Non-radioactive *in situ* hybridization for the detection and monitoring of trisomy 12 in B-cell chronic lymphocytic leukaemia

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Summary. Non-radioactive *in situ* hybridization (NISH) with a chromosome 12-specific alpha satellite probe was performed on 20 patients with chronic lymphocytic leukaemia (CLL) with normal karyotype (15 cases) or with inadequate mitotic yield (5 cases) from mitogen-stimulated cultures.

All patients had over 70% lymphocytes coexpressing the CD5/CD23 antigens. While less than 1% interphase nuclei showed three fluorescent spots in 16/20 patients, evidence of trisomy 12 in 15–25% interphase cells was detected in four patients. According to the FAB classification the diagnosis in these patients was typical B-CLL, stage III (Rai's staging system) in one case, CLL/PLL, stage II and III in two cases, PLL, stage III in one case. In order to confirm these results,

NISH was repeated after 1 month in one patient and after 2 years in three patients. All patients had been treated with chemotherapy in the period between the two NISH experiments. In all cases a 1·8–3-fold increase of percentage of trisomic interphase cells was detected.

These findings suggest that in B-CLL clones with trisomy 12 may have proliferative advantage over clonal B-lymphocyte without +12 and, possibly, that they may be more resistant to chemotherapy.

We conclude that NISH is a sensitive technique allowing for the detection and monitoring of trisomy 12 in a fraction of B-CLL patients with normal karyotype or with no analysable mitoses despite employment of polyclonal B-cell mitogens.

Because abnormal lymphocytes in B-cell chronic lymphocytic leukaemia (B-CLL) often fail to divide in culture, stimulation with T- and B-cell mitogens has been widely adopted over the last decade in most cytogenetic laboratories (Han et al, 1984; Pittman & Catovsky, 1984; Bird et al, 1989). While optimal protocols to induce cell proliferation are currently under investigation (Ueshima et al, 1989), an adequate number of mitoses is generally obtained in 70–80% of patients, of which about a half show normal karyotype. Since several reports (Drexler, 1988; Kluin-Nelemans & Jansen, 1989) indicate that normal T-lymphocytes may proliferate under mitogenic stimulation, techniques permitting the cytologic identification of metaphase cells (Autio et al, 1987), as well as molecular genetic studies (Einhorn et al, 1990) have been employed to discriminate whether normal karyotype in B-CLL is a real finding or whether it reflects inadequacy of culture conditions. This issue is drawing increasing attention in view of the prognostic significance of

chromosomal aberrations, especially trisomy 12, in B-CLL (Juliusson et al, 1990). While the above-mentioned studies showed that no chromosome aberrations were present in CLL patients with normal karyotype, a recent study employing the non-radioactive *in situ* hybridization (NISH) for the detection of trisomy 12 (Perez Losada et al, 1991), showed 2/13 CLL patients cytogenetically normal, to be trisomic in interphase cells by using a chromosome 12-specific alpha satellite DNA probe.

This finding prompted us to further evaluate the role of NISH in the detection of trisomy 12 in B-CLL. We therefore studied 20 patients with normal karyotype (15 cases) or with inadequate mitotic yield (5 cases) and documented the presence of trisomy 12 in four. Sequential NISH experiments were performed in these four patients in order to monitor the frequency of trisomy 12 in interphase nuclei during the course of the disease.

PATIENTS AND METHODS

Twenty patients with B-CLL with normal karyotype (15 cases) or with no analysable mitoses (5 cases) are included in

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the present report. These patients belong to a series of 35 consecutive B-CLL admitted to our Institute between January 1988 and December 1990 submitted to cytogenetic investigation. Besides the classical staging procedures (Silber & Stahl, 1990), immunologic studies were performed on admission for each patient on peripheral blood (PB) mononuclear cells with a standard immunofluorescence technique (Cuneo *et al*, 1992). A panel of monoclonal antibodies detecting T-cell (CD2, CD5) and B-cell antigens (CD19, CD23, CD24) was employed. Double labelling with monoclonal antibodies detecting CD23 and CD5 antigens was performed on all patients. Fluorescence was analysed using a FACScan flow cytometer (Becton-Dickinson) gating primarily on lymphocytes. Expression of surface immunoglobulins (Ig) (heavy and light chains) was tested under a Diaplan fluorescence microscope (Leitz) (200 cells observed).

Cytogenetic studies. On admission 2×10^6 PB cells/ml were incubated in RPMI 1640 with 10% fetal calf serum added as previously described (Castoldi *et al*, 1987). The following mitogens were employed in all patients: lipopolysaccaride (LPS) (0·1 mg/ml), pokeweed mitogen (PWM) (0·1 mg/ml), phytohaemagglutinin (PHA) (0·1 mg/ml). Cells were harvested after 3–4 d according to standard procedures. At least 10 metaphases, and usually more than 20, obtained from two or three mitogen stimulated cultures were karyotyped before a patient was classified as cytogenetically normal.

Non-radioactive in situ hybridization (NISH). For the detection of trisomy 12 in interphase cells the chromosome 12-specific alpha satellite probe pBR12 was used (courtesy of Dr M. Rocchi, Istituto Gaslini, Genova). The probe was biotinylated using the BRL kit (Gaithersburg, Maryland) following the manufacturer's instructions.

NISH experiments were performed on methanol/acetic acid-fixed cell suspensions from patients without analysable metaphases or with normal karyotype. In all experiments two control slides from a normal volunteer donor and from a patient with trisomy 12 documented cytogenetically were included. Slides for conventional cytogenetic analysis, aged at room temperature for 5–15 d, were treated with RNAse (RNAse A, 100 µg/ml in $2 \times$ SSC), dehydrated in alcohol and denatured in 70% formamide at 70°C for 2 min. The biotinylated probe (100 ng/slide) was dissolved in 45 µl hybridization mixture (total volume) consisting of 60% formamide in $2 \times$ SSC, 10% wt/vol dextrane sulphate and 30 µg salmon sperm DNA. Following denaturation at 75°C for 5 min, 45 µl hybridization mixture were layered onto each slide. Slides were covered with a 24×50 mm coverslip and sealed with rubber cement (Sanford, Bellwood). Hybridization was allowed to proceed overnight at 37°C in a moist chamber. Slides were washed in four changes of 50% formamide at 45°C for 4 min and in four changes of $2 \times$ SSC at 45°C for 3 min. Following a 20 min blocking step with 5% non fat dry milk powder in $2 \times$ SSC, slides were incubated with FITC-avidin (Vector, Burlingame) (5 µg/ml in $4 \times$ SSC/5% non fat dry milk) for 20 min. Signal amplification was obtained with a layer of biotinylated anti-avidin monoclonal antibody (Vector, Burlingame; 5 µg/ml) followed by incubation with FITC-avidin. Propidium iodide (0·5 µg/ml) was used as counterstain.

Slides were examined under a fluorescence microscope (Diaplan, Leitz); at least 200 single interphase cells with well-delineated fluorescent spots were counted in each patient for signal screening.

RESULTS

Clinical and immunologic features

According to the FAB criteria (Bennett *et al*, 1989) the diagnosis was B-CLL in 15 cases, CLL/PLL (10–50% prolymphocytes in the PB smear) in four cases, prolymphocytic leukaemia (PLL) (> 50% prolymphocytes) in one case. The diagnosis had been made 1–3 years before admission in 15 patients; five patients were newly diagnosed. At the time of referral to our hospital, four patients were in stage I, seven in stage II, eight in stage III, one in stage IV according to Rai. Twelve patients had been previously treated either with chlorambucil or with multiagent chemotherapy including vincristine, cyclophosphamide and prednisone.

Positivity for the CD19 B-cell marker was detected in over 80% of the mononuclear cells in all cases. Less than 20% PB lymphocytes expressed the CD2 T-cell associated antigen. All patients had over 70% cells coexpressing CD5 and CD23 antigens in the lymphocyte gate with monotypic expression of surface Ig. Of the four patients with trisomy 12 in interphase nuclei, one had CLL, two had CLL/PLL, one had PLL. One patient was in stage II, three were in stage III. Salient clinical and immunologic findings in these patients are reported in Table I.

Cytogenetic and NISH studies

Twenty patients had either no analysable mitoses (5 cases) or had normal karyotype (15 cases) from at least two mitogen-stimulated cultures. In seven patients metaphases from LPS-, PWM- and PHA-stimulated cultures could be studied. In eight patients no analysable mitoses were obtained from LPS-stimulated cultures. Data concerning cytogenetic analysis in the four patients with trisomy 12 in interphase cells are reported in Table I. Of the 20 patients studied by NISH, 16 showed two fluorescent spots in over 85% of interphase cells. Fluorescence was localized in the paracentromeric region of chromosome 12 in metaphase cells and only minimal non-specific binding to other chromosomes was detected. In these patients 5–15% interphase nuclei showed one signal only, while three signals were detected in less than 1% interphase cells and were not seen in metaphase chromosomes.

Four patients showed three fluorescent signals in 15–25% interphase nuclei. Metaphases with three signals were not seen in these patients (Fig 1). Results are detailed in Table II. In order to confirm these results and to monitor the frequency of trisomy 12 during the course of the disease, NISH as well as cytogenetic studies were repeated in these four patients on PB samples collected 1 month later (patient 1) and 2 years later (patients 2–4). All four patients had been treated in the period between the two NISH experiments. Three patients showed persistence of normal metaphases (nos. 1, 3 and 4) while 7/12 metaphases with trisomy 12 were detected in patient 2. Percentage of interphase cells with

Table I. Clinical and immunologic findings at the time of the first NISH experiment in four B-CLL patients cytogenetically normal with trisomy 12 in interphase cells.

Patient, sex, age	Diagnosis (date)	WBC ($\times 10^9/l$)	Stage	Immunophenotype (% positive cells)	Previous therapy	No. of karyotypes (mitogen)
1, m, 70	CLL/PLL (August 1990)	120	II	CD19 (84%) CD2 (15%) IgM/IgD/k	No	21 (PWM) 10 (PHA)
2, f, 50	PLL (July 1985)	50	III	CD19 (87%) CD2 (8%) IgM/IgD/k	Chlorambucil COP	16 (PWM) 12 (PHA)
3, f, 72	CLL/PLL (June 1985)	48	III	CD19 (85%) CD2 (10%) IgM/IgD/k	Chlorambucil COP	26 (PWM) 8 (PHA)
4, m, 68	CLL (May 1988)	26	III	CD19 (89%) CD2 (10%) IgM/IgD/IgG/IgA/k	Chlorambucil	10 (PWM) 7 (PHA)

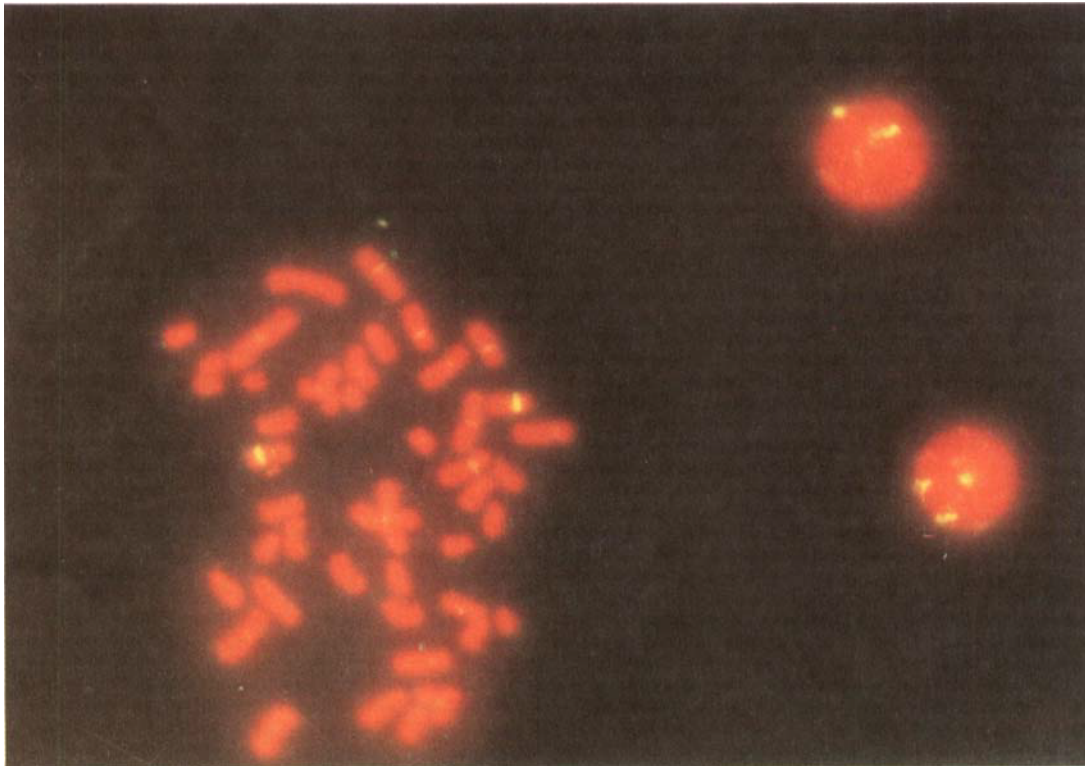*Abbreviations: COP: cyclophosphamide, vincristine and prednisone.*

Fig 1. NISH with a chromosome 12-specific alpha satellite DNA probe in a patient with B-CLL with normal karyotype. In the same microscopic field a metaphase with two copies of chromosome 12 as well as two interphase cells with three (lower right) and two (upper right) fluorescent spots are shown.

three signals was found to be increased in all patients, ranging between 28% and 50% in this second NISH experiment. Percentage of trisomic cells along with pertinent clinical information is summarized in Table II.

DISCUSSION

This study shows that NISH is a sensitive technique allowing for the detection of trisomy 12 in a fraction of B-CLL patients

Table II. Results of hybridization signal screening in interphase nuclei in four patients with B-CLL (200 cells observed)*

Patient	Date	No. of fluorescent spots in interphase cells (%)			WBC ($\times 10^9$/l)/ % CD23/CD5 +ve cells	Stage	Therapy†
		1	2	3			
1	August 1990	8	67	25	120/81%	II	Clorambucil
	October 1990	9	46	45	52/80%	II	
2	December 1988	11	74	15	50/83%	III	COP, CHOP
	December 1990	8	42	50	72/90%	III	(6 courses)
3	December 1988	12	67	21	48/85%	III	Clorambucil,
	December 1990	5	55	40	58/92%	III	COP (3 courses)
4	October 1988	7	68	25	26/87%	III	COP
	November 1990	9	63	28	13/48%	IV	(9 courses)

Abbreviation: CHOP: cyclophosphamide, daunorubicin, vincristine and prednisone.

* Less than 1% cells with three fluorescent spots were detected in a control slide from a normal donor; over 80% trisomic cells were observed in a control slide from a patient with a 47 XX, +12 karyotype.

† Therapy given in the period between the two NISH experiments.

not evaluable cytogenetically or with normal karyotype from mitogen stimulated cultures. In our experience the discrepancies between cytogenetics and NISH studies in detecting trisomy 12 were only encountered when the trisomic clone accounted for less than 45% interphase cells. In patient 2 some metaphases with trisomy 12 were detected when the second NISH experiment showing 50% trisomic interphase cells was performed. Notably, in our four patients with trisomy 12 in interphase cells, no metaphases from LPS-stimulated cultures could be studied, whereas no evidence of trisomy 12 was detected by NISH in seven patients with adequate number of mitoses from LPS-stimulated cultures.

We should thus conclude that NISH is important in detecting trisomy 12 in B-CLL, especially when the abnormal clone represents less than 50% of interphase cells and when no metaphases from cultures with LPS are available. Of course these findings await confirmation on large series.

Perez Losada *et al* (1991) found trisomy 12 in interphase cells in 2/13 patients with normal karyotype; thus, of a total of 33 B-CLL patients studied so far by NISH, six (18%) were shown to carry trisomy 12 in non-dividing cells. However, molecular genetic studies employing probes for the detection of restriction fragment length polymorphism on chromosome 12 failed to provide evidence of trisomy of this chromosome in 13 CLL cytogenetically normal patients. Possibly, heterogeneity of patient population in different series may account for these differences. Indeed, while the majority of patients reported by Einhorn *et al* (1990) were in stage 0 or I according to Rai, patients included in the present study were more frequently in stages II–IV and were submitted to NISH at the time of hospitalization for disease progression. The frequency of trisomy 12, especially in the context of complex karyotypes, may increase in advanced stages of B-CLL (Castoldi *et al*, 1987; Oscier *et al*, 1990).

An interesting finding in our study is the presence of trisomy 12 in 15–25% of interphase nuclei despite the leukaemic cells being over 80% mononuclear cells in all

samples. Expansion of residual normal B- or T-lymphocytes under mitogen stimulation was excluded in two cases showing monotypic expression of surface immunoglobulins by immuno-cytochemical technique (APAAP) (Fagioli *et al*, 1990) in cytospin preparations obtained after 72 h cultures (data not shown). Thus, the most likely explanation for our finding is that clonal B-lymphocytes with and without trisomy 12 may coexist in some patients with B-CLL.

This, in turn, may suggest that the acquisition of an extra copy of chromosome 12 is not an early event in the genesis of B-CLL and that the development of some chromosome abnormalities in this disease may result from genetic instability of already transformed B-lymphocytes. The recent demonstration of the existence of oligoclonal patterns of Ig gene rearrangement in some cases of B-CLL (Rechavi *et al*, 1989) supports this argument.

Because trisomy 12 has been shown to be an independent prognostic indicator (Juliusson *et al*, 1985, 1990), it was interesting to assess the percentage of interphase cells carrying trisomy 12 in our patients at different times during the course of the disease. An increase of 3:2 signal ratio in interphase nuclei was detected after a 2-year interval in two patients (nos. 2 and 3) and after a 1-month interval in one patient (no. 1). In one patient (no. 4) the absolute percentage of trisomic cells had not increased significantly over a 2-year period; however, the second NISH experiment was performed when a reduction of clonal B-lymphocytes to 48% of mononuclear cells had been obtained by chemotherapy, thus making the percentage of trisomic cells calculated over B-lymphocytes much higher than the one detected at presentation (see Table II).

Since all four patients had been treated with chemotherapy between the two NISH studies it is not clear whether cells with trisomy 12 had a proliferative advantage over cytogenetically normal cells, or whether their increase over the time has to be ascribed to different susceptibility to treatment of the genetically distinct populations of transformed lymphocytes.

The clear-cut increase of trisomic cells observed in patient 1 over a 1-month period following cytoreduction obtained by clorambucil, would indicate that B-lymphocytes with trisomy 12 may be more resistant to this alkylating agent than clonal lymphocytes, without trisomy 12.

In conclusion, we have confirmed that NISH may be of use in detecting clones with trisomy 12 in B-CLL; data from sequential studies in four patients seems to indicate a possible role for NISH in monitoring the size of the trisomic clone during the natural history of the disease and following cytoablative treatment.

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