

Interphase Cytogenetics of Hematological Cancer: Comparison of Classical Karyotyping and *in Situ* Hybridization Using a Panel of Eleven Chromosome Specific DNA Probes¹

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ABSTRACT

Numerical chromosome aberrations were detected in hematological cancers by nonradioactive *in situ* hybridization (ISH) procedures, using centromere specific probes for chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y. All 15 cases could be evaluated by ISH for these 11 probes. Our experiments show that in seven of these randomly selected leukemia bone marrow cell suspensions numerical aberrations for one or two chromosomes could be detected by this method. The results of ISH on interphase nuclei and in some cases on metaphase preparations were compared with karyotyping data. Seven cases of chromosomal aberrations observed with ISH (three for monosomy and four for trisomy) were confirmed by this classical cytogenetic technique, whereas in five instances an aberration was found only with ISH (twice for monosomy, twice for trisomy, and one disomy for the Y-probe). One case of a trisomy for chromosome 1 observed by ISH on interphase nuclei could be explained by a marker chromosome, a finding that was further substantiated by ISH on metaphase spreads. In this case double-target ISH on interphase cells with the probes for chromosomes 1 and 16 strongly suggested a translocation between these chromosomes. Also, in one case a marker chromosome could be characterized as a translocation between chromosomes 7 and 17. In this latter case the cytogenetic examinations revealed only monosomy for chromosomes 7 and 17 in addition to noncharacterized marker chromosomes.

Our results indicate that the nonradioactive ISH procedure in combination with chromosome specific repetitive centromeric probes is a powerful tool for studying both numerical and structural chromosomal aberrations in interphase nuclei of leukemias. It may therefore become a valuable and routine diagnostic tool in addition to the existing karyotyping procedures.

INTRODUCTION

Cytogenetic analysis, using karyotyping on basis of chromosome banding techniques, plays an important role in the detection of chromosomal abnormalities in human solid tumors (1). In some cases these aberrations can be correlated with diagnosis and/or prognosis of the malignant disease and may thus dictate therapeutic modalities. However, the interpretation of the chromosome banding patterns is often difficult due to the small number of recognizable metaphases, minimal chromosome spreading, poor banding quality, and condensed or fuzzy appearance of the chromosomes. A further limitation of this technique is that it requires dividing cells, and as a result the majority of the tumor cells are excluded from such analyses (2). Furthermore, in order to obtain analyzable metaphase chromosomes, culturing of tumor cells may be needed. This can introduce an additional unwanted parameter, *i.e.*, the selection of a certain highly proliferative subpopulation of tumor cells.

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Karyotyping of bone marrow cells from patients with hematological cancers has revealed many specific chromosomal abnormalities, which provide diagnostic as well as prognostic information (3-5). A classical example of such a marker in CML³ is a translocation, resulting in the Philadelphia chromosome (6). However, in cases of terminally differentiated (tumor) cells, such as segmented neutrophils, or cells that have a low proliferative capacity, such as cells from patients with chronic lymphatic leukemia, karyotyping is often difficult or sometimes even impossible.

The cytogenetic analysis by ISH of nonmitotic cells was introduced by Cremer *et al.* (7) and is generally referred to as "interphase cytogenetics." The use of chromosome specific DNA probes in combination with the ISH technique enables the detection of numerical and structural chromosome aberrations in both metaphase spreads and interphase nuclei. As described previously by our group and others (8-12), this approach is of great importance in studies of solid tumors, where direct chromosome analysis is frequently hampered by the small number of recognizable metaphases or disturbed by the fact that the tested metaphases are not representative of the tumor because of *in vitro* selection.

Until now, only a few ISH studies have been performed in cases of leukemia and even fewer studies have correlated ISH results with classical karyotyping data (13-16). In the underlying study we have analyzed bone marrow specimens from 15 randomly selected leukemia patients with the ISH procedure, using 11 DNA probes recognizing highly repetitive sequences in the (peri-)centromeric region of specific chromosomes. These results were correlated with cytogenetic analyses of G-banded metaphase chromosomes.

MATERIALS AND METHODS

Sample Preparation. Bone marrow aspirations of three cases of CML, eight cases of acute leukemia (AML/AUL/ALL) (17), and four cases of myeloid dysplastic syndromes (MDS/CMMoL) (18) were randomly selected for our study. Cell preparations for ISH were made with freshly obtained bone marrow or peripheral blood. The samples were layered onto Ficoll-Hypaque (Pharmacia/LKB, Uppsala, Sweden; specific density, 1077 g/ml) and centrifuged at 1200 rpm for 30 min. The low density cells were recovered, washed in PBS, fixed in 70% ethanol (-20°C), and stored at -30°C.

Metaphase spreads were obtained from bone marrow cells that had been cultured in RPMI 1640 for 1 or 24 h. Colcemid was present during the last hour of the culture. Before fixation in methanol:glacial acetic acid (3:1), the cells were exposed to a hypotonic solution (0.075

³ The abbreviations used are: CML, chronic myeloid leukemia; ISH, *in situ* hybridization; AML, acute myeloid leukemia; AUL, acute undifferentiated leukemia; ALL, acute lymphatic leukemia; MDS, myeloid dysplastic syndrome; CMMoL, chronic myelomonocytic leukemia; PBS, phosphate buffered saline; GTG, trypsin-Giemsa; SSC, 0.3 M NaCl-30 mM sodium citrate; FITC, fluorescein isothiocyanate; CMMoL-t, chronic myelomonocytic leukemia with transformation.

M KCl) for 15 min. Slides were made according to routine cytogenetic procedures. Karyotyping was performed using the GTG technique.

Tumor Cell Processing for *in Situ* Hybridization. For ISH, 5 μ l of a cell suspension were dropped onto poly-L-lysine (Sigma Chemical Co., St. Louis, MO; M_r 150,000–300,000) coated slides, air dried, and heated at 80°C for 1 h. To obtain an optimal recovery of cells and proper removal of cellular protein for improvement of DNA probe and antibody penetration, a proteolytic digestion step was applied as described before (12). The digestion was performed with pepsin (Sigma) at a concentration of 100 μ g/ml in 0.01 M HCl for 20 min at 37°C. After subsequent dip washes in H₂O and PBS, the nuclei were postfixed in 4% formaldehyde in PBS for 20 min at 4°C. Then the slides were subsequently washed in PBS and H₂O, respectively, and equilibrated in 60% formamide-2 \times SSC, pH 7.0, for 10 min at room temperature.

DNA Probes. For the detection of the target sequences on chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y, the plasmid probes (as summarized in Table 1) were used. The probes were labeled by nick-translation with biotin-11-dUTP (Sigma) (19) or modified with digoxigenin (Boehringer, Mannheim, Germany) according to the instructions of the supplier. Several of these probes are commercially available from ONCOR (Gaithersburg, MD).

***In Situ* Hybridization.** The DNA probes were hybridized to the (tumor-) cell preparations (in single- and double-target ISH) as described before (12, 20) in 60% formamide, 2 \times SSC, and 10% dextran sulfate at a probe concentration of 1 ng/ μ l hybridization mixture. Under these stringency conditions, hybridization to minor binding sites was avoided. Of the hybridization mixture, 8 μ l were applied to the slides under a coverslip (18 \times 18 mm). Denaturation of probe and target DNA was carried out by heating the slides in a moist chamber to 70°C for 2.5 min. Hybridization was then performed overnight at 37°C. The coverslips were removed by immersing the slides in 60% formamide-2 \times SSC, pH 7.0. Next, the slides were washed three times for 5 min in the same buffer at 42°C and subsequently three times for 5 min in 2 \times SSC, pH 7.0 at 42°C.

Detection of hybrids was accomplished with fluorescent reporter molecules as described previously (20). The single-target ISH reactions were performed using biotinylated probes and immunocytochemically detected in 4 \times SSC-0.05% Tween 20-0.5% Boehringer milk, using FITC conjugated avidin (Vector Laboratories, Burlingame, CA) and if necessary immunologically amplified using biotin labeled goat anti-avidin (Vector) followed by a second layer of FITC conjugated avidin (21). Double-target ISH using biotin-labeled and digoxigenin labeled probes, which were hybridized simultaneously, were immunocytochemically detected using monoclonal anti-digoxin (Sigma) in PBS-0.05% Tween 20-0.5% Boehringer milk, followed by an incubation of FITC conjugated rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark) in the same buffer. After a brief wash in 4 \times SSC-0.05% Tween 20, the biotinylated probes were immunocytochemically detected using Texas red-conjugated avidin (Vector), and if necessary amplified with biotin labeled goat anti-avidin and a second layer of Texas red-conjugated avidin. All the immunocytochemical steps were performed for 30 min at 37°C.

Evaluation of ISH Results. The ISH procedure was optimized with respect to sensitivity and specificity for the chromosome specific centromeric probes used in this study. Evaluation and counting of fluorescent ISH signals were done by two trained observers according to

criteria described before (10–12, 14). Evaluation of the preparations was performed by counting 200 nuclei/slide. The analyses of the ISH preparations and karyotyping were done without previous knowledge of the results obtained by the complementary technique.

Using this ISH procedure, at least 98% of the interphase nuclei of the human lymphocytes, the bone marrow cells and the leukemic cells showed one, two, or three ISH signals in all preparations. After the proteolytic digestion step with pepsin, which allowed a good penetration of the DNA probes and reporter molecules, the cells retained a good morphology, while discrete ISH signals with a high fluorescence intensity were obtained. By counting the number of hybridization spots in 200 nuclei of individual control specimens from normal human lymphocytes and bone marrow of healthy donors, the percentages of cells containing the euploid number of spots for each of the eleven probes varied between approximately 90% and 98% (Table 2; Fig. 1, A and B). Intra- and interobserver studies revealed limited variability (<2%) in the percentages of detected spot numbers (see also Refs. 11 and 12). The percentage of cells with one signal can be explained partly by close juxtaposition or overlapping of two signals. For the specimens used in our study we therefore decided upon a monosomy or trisomy for a certain chromosome only when the percentage of cells with one or three spots, respectively, was greater than the mean \pm 3 \times SD of the controls. The detection of three spots in control cells was the case in less than 1% of the cells for all the probes. A diagnosis of trisomy in leukemic specimens could theoretically be made if more than 2% of the nuclei showed three distinct hybridization signals. Paired or split spots, as described previously (11, 14, 15), were normally counted as one signal. Finally, to avoid misinterpretations with regard to monosomy or trisomy, we performed double-target hybridization experiments with the DNA probe showing the presumed aberration, in combination with a DNA probe, that revealed the euploid number of spots in more than 90% of the nuclei.

RESULTS

The clinical data, cytogenetic analyses on GTG-banded metaphase spreads, and ISH results of the 15 patients are summarized in Table 3.

Twelve of 15 patients, which can be subdivided in three different groups, *i.e.*, those with chronic leukemia (patients 1 to 3), acute leukemia (patients 4 to 11), and myelodysplastic syndrome (patients 12 to 15), showed structural and/or numerical chromosome aberrations on basis of karyotyping assays. For one patient with AML (case 5), no metaphases were found for karyotyping, although a monosomy 16 was observed by ISH. Therefore, a comparison of numerical chromosome aberrations detected by cytogenetic analyses of bone marrow cells with the results obtained by *in situ* hybridization to interphase nuclei, was possible for 14 patients screened with the 11 probes (154 of 165 probe hybridizations). In 144 of these 154 probe hybridizations a good correlation between results of both meth-

Table 1 Chromosome specific centromeric probes used in this study

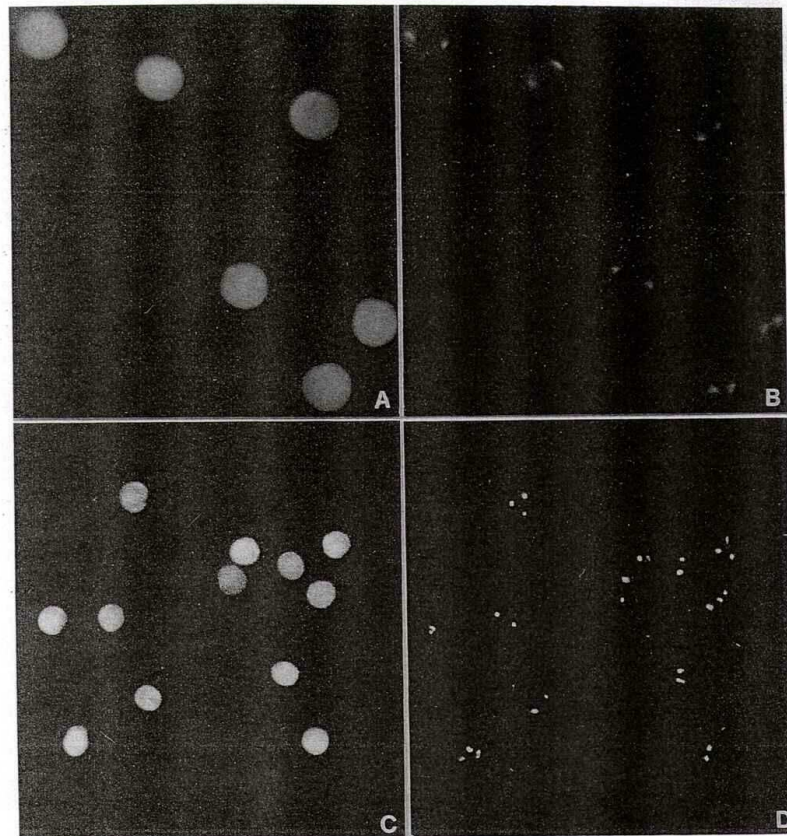
Chromosome	Name	Vector	Insert (kilobases)	Sequence type	Ref.
1	pUC1.77	pUC12	1.77	Sat. III	25
7	pTt.1	pUC9	0.68	Alphoid	26
8	D8Z2	pBS	2.55	Alphoid	27
9	pHUR98	pBR322	0.16	Sat. III	28
10	p10.1	pUC9	0.95	Alphoid	29
11	pLC11A	pSP65	0.85	Alphoid	30
16	pHUR195	pBR322	1.20	Sat. II	28
17	p17H8	pSP65	2.70	Alphoid	31
18	L1.84	pAT153	0.68	Alphoid	32
X	pBAMX5	pBR322	2.00	Alphoid	33
Y	DYZ3	pSP65	2.45	Sat. III	34

Table 2 Hybridization data of normal bone marrow and peripheral blood samples from male controls (n = 6)

Of each sample 200 nuclei were counted. Given are the mean percentages of the number of ISH spots per nucleus \pm SD.

Probe for chromosome	No. of spots per nucleus			
	0	1	2	3
1	0.5 \pm 0.3	6.7 \pm 2.8	92.8 \pm 2.7	0.5 \pm 0.8
7	0	5.5 \pm 1.5	94.0 \pm 1.7	0.5 \pm 0.8
8	0	4.5 \pm 1.5	95.0 \pm 1.2	0.5 \pm 0.5
9	0	6.2 \pm 2.0	93.3 \pm 1.6	0.5 \pm 0.5
10	0.5 \pm 0.6	7.1 \pm 2.0	92.3 \pm 2.1	0.3 \pm 0.5
11	0	5.0 \pm 1.1	94.5 \pm 1.2	0.5 \pm 0.8
16	0.5 \pm 0.3	10.1 \pm 1.1	89.3 \pm 0.8	0.3 \pm 0.5
17	0.5 \pm 0.3	5.3 \pm 2.1	94.0 \pm 2.6	0.5 \pm 0.6
18	0.5 \pm 0.3	8.1 \pm 2.7	92.9 \pm 2.5	0.7 \pm 0.8
X	0.5 \pm 0.5	98.6 \pm 0.5	1.0 \pm 0.6	0
Y	1.1 \pm 0.8	98.6 \pm 1.8	0	0

Fig. 1. *In Situ* hybridization results on interphase nuclei from normal lymphocytes with the probe for chromosome 1 (B) and to nuclei of bone marrow from case 3 with the probe for chromosome 10 (D). (A and C, DNA counterstaining with 4',6-diamidino-2-phenylindole). In A and B more than 98% of the interphase nuclei exhibit two spots. In C and D, 31% of the nuclei show three spots, indicated as a trisomy.



ods is found. In 7 of the 144 probe hybridizations numerical chromosome aberrations were observed with both procedures, *i.e.*, three instances of monosomy and four instances of trisomy. In 10 of the 154 probe hybridizations there was no correlation between the karyotyping and ISH. In 5 of these 10 tests an

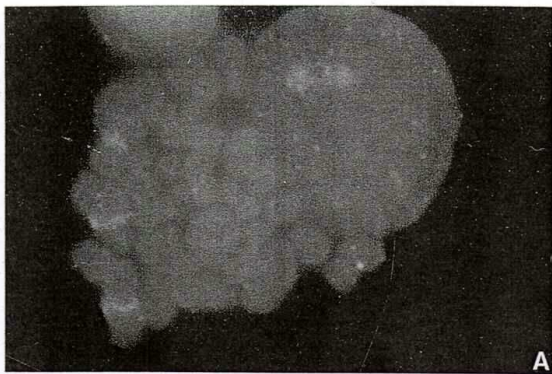
aberration was found only with ISH (two instances of trisomy, two instances of monosomy, and one instance of disomy for the Y-probe). On the other hand in the classical karyotyping procedure 5 numerical aberrations were observed (one trisomy, three monosomy, one nullisomy), which were not detected by

Table 3 Clinical data, cytogenetic analyses, and ISH results on interphase nuclei of bone marrow from patients with leukemia

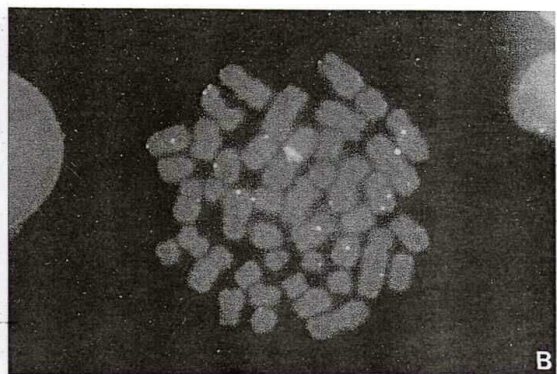
Case	Sex	Age (yr)	Diagnosis	Karyotype	Numerical and structural chromosome aberrations detected by ISH using probes for chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y.
1	M	39	CML	46,XY,t(9;22)(q34;q11),i(17q),i(9q),+8,-9,-16,-16,-17,+mar1	Trisomy 1 (65%); trisomy 8 (87%); t(1;16) (60%)
2	M	51	CML	46,XY,t(9;22)/44,Y,t(9;22),-X ^a	None
3	F	50	CML	46,XX,t(9;22)/47,XX,t(9;22),+Ph'	Trisomy 10 (31%); monosomy 16 (16%)
4	M	40	AML, M1-M2 ^b	46,XY	None
5	F	43	AML-M0	No metaphases found	Monosomy 16 (18%)
6	M	43	AML-M4	46,XY	None
7	F	41	AML-M5	46,XY,10p+,11p-, many additional nonclonal aberrations	None
8	M	44	AUL	46,XY/46,XY,t(9;22)/46,XY,t(9;22),-7,+der(7)t(7p;?)	None
9	F	44	AUL	46,XX,t(9;22)(q34;q11)	None
10	M	42	ALL	46,XY,t(9;22)/46,XY,t(9;22),dup(7q)/47,XY,t(9;22),9p+,+5,+8,-6,-7,+mar1	Monosomy 7 (65%); trisomy 8 (67%)
11	M	61	AML-M6 <RAEB.t	46,XY/44,XY,-1,-5,-7,-17,+mar1,+mar2,+2xmar3	Monosomy 1 (71%); t(7;17) (65%)
12	F	62	CMoL	46,XX	None
13	M	70	CMoL.t	46,XY/45,XY,-16/46,XY,+11,-16	Trisomy 11 (72%); monosomy 16 (97%)
14	M	47	RA	46,XY/47,XY,+8	None
15	M	58	RAEB.t	46,XY/44,XY,del(3q),+8,-5,-6	Monosomy 17 (96%); disomy Y (20%); Trisomy 8 (94%)

^a In all three cells with 44 chromosomes one X-chromosome was missing next to other different aberrations in the different individual cells.

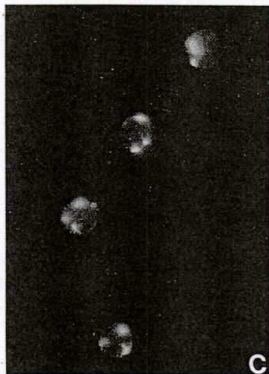
^b M0-M6 = classification according to French-American-British classification (17); RA, refracting anemia; RAEB.t, RA with excess of blast in transformation.



A



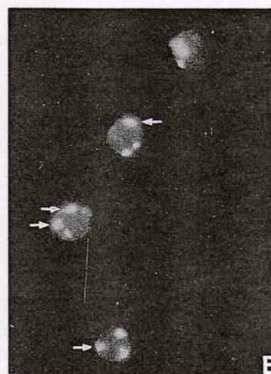
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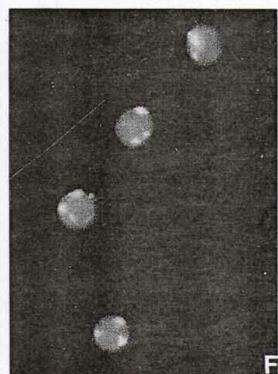
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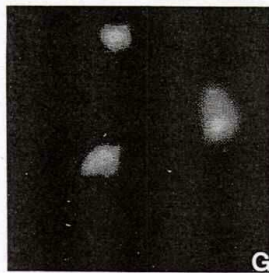
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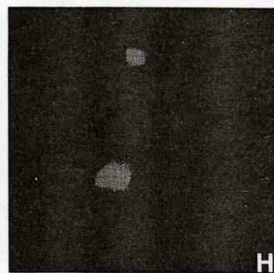
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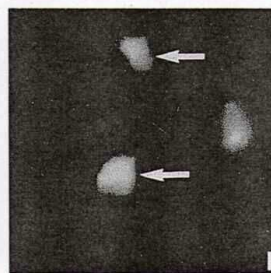
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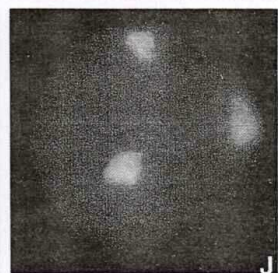
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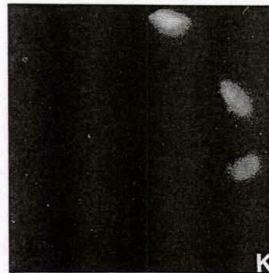
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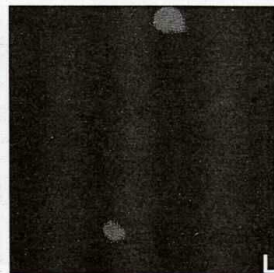
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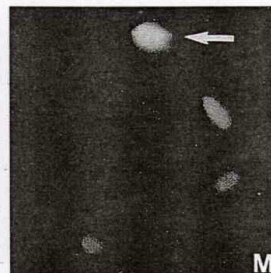
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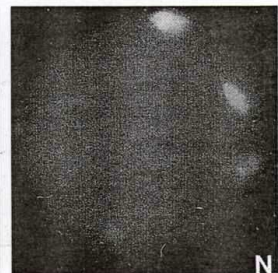
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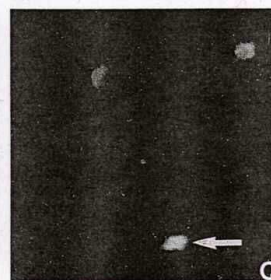
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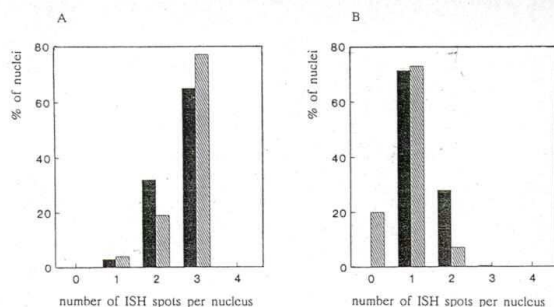


Fig. 3. Evaluation of *in situ* hybridization reactions with the probe for chromosome 1 in case 1 (A) and case 11 (B). A comparison of the spot number distribution between interphase nuclei and metaphase spreads is shown. ■, percentages of interphase nuclei with the indicated spot number; ▨, percentages of metaphase spreads with indicated spot number. In A 400 nuclei and 26 metaphase spreads and in B 400 nuclei and 15 metaphase spreads are evaluated.

ISH of interphase nuclei.

Those cases in which either the conventional cytogenetic analysis or the ISH procedure revealed aberrations for the chromosomes, detectable by the probes used in this study, will be discussed in detail.

Case 1. ISH on interphase nuclei with the panel of probes revealed significant aberrations for chromosome 1 (65% three spots) (Fig. 2, C, G, and H) and chromosome 8 (87% three spots). Trisomy 8 could be confirmed by karyotyping.

Hybridization on metaphase spreads with the probe for chromosome 1 (Fig. 2A) showed a similar number and distribution of ISH spots, as shown in Fig. 3A. The karyotype observed in the bone marrow cells of this patient was extremely complex (Table 3). The alterations of chromosome 9, 16, and 17, detected by karyotyping, were not seen by our ISH assays. However, the appearance of an i(9q) and i(17q) can explain the differences between karyotyping and ISH on chromosomes 9 and 17. Apparently these isochromosomes still contain the aliphoid centromeric regions which are recognized by the probes used. The extra ISH spot with the probe for chromosome 1 as well as the normal number of spots with the probe for chromosome 16 could be explained after double-target ISH experiments, in which we combined the DNA probes for the chromosomes 1 and 16. The results showed a colocalization of ISH signals for chromosomes 1 and 16 in approximately 60% of the interphase nuclei (see Fig. 2, C-N), suggesting a translocation between (parts of) chromosomes 1 and 16. Reexamination of metaphases hybridized with the probe for chromosome 1 showed a marker chromosome (mar1) with two or perhaps even three centromeric regions as detected by 4',6-diamidino-2-phenylindole counterstaining (Fig. 4A). This marker could, however, not be characterized as being derived from chromosome 1 and/or chromosome 16 by karyotyping.

Case 2. Cytogenetic analysis of bone marrow from this patient with CML showed 2 cell populations, although only 8 mitoses were available for examination. In 5 of the analyzed metaphases

46 chromosomes were counted, whereas in the other 3 metaphases only 44 chromosomes were counted. In these three cells an X-chromosome was missing next to other aberrations in the different individual cells. Virtually all the nuclei contained one fluorescent ISH spot after incubation with the X-probe. The fact that karyotyping showed no marker chromosomes in the latter cell populations suggested that for this case a discrepancy in the results of the two cytogenetic procedures exists. Double-target ISH on interphase cells with the probes for the chromosome X and Y showed that virtually all cells had one fluorescent signal per nucleus for each of these centromeric probes in both interphase nuclei as well as metaphase spreads. The absence of the X chromosome was probably a preparation artifact, since in these metaphase spreads also another chromosome was missing (see Table 3).

Case 3. The nuclei of this patient with CML showed a significantly different distribution for chromosome 10 in ISH (31% three spots, 65% two spots, and 4% one spot) as compared to karyotyping results. Furthermore, with the probe for chromosome 16 we detected a statistically significant percentage (16%) of cells with one fluorescent ISH spot. No numerical and/or structural chromosome aberrations for one of these two chromosomes was detected by cytogenetical analysis (Fig. 1, C and D). The ISH results could not be examined on metaphase spreads because no samples were available.

Case 7. Cytogenetic analysis of bone marrow cells from this patient with AML showed a normal number of chromosomes, but with many structural aberrations, including 10p+ and 11p-. ISH with the probes for chromosomes 10 and 11 showed that almost all cells contained two ISH fluorescent signals per nucleus (respectively, 96 and 93%).

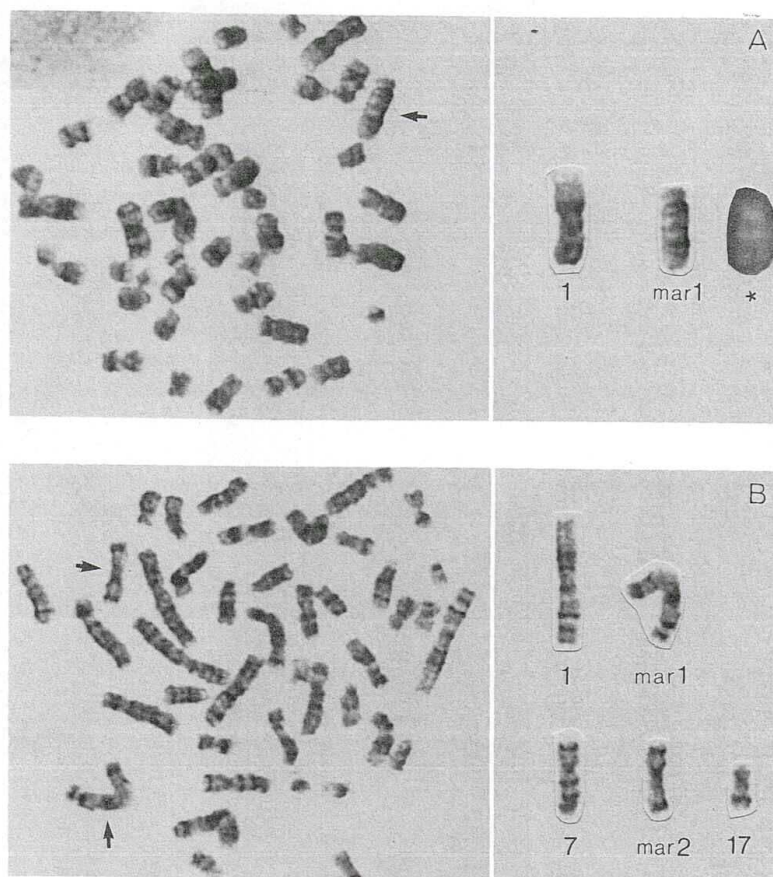
Case 8. ISH on interphase nuclei from this patient with AUL with the panel of probes revealed no aberrant distribution of spot numbers as compared to the control cells. The cytogenetic data showed a monosomy of chromosome 7, in combination with an additional marker chromosome, which contained parts of the lacking chromosome 7.

Case 10. Hybridization to interphase nuclei from this patient with ALL, using the panel of probes, showed a significant number of cells that contain one ISH fluorescent signal (65%) with the probe for chromosome 7, and three ISH signals (67%) with the probe for chromosome 8. The cytogenetic analysis also observed a significantly high number of metaphases with only one chromosome 7 and three No. 8 chromosomes (73%). As a result, in this case a good correlation between the two analytical procedures could be demonstrated.

Case 11. ISH on interphase nuclei with the panel of probes revealed that about 70% of the bone marrow nuclei from this patient with AML showed one fluorescent ISH signal with the probe for chromosome 1 (not shown). The same result was observed by ISH on metaphase spreads (Fig. 2B). The distribution of spots in interphase nuclei and metaphase spreads are shown in Fig. 3B. The cytogenetic analysis from bone marrow of this patient demonstrated two cell lines: one with a normal karyotype (46,XY, in two metaphases); and one with a complex

Fig. 2. Results of single-target (A, B) and double-target (C-R) *in situ* hybridization on interphase nuclei and metaphase spreads of specimens from case 1 (A, C-N) and case 11 (B, O-R). Blue (J, N, R) or red (A, B) counterstaining of DNA was done with 4',6-diamidino-2-phenylindole or propidium iodide, respectively. In A, three hybridization signals are demonstrated on a metaphase spread of a malignant cell from case 1 after incubation with the probe for chromosome 1. In B, one hybridization signal with probe pUC1.77 is seen on a metaphase spread of case 11, indicating a monosomy for chromosome 1. In C-N, a double-target hybridization was performed with the probe for chromosome 1 (green spots) and the probe for chromosome 16 (red spots). Colocalization of red and green spots, resulting in yellow/white signals in E, F, I, J, M, and N, suggest a translocation between a (part of) chromosome 1 and chromosome 16 (see arrow). In O-R, double-target hybridization was performed with the probe for chromosome 7 (green spots) and the probe for chromosome 17 (red spots). Again, colocalization of the red and green spots (resulting in the yellow signal in Q) indicates the translocation between chromosomes 7 and 17. The arrows in E, I, M, and Q indicate a colocalization of red and green spots.

Fig. 4. Representative partial karyotypes from case 1 (A) and case 11 (B). Cytogetic investigations were carried out from bone marrow as described in "Materials and Methods." In case 1 (A) a very complex karyotype was found (Table 3) with one marker chromosome (mar1), showing two centromeric regions in the 4',6-diamidino-2-phenylindole counterstain (*). Double-target ISH proved that chromosomes 1 and 16 were involved (see Fig. 2, C-N). In case 11 (B), one chromosome 1 was absent in 34 of 36 metaphases, while several marker chromosomes were present, as a result of alterations, for chromosomes 1, 7, 17, 20, and 21. Arrows, several marker chromosomes that were also identified by ISH.



karyotype (in 34 metaphases). A monosomy for chromosomes 1, 5, 7, 17, 20, and 21, and several marker chromosomes mar1, mar2 and 2xmar3 were found. mar1 was cytogenetically characterized with a part of chromosome 1, mar2 was a translocation between a chromosome 17 and 7p, and mar3 was a translocation product of chromosome 20p and 21.

The single-target ISH results obtained with probes for chromosomes 7 and 17 showed two spots per nucleus in, respectively, 96 and 91% of the cells. Double-target ISH with the centromeric probes for chromosomes 7 and 17, however, demonstrates the translocation between chromosomes 7 and 17 in approximately 65% (Fig. 2, O-R), as also characterized by the cytogeneticist (Fig. 4B). Double target ISH on metaphase spreads could not be performed because of lack of samples.

Case 13. ISH on bone marrow cells from this patient with MDS (CMol.t) revealed significant aberrations for chromosome 11 (3% four spots, 72% three spots, 23% two spots, 2% one spot) and chromosome 16 (97% one spot, 3% two spots) (Fig. 5, A-D). Both types of aberrations were confirmed by karyotyping. From the 19 analyzed metaphases, one had a normal chromosome number, 9 showed only a monosomy for chromosome 16, and 9 spreads revealed a monosomy for chromosome 16 and a trisomy for chromosome 11 (i.e., 93% monosomy 16, 47% trisomy 11). The lower number of cells with trisomy 11 found with the karyotyping could indicate that these

cells show a lower proliferative capacity to the other cell population(s). In this case the interphase cytogenetic analysis gives a better estimation of the real number of cells than the conventional karyotyping procedure.

Case 14. ISH on interphase nuclei from this patient with MDS (refracting anemia) using the panel of probes revealed no aberrant distribution of spot numbers as compared to the control cells. The cytogenetic analysis, however, showed two cell lines: one with a normal karyotype (46,XY), and one with a trisomy 8 (in 4 of 38 metaphases). The discrepancy between the two cytogenetic analyses is most probably due to the fact that the sample used for karyotyping was analyzed 10 years before the ISH data were obtained. To be able to perform these ISH reactions for our study, a new sample had to be used, which could not be karyotyped.

Case 15. Cytogenetic analysis of bone marrow cells from this patient with MDS (refracting anemia with excess of blast in transformation) showed two cell lines, one with a normal karyotype (46,XY; in five of the analyzed metaphases) and one with many chromosomal aberrations (44,XY, del(3q),+8,-5,-6; in five analyzed metaphases). ISH on interphase nuclei revealed a trisomy with the probe for chromosome 8 in 93% of the nuclei, and a monosomy for chromosome 17 in 95% of the tumor cells (Fig. 5, E-G). The trisomy 8 could be confirmed by karyotyping, but the monosomy 17 was in contrast to the

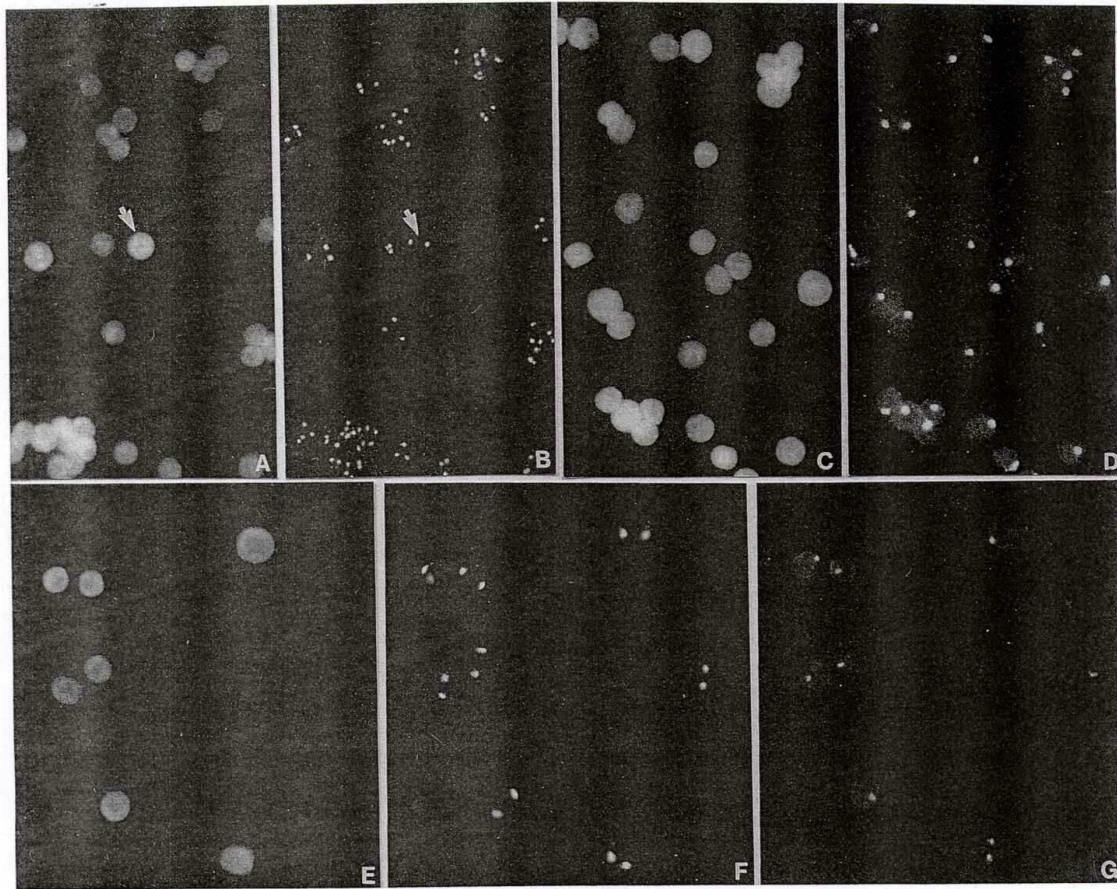


Fig. 5. Results of single-target (B, D) and double-target (F, G) *in situ* hybridization on bone marrow specimens from case 13 (A-D) and case 15 (E-G). In A, C, and E, the DNA was counterstained with 4',6-diamidino-2-phenylindole. In A and B, a significant number of nuclei showed trisomy for the chromosome 11 probe, while in C and D a significant number of interphase nuclei showed monosomy for chromosome 16. Both aberrations were also characterized by karyotyping (see Table 3). Arrow, cell with two spots per nucleus. In E-G, a double-target *in situ* hybridization procedure was performed with the chromosome 1 probe (F, two spots/nucleus) and the chromosome 17 probe (G, 95% of the nuclei show one spot), suggesting monosomy for chromosome 17.

cytogenetic analysis, where two normal chromosomes 17 were observed. Also, with the probe for chromosome Y there is a significant number of cells with two spots per nucleus, suggesting a disomy (in 20% of the tumor cells). Double-target *in situ* hybridization experiments on interphase nuclei with the X and Y probe supported these results (not shown).

DISCUSSION

Several studies have demonstrated that DNA probes, recognizing specific repetitive sequences in the (peri)-centromeric region of a particular chromosome, can be used to detect and quantify their respective chromosomes or chromosome areas in the interphase nucleus (8-16). Recently, some studies were published, comparing the interphase cytogenetic procedure using nonradioactive ISH with conventional cytogenetic analyses of cell lines derived from solid tumors, in neoplastic cells from bone marrow and peripheral blood (13-16). However, only a few DNA probes were used; furthermore, the number of specimens from patients with leukemia was rather small (13, 16). In the present study we analyzed a group of 15 randomly selected

patients suffering from leukemia with the ISH procedure, using 11 DNA probes, specific for repetitive target sequences on human chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y (see Table 1) and compared these results to data obtained through conventional karyotyping methods.

Using the ISH procedure as described in "Materials and Methods," at least 98% of the interphase nuclei of a human lymphocyte preparation, bone marrow cells, and the leukemic cells showed one, two, or three ISH signals in all preparations. After the proteolytic digestion step with pepsin, which guarantees a good penetration of the DNA probes and reporter molecules, the cells retained a good morphology, while discrete ISH signals with a high fluorescence intensity were obtained. With ISH we detected 13 numerical chromosome aberrations in 165 analyses. Seven were in good correlation with the classical karyotyping procedure (cases 1, 10, 11, 13, and 15). However, the trisomy for chromosome 10 (case 3; Fig. 1, C and D) and monosomy for chromosome 16 (cases 3 and 5), and the monosomy for chromosome 17 and disomy for chromosome Y (case 15, male) could not be confirmed by the cytogenetic

analysis, although the double-target ISH results further supported these aberrations (Fig. 5, E-G). This can be explained by the fact that the difficulties of the classical karyotyping techniques are often a consequence of the lack of mitoses and/or poor banding quality as a result of condensed or fuzzy appearance of the chromosomes (1, 2). On the other hand, in addition to the 7 correlated aberrations, 14 abnormalities for the 11 examined chromosomes were found only with the classical karyotyping. Some of these were structural aberrations, as in case 1 [i(9q) and i(17q), t(16q;16p)?], case 7 [10p+,11p-], case 8 [-7,+der(7)t(7p;?) and case 10 [9p+]. The nature of the probes used for the ISH in this study was such that they did not allow detection of these aberrations. Trisomy 8 as detected by karyotyping in case 14 and the discrepancy with the ISH results is due to the fact that the sample used for karyotyping was analyzed 10 years before the ISH data were obtained. The new sample which had been used for our ISH studies could not be karyotyped. The nullisomy for chromosome X as detected by the cytogeneticist in 3 of 8 analyzed metaphases in case 2 (male) was not in accord with the results obtained by single- and double-target ISH experiments, where one spot per nucleus for the X centromeric region was detected (94%). This finding can probably be explained by a preparation artifact, since other different chromosomes also were missing in these three individual cells. This result illustrates an advantage of the ISH procedure, in which more cells can be analyzed, as compared to karyotyping.

Although centromeric DNA probes are already able to detect numerical chromosome aberrations, our results suggest that ISH with centromeric probes is also capable of detecting structural aberrations. The translocation in case 11 between chromosomes 7 and 17 as observed by karyotyping (Fig. 4B) could also be demonstrated by ISH in interphase nuclei (Fig. 2, O-R). The trisomy for chromosome 1 in case 1 (Fig. 2A) could not be demonstrated with the classical karyotyping. Double-target ISH experiments on interphase nuclei suggested that there might be a translocation between a chromosome 16 and a chromosome 1 (Fig. 2, C-N). Reexamination of the GTG-banded metaphase spreads of this case revealed a marker chromosome which contained two or three centromeric regions, probably derived from chromosome 1 and both No. 16 chromosomes (Fig. 4A). Detection of chromosomal abnormalities in leukemia, using cytogenetic analyses on the basis of chromosome banding techniques, plays an important role in determining diagnosis, prognosis, and treatment protocols of some specific types of cancer such as CML and AML.

The DNA probes, specific for the (peri)-centromeric targets of chromosomes, enable the detection of part of a chromosome; only the copy number of the target sequence is obtained. The development of chromosome specific library probes (22-24) offers the opportunity to study structural aberrations in more detail. Also the identification of marker chromosomes and analyses of complex karyotypes may in the future be solved by using the nonradioactive ISH with centromeric probes, single copy sequences, and total chromosomal libraries.

It can be concluded that detection of numerical and structural chromosome aberrations as detected by ISH on interphase nuclei will in the future become an important additional technique to support and complement the classical karyotyping technique.

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