminobenzidine tetrahydrochloric acid (DAB; Sigma). The cells were counterstained with hematoxylin. All analyses of interphase signals were performed using slides with good cell morphology and complete, nonoverlapping slides were washed in three changes of hybridization buffer. Denaturation of the probe and cells was performed on fresh conventional cytogenetic preparations. In these patients, previous chromosome studies had shown a normal karyotype. Table 8, in situ hybridization was performed on fresh conventional cytogenetic preparations. In the six patients with CLL, in the case of patient nos. 14 and 15 were 47,XX,+12; t(7;13)(q21;q14) and 46,XY;12p+; 13q−; 17q−, respectively.

RESULTS

In situ hybridization was performed on specimens from 15 patients with CLL. In the case of patient nos. 1 through 8, in situ hybridization was performed on fresh conventional cytogenetic preparations. In these patients, previous chromosome studies had shown a normal karyotype. Table 2 shows the proportions of cells with zero, one, two, or three signals. In patient nos. 1, 2, 4, 6, 7, and 8, two hybridization signals in interphase nuclei were detected in 76% to 98% of the cells analyzed. No signals or only one signal were seen in the remaining cells. Patient no. 3 had three signals in 5% (12 of 250 cells), two signals in 85%, and one or no signals in 10% of the cells studied. In patient no. 5, three signals were present in 56% (140 of 250 cells), two signals in 34%, and only one or no signals in 10% of the cells (Fig 1).

In the case of seven patients, in situ hybridization was performed on MAC preparations. Previous G-banding analyses had shown a normal karyotype in five of these patients (nos. 9 through 13); patient no. 14 exhibited trisomy 12 in 50% of the metaphases analyzed, and patient no. 15 had a structural aberration of chromosome 12 in 15% of the metaphases analyzed. Of these seven patients, only patient no. 14 had cells with three in situ hybridization signals. In this patient, the frequencies of cells with three and two (or fewer) were 81% and 19%, respectively, when counted without regard to cell subset. In this patient in situ hybridization signals were also analyzed from CD3, κ, and λ positive cells. Eighty-seven percent of the λ positive B cells had three signals, whereas in none of the cells positive for κ and T-cell markers three signals were seen (Table 3).

In MAC preparations from the three controls the frequency of two signals was 90% to 93%. The corresponding figure in conventional preparations from the three other controls was 73% to 95%. These figures demonstrate that MAC preparations are equally suitable for in situ hybridization as are conventional cytogenetic preparations. In the six controls, none of the cells contained three hybridization signals.

<table>
<thead>
<tr>
<th>No. of In Situ Hybridization Spots</th>
<th>Patient No.</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional cytogenetic preparations</td>
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<td>6</td>
<td>13</td>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>85</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>34</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
<td>15</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>96</td>
<td>0</td>
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<tr>
<td></td>
<td>8</td>
<td>11</td>
<td>13</td>
<td>76</td>
<td>0</td>
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<tr>
<td>Control 1</td>
<td>4</td>
<td>9</td>
<td>87</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control 2</td>
<td>2</td>
<td>3</td>
<td>95</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td>13</td>
<td>14</td>
<td>73</td>
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<tr>
<td>MAC preparations</td>
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<td>5</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
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<td>1</td>
<td>99</td>
<td>0</td>
</tr>
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<td></td>
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<td>0</td>
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<td>93</td>
<td>0</td>
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<tr>
<td>Control 5</td>
<td>2</td>
<td>6</td>
<td>92</td>
<td>0</td>
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</tr>
<tr>
<td>Control 6</td>
<td>1</td>
<td>9</td>
<td>90</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Karotypes of patient nos. 1 through 13 were normal. Karyotypes of patient nos. 14 and 15 were 47,XX,+12; t(7;13)(q21;q14) and 46,XY;12p+; 13q−; 17q−, respectively.

DISCUSSION

The present study shows that the characterization of human chromosomes in interphase nuclei by in situ hybridization is a powerful method in the study of chromosomal aneuploidy. Especially in CLL, in which the neoplastic B cells often fail to proliferate actively despite polyclonal B-cell activators, the advantages of this technique become evident. Thus, in a study using in situ hybridization, one can easily analyze up to 250 cells, which would be very laborious in conventional karyotype analysis. In our study the possibility to use large numbers of cells provided new information about the frequency and lineage specificity of trisomy 12 in CLL.

Our results show that trisomy 12 is more common than expected on the basis of standard cytogenetic G-banding analyses. Results by Einhorn et al., who used Southern blotting analysis with probes that detect RFLPs on chromosome 12, corroborate our findings indicating that the proportion of normal metaphases does not match the proportion of cells lacking the trisomy. However, these investigators were unable to find any trisomy 12 patients not detected in conventional karyotype analysis. In our small patient material we were able to find two cases of trisomy 12 previously undetected by karyotype analysis. Therefore, standard chromosome banding analysis of metaphase cells does not appear to be a sensitive method of detecting aneuploidy in CLL. Discrepancies between various studies...
concerning the prognostic significance of trisomy 12 might be explained by this methodologic inadequacy. As in situ hybridization technique is rapid and relatively easy to use, more CLL cases should be studied using the chromosome 12-specific DNA probe before one can reliably estimate the prognostic importance of this trisomy. To fully elucidate the clinical and biologic significance of trisomy 12, interphase cytogenetics should be used simultaneously with metaphase cytogenetics.

In addition to trisomy 12, there are other chromosome abnormalities, some with prognostic value, eg, of chromosomes 13 and 14, often detected in CLL. Because only a 12-specific probe was used in our interphase cytogenetic study, we cannot rule out the possibility that the patients might also have had other chromosomal abnormalities. Thus, interphase cytogenetics cannot replace karyotype analysis of metaphase cells. The methods are complementary.

None of our healthy controls showed three signals for chromosome 12 in 200 to 250 cells scored from a predetermined area of the slide. The proportion of control cells with two signals was 73% to 95%, but never 100%. Whether cells of zero or one spot represent nullisomy or monosomy 12, or whether they are due to poor penetration of the probe, incomplete denaturation of target DNA or possible damage in DNA during long storage of the cells in fixative is not known. With chromosome 7 or 9 specific probes, the frequencies of cells with zero or one hybridization spot has been reported to be as high as 11%. Thus, it is evident that this technique should be used with great caution for analysis of monosomy of low frequency. Even though the frequency of the cells with only one signal was as high as 13% to 19% in patient nos. 1, 6, 8, and 15 and in control no. 3, we do not consider these patients necessarily as cases of monosomy 12. Similarly, it should be noted that cases with a very low frequency of trisomic cells may remain undetected for the reasons mentioned above, and finally, that in patient no. 3 any definite conclusions are also hindered by the fact that light chain clonality was not determined, and thus it is possible that some of the B cells are not clonal neoplastic cells. Further studies are needed to confirm the existence of "preneoplastic" cells without the trisomy.

The restriction of trisomy 12 to clonal B cells in patient no. 14 confirms our earlier findings made by MAC study of metaphase cells. Because only metaphase cells were analyzed in the previous study, we did not find out whether nonclonal B cells also had the abnormality. In the case of patient no. 14, who exhibited \( \lambda \) clonality, \( \kappa \) positive cells accounted for less than 1% of all cells. Nevertheless, it was not difficult to find 200 cells for analysis. None of these cells contained trisomy 12. The frequency of \( \lambda \) positive cells with two signals was 8%. Whether these "disomic" cells represent normal, nonclonal positive B cells or disomic "preneoplastic cells" cannot be solved because the hybridization is never 100% effective.

In conclusion, the combination of MAC and in situ hybridization proved useful in the study of CLL, showing that standard karyotype analysis fails to show all patients with trisomy 12, and that trisomy 12 is restricted to cells with Ig light chain clonality. The combination is superior to karyotype analysis in the study of trisomy 12 in CLL because it allows the study of all cell populations of both interphase and metaphase cells, whether neoplastic, or normal. No mitogens are required. On the other hand, the method can be used to study the effect of polyclonal B-cell activators or B-cell growth factors on the different lympho-

### Table 3. In Situ Hybridization Spot Frequencies (%) for Chromosome 12-Specific \( \alpha \) Satellite DNA Probe Among B and T Lymphocytes of Patient No. 14

<table>
<thead>
<tr>
<th>No. of In Situ Hybridization Spots</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>B lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \kappa ) positive</td>
<td>3</td>
<td>5</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>( \lambda ) positive</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>87</td>
</tr>
<tr>
<td>T lymphocytes (CD3)</td>
<td>4</td>
<td>7</td>
<td>89</td>
<td>0</td>
</tr>
</tbody>
</table>

At least 200 cells were analyzed in each subset.
cyte subpopulations.\textsuperscript{27} The method also has the advantage of enabling the study of the role of chromosome abnormality in the process of malignant transformation. The possibility that part of the neoplastic cell population in CLL might carry trisomy 12 while some cells do not presents a chance to study cancer genes in these different neoplastic cell populations. Finally, in view of the suggested use of trisomy 12 in CLL as an indication for treatment,\textsuperscript{3} it is now possible to monitor the frequency of trisomy 12 in relation to the clinical response to treatment.

REFERENCES

Trisomy 12 in chronic lymphocytic leukemia: an interphase cytogenetic study

AP Losada, M Wessman, M Tiainen, AH Hopman, HF Willard, F Sole, MR Caballin, S Woessner and S Knuutila