FLOW KARYOTYPING AND FLUORESCENCE IN SITU HYBRIDIZATION

NEW CYTOGENETIC APPROACHES FOR THE DETECTION OF LEUKEMIA CELLS

FLOW KARYOTYPERING EN FLUORESCERENDE IN SITU HYBRIDIZATIE
NIEUWE CYTOGENETISCHE BENADERINGEN TER OPSPORING VAN LEUKEMIECELLEN

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Chapter I

General Introduction

1.1. History of cytogenetics

Cytogenetic analysis of tumors has become increasingly important in relation to diagnosis, biological behavior and prognosis. Today's cytogenetic knowledge of malignant cells is based on a solid foundation obtained in a research field that has developed extremely rapidly in the last four decades.

Mitotic abnormalities were recognized as such as early as 1879 (Arnold 1879). The first, to suggest that a relation might exist between chromosome abnormalities and the transition to malignancy was Theodor Boveri in 1914 (Boveri 1914), whose observations were mainly based on observations of mitoses in sea urchin eggs. There was no way, however, to confirm his hypothesis. Though mitotic figures could be observed in biopsies from tumors, techniques to perform detailed chromosomes studies were not available. Therefore, determination of the chromosome composition was impossible.

In 1956 the correct number of the human chromosome complement was established (Tjio and Levan 1956). The increasing knowledge of tissue culture, the discovery that colchicine treatment resulted in metaphase accumulation and the observation that lectins like phytohemagglutinin (PHA) could initiate lymphocyte proliferation and thereby increase the number of mitotic cells in tissue culture (Nowell 1960) greatly facilitated cytogenetic research. The improvements in techniques were of benefit to study cancer cytogenetics. In patients with chronic myelocytic leukemia (CML) a small chromosomal marker was discovered in the metaphases of their bone marrow cells (Nowell and Hungerford 1960). This marker was called the Philadelphia chromosome. Since virtually all CML cases appeared to have this chromosomal marker it was regarded as a strong indicator for the hypothesized correlation between changes in the DNA, as visualized by chromosomal aberrations, and malignant transformation of cells. The discovery led to a more intensified search for tumor specific cytogenetic aberrations in various other tumors. However, still a major drawback was the fact that recognition of the (altered) chromosomes had to be performed on the basis of relative chromosome length and centromere index.

A new important step in the recognition of chromosomes was introduced when banding techniques became available. As a result of this, unique banding patterns could be created on chromosomes, on the basis of which individual chromosomes could be identified. The first differential staining technique of chromosomes was based on the dye quinacrine mustard (Q-banding) (Caspersson et al. 1970). This technique allowed the identification of the complete set of chromosomes in a metaphase. For example the above mentioned Philadelphia chromosome could now be identified as a reciprocal translocation between chromosomes 9 and 22 (Rowley 1973). Q-banding was soon followed by other techniques and refinements. Nowadays a number of

different banding techniques is available which allow high resolution chromosome banding (Hagemeijer et al. 1979, Yunis 1981, Testa 1984) .

These improvements have led to the establishment of a list of chromosomal defects that are non-randomly associated with several types of tumors (Yunis 1983, Sandberg 1990). The usefulness of the clinical application became evident after the development of banding techniques, in particular for the leukemias. Large clinical investigations on the prognostic significance of particular karyotypic abnormalities were undertaken (Third International Workshop on Chromosomes in Leukemia, 1981; Fourth International Workshop on Chromosomes in Leukemia, 1984)

In some types of tumors there appears to be a clear correlation between the rearrangement in the DNA, as visualized by chromosomal changes, and the onset of the malignancy (e.g. in CML). In various others, however, the relation between chromosome aberrations and the development of a tumor remains unclear. Even with the use of high resolution banding techniques there are neoplasms in which genetic alterations cannot be observed at the level of the light microscope. This indicates that it is not the chromosomal aberration per se, but the underlying genetic event that is causative for the onset of a malignancy. When changes in the chromosomes have taken place in tumor cells, they are merely a visual reflection of the fundamental changes in the DNA. In this respect the development of molecular biology became useful in the mid- seventies.

It soon became apparent that the uncontrolled growth of cancer cells is caused by a deregulation at the DNA level in which the cellular oncogenes play an important role. A number of oncogenes have been mapped near or at the breakpoints where translocations takes place. Some translocations are consistently associated with specific types of leukemia or lymphoma. (For an overview see: (De Klein 1986, Hagemeijer 1992). Because of this the expression of cellular oncogenes can be elevated, suppressed, or might result in a chimeric gene product. This can affect proliferation in a number of ways, as will be illustrated with the examples given below.

One of the first translocations in which the role of oncogenes was elucidated was the translocation t(8;14) that occurs in Burkitt's lymphoma (Zech et al. 1976). Later two other translocations were described, involving the same breakpoint on chromosome 8 translocated to chromosomes 2 or 22. (Van den Berghe et al. 1979, Bernheim et al. 1981) With each of the three translocations the breakpoint involved the c-*MYC* gene from chromosome 8 and one of the three immunoglobulin loci on chromosomes 2, 14 and 22 (for review see Croce 1993). In cells with the translocation t(8;14) the c-*MYC* gene from chromosome 8q24 is deregulated due to the juxtaposition with the immunoglobulin heavy chain (IGH) locus on chromosome 14. Exactly how the IGH locus contributes to the deregulation is unclear. One hypothesis is that IGH gene enhancers, when moved near the c-*MYC*, might stimulate inappropriate

transcription of this gene. It was demonstrated that, for each of the three types of translocations, the c-*MYC* on the unaffected chromosome 8 is transciptionally silent while the c-*MYC* gene associated with the translocation was expressed at high levels (ar-Rushdi et al. 1983, Nishikura et al. 1983) resulting in an increase of proliferation of the affected cells.

As in Burkitt's lymphoma, in low grade malignant follicular lymphoma the chromosome region 14q32 is involved in a translocation. In this case the t(14;18) translocation causes a transposition of the bcl2 gene (Tsujimoto et al. 1984, Bakhshi et al. 1985). Overexpression of this gene results in increased cell survival (Vaux et al. 1988, Nunez et al. 1990, Fairbairn et al. 1993) induced by prevention of apoptosis and creates a resistance to chemotherapy

For the myelocytic leukemias, the translocation between chromosomes 9 and 22 in CML patients (Rowley 1973) was the first in which the involvement of a cellular oncogene was described in detail. As a result of the translocation, the oncogene c-*ABL* is translocated from chromosome 9q34 to sequences on chromosome 22q11(De Klein et al. 1982, Bartram et al. 1983, Heisterkamp et al. 1983, Groffen et al. 1984) called the breakpoint clustering region (*BCR*). The fusion gene that is created in this way is capable of producing a 8.5 kb mRNA (Shtivelman et al. 1985, Grosveld et al. 1986) resulting in a 210 kD fusion protein (Konopka et al. 1984, Kloetzer et al. 1985, Konopka et al. 1985, Ben-Neriah et al. 1986). The normal c-*ABL* gene product of 145 kD has a tyrosine kinase activity with little or no autophosphorylation activity in vitro. The 210 kD fusion product from the *BCR -ABL* translocation, however, is readily phosphorylated by itself in vitro (Konopka et al. 1984, Davis et al. 1985, Konopka et al. 1985, McWhirter and Wang 1991). The uncontrolled tyrosine kinase activity, the fact that in almost all CML cases the translocation between chromosomes 9 and 22 has taken place and the observation that the introduction of the chimeric gene into mice induces leukemia (Heisterkamp et al. 1990), makes it very likely that the 210 kD fusion protein plays a role in the genesis, maintenance and/or the progression of CML.

1.2 Cytogenetics of leukemia

1.2.1 Leukemia in relation to normal hemopoiesis

The pluripotent hemopoietic stem cells provide a continuous source of differentiated cells. The development of such cells occurs in the bone marrow. A program of proliferation and differentiation has to take place to yield sufficient numbers of fully differentiated functional blood cells. The lineage map that describes the hemopoietic system is shown in Figure 1.1.[A]. The hemopoietic stem cell is capable of self renewal and gives rise to lymphoid and myeloid progenitors. The lymphoid progenitors produce cells that differentiate to fill the lymphoid compartment (T- and B-lymphocytes). The myeloid progenitors give rise to

progenitors with the capacity to differentiate in the granulocytic, erythroid, macrophage or megakaryocytic direction (CFU-GEMM).

The direction of differentiation from the pluripotent hemopoietic stem cell is driven by growth factors. Cell proliferation takes place among most of the line of differentiation, so finally a sufficient number of functional cells can be delivered to the blood circulatory system.

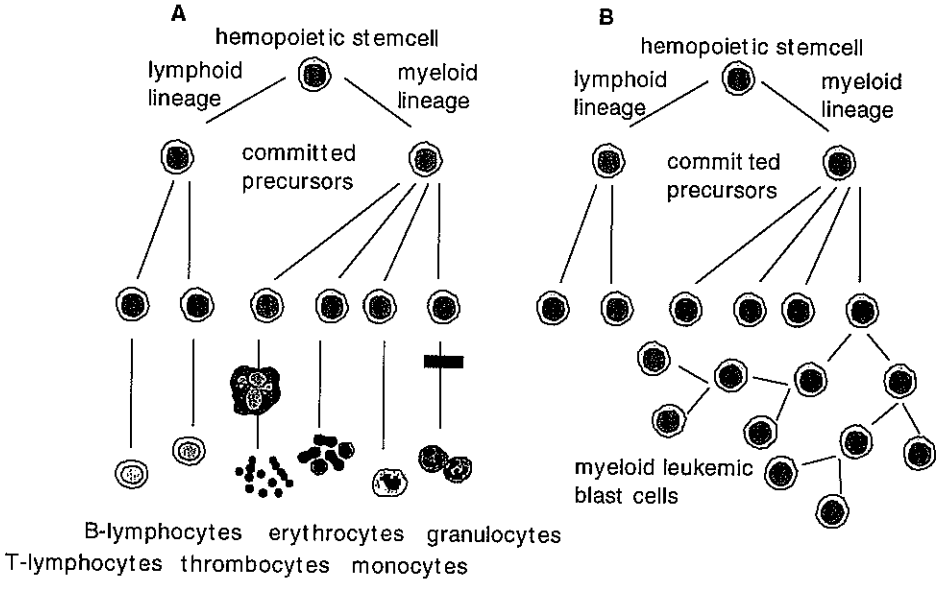

Figure 1.1
Hemopoietic lineage map with postulated leukemia development. Leukemia develops when a block occurs that prevents normal differentiation to fully functional blood cells (as indicated by a solid black bar in the differentiation pathway of granulocytes in panel A). Leukemic cells, from which a fraction is clonogenic, distort and finally suppress normal hemopoiesis (panel B).

In the case of malignant hemopoietic disorders, cells are disregulated somewhere in this process of differentiation. At this stage their further normal differentiation is blocked and uncontrolled proliferation of mainly immature cells ensues. The accumulation of these cells will disturb normal hemopoiesis and finally render patients lacking functional blood cells (see Figure 1.1B).

1.2.2 Non-random chromosome aberrations associated with leukemia

A high percentage of leukemias display non-random cytogenetic aberrations (Rowley 1994) and specific chromosomal aberrations are correlated to prognostic outcome (Arthur et al. 1989, Bloomfield et al. 1989, Pierre et al. 1989). In table 1.1 an overview is given with the most frequently recurring structural chromosomal aberrations.

Table 1.1 Recurring structural chromosomal rearrangements in hematological malignancies

disease	chromosome rearrangement	involved genes
Malignant myelocytic diseases		
Chronic myelocytic leukemia	t(9;22)(q34;q11)	*BCR;ABL*
blast crisis	t(9;22)(q34;q11),	*BCR;ABL*
	i(17q)	
Acute myelocytic leukemia		
M2	t(8;21)(q22;q22)	*ETO;AML1*
M3	t(15;17)(q22;q12)	*PML;RARA*
M4 Eo	inv(16)(p13;q22) or t(16;16)(p13;q22)	*MYH11;CBFB*
M4/M5	t(9;11)(p22;q23)	*AF9;MLL*
	t(10;11)(p11-p15;q23)	*AF10;MLL*
	t(11;17)(q23;q25)	*MLL;AF17*
	t(11;19)(q23;p13)	*MLL;ENL*
	other t(11q23)	*MLL*
	del(11)(q23)	
AML (all FAB classifications)	t(6;9)(p23;q34)	*DEK;CAN*
	t(3;3)(q21;q26) or inv(3)(q21;q26)	*?;EVI1*
	del(7q)	
	del(5q)	
	del(20q)	
	t(12p) or del(12p)	
Therapy related AML	del(7q) and/or del(5q)	*IRF1?*
	t(11q23)	*MLL;?*
	t(3;21)(q26;q22)	*EAP/MDS1/EVI1;AML1*
	der(1)t(1;7)(q10;p10)	
Malignant B-lymphocytic diseases		
Acute lymphocytic leukemia		
Pre-B	t(1;19)(q23;p13)	*PBX1;TCF3(E2A)*
B(SIg+)	t(8;14)(q24;q32)	*MYC;IGH*
	t(2;8)(p12;q24)	*IGK;MYC*
	t(8;22)((q24;q11)	*MYC;IGL*
B or B-myelocytic	t(9;22)(q34;q11)	*BCR;ABL*
	t(4;11)(q21;q23)	*AF4;MLL*
Other		
	t(5;14)(q31;q32)	*IL3;IGH*
	del(9p),t(9p)	
	del(12p),t(12p)	
non-Hodgkin's lymphoma		
Burkitt type	See SIg+ALL	*MYC;IGH;IGK;IGL*
Follicular	t(14;18)(q32;q21)	*IGH;BCL2*
Mantle cell	t(11;14)(q13;q32)	*CCND1;IGH*

Malignant B-lymphocytic diseases (continued)		
Diffuse large cell	t(3;14)(q27;q32)	*BCL6;IGH*
	t(10;14)(q24;q32)	*LYT10;IGH*
Chronic lymphocytic leukemia	t(11;14)(q13;q32)	*CCND1;IGH*
	t(14;19)(q32;q13)	*IGH;BCL3*
	t(2;14)(p13;q32)	*ICH*
	t(14q)	
Multiple myeloma	t(11;14)(q13;q32)	*CCND1;IGH*
Malignant T-lymphocytic diseases		
Acute lymphocytic leukemia	t(1;14)(p32;q11)	*TAL1;TCRD*
	t(11;14)(p15;q11)	*RBTN1;TCRA*
	t(11;14)(p13;q11)	*RBTN2;TCRA*
	t(8;14)(q24;q11)	*MYC;TCRA*
	inv(14)(q11q32)	*TCRA;IGH*
	t(10;14)(q24;q11)	*HOX11;TCRA*
	t(1;14)(p34;q11)	*LCK;TCRD*
	t(7;9)(q35;q32)	*TCRB;TAL2*
	t(7;9)(q35;q34)	*TCRB;TAN1*
	t(7;7)(p15;q11)	*TCRG;?*
	t(14;14)(q11;q32)	*TCRA;IGH*
	t(7;14)(q35;q11)	*TCRB;TCRD*
	t(7;14)(p15;q11)	
Non-Hodgkin lymphoma		
T	see T-cell ALL	
	t(14;16)(q26;p13.1)	*IL2;BCM*
T or B (Ki-1+)	t(2;5)(p23;q35)	*NMP;ALK*
Chronic Lymphocytic Leukemia	t(8;14)(q24;q11)	*MYC;TCRA*
	inv(14)(q11q32)	*TCRA/D;IGH*
Adult T-cell leukemia	t(14;14)(q11;q32)	*TCRA;IGH*
	inv(14)(q11q32)	*TCRA/D;IGH*

Data derived from Rowley 1994

Through cloning of breakpoints more insight is provided into the nature and biological behavior of the hemopoietic disorders, which in turn may influence the choice of optimal treatment strategies. For example: the involvement of c-*MYC* causes aggressive growth in a number of T cell leukemias and Burkitt's lymphoma, while the involvement of the bcl-2 gene in low grade malignant follicular lymphoma(t(14;18) causes a growth pattern that is correlated with restriction of apoptosis. Another well documented example is the t(15;17) translocation in AML M3 which affects the retinoic acid receptor. In these patients effective treatment is achieved by administration of high dosis all-trans-retinoic acid.

1.3 New cytogenetic techniques

The analysis of metaphase chromosomes is time-consuming work performed by skillful and well trained scientists. Automation as developed by slide scanning and computer assisted analysis was and is performed with moderate success. The 'eye of the master' is still required to accurately analyze the individual metaphases. In a large number of routine cytogenetic laboratories therefore, this type of automation has not lead to a major breakthrough with respect to resolution, speed or productivity. Quite a different approach of analyzing chromosomes has been found in the area of flow cytometry.

1.3.1 Flow karyotyping

Flow cytometry enables one to measure particles with a size ranging from approximately 0.1 to 100 micrometer one by one at a relatively high speed, i.e. in the order of thousands of particles per second. These particles pass one or more laser beams one by one in a fluid jet stream (Figure 1.2).

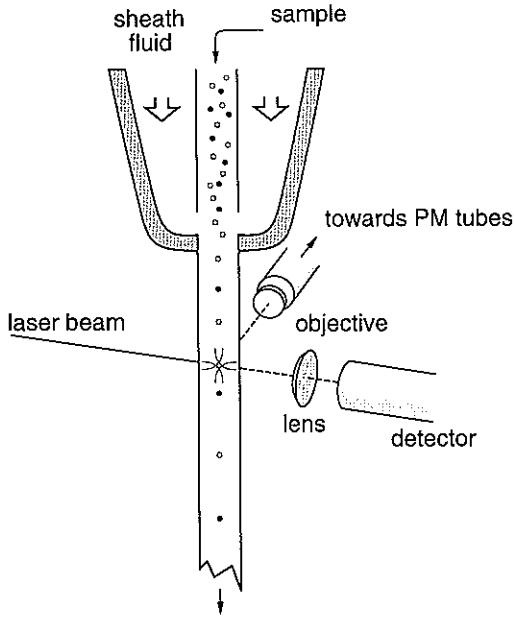

Figure 1.2
Principle of flow cytometric measurement. Particles from the sample are focused hydro-dynamically in the nozzle. One by one they pass one or two lasers at high speed. At the laser interception points light scatter and fluorescence parameters provide information about size, complexity and amount of dye bound.

PM = photomultiplier

When the particles pass the laser beams, scattering of laser light in the near forward and the perpendicular direction can be measured thus providing information about size and complexity of the particles. Fluorescent dyes can be bound to the particles that can than be excited by the laser light. The emitted fluorescence will provide additional information. Another important aspect is the possibility to sort out

particles on the basis of light scatter and fluorescence properties. In the nozzle compartment a crystal, vibrating at a frequency of 20 to 40 kHz, drives controlled droplet break-off. When a particle, that meets the specified light scatter and/or fluorescence criteria that are set by the operator, traverses through the fluid stream to the point where droplet break-off occurs the droplet in which it is enclosed will be electrically charged. On its way down the droplet will traverse through a static electrical field and subsequently be deflected. (See Figure 1.3)

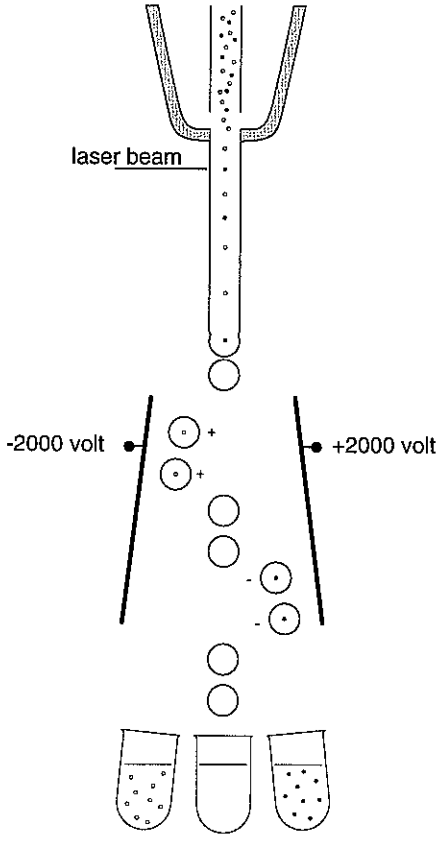

Figure 1.3
Principle of flow sorting. Particles enclosed in droplets can either be sorted to the left or the right when the droplets are charged and thereby deflected when they pass the electro-magnetic field created by the deflection plates.

Deflected droplets are collected in sample tubes, resulting in highly purified subpopulations of the original sample.

1.3.1.1 Univariate flow karyotyping

Studies have show that colorimetric quantitation of dye binding is indicative for the nuclear DNA content (Den Tonkelaar and Van Duijn 1964). Differences occur when variations in the compactnes in DNA leads to variations in dye binding (Mayall 1969, Mayall and Mendelsohn 1970, Dreskin and Mayall 1974). For chromosomes a relation between size and DNA content was demostrated using Feulgen stained metaphase

preparations (van der Ploeg et al. 1974b, van der Ploeg et al. 1974a). Theoretically the constantness of DNA content per particle (i.e. chromosome or nucleus) will lead to uniform patterns when suspension are run through a flow cytometer. Using fluorescent dyes the DNA content of cell nuclei could be determined by flow cytometry (Van Dilla et al. 1969). These developments greatly influenced studies concerning tumor aneuploidy (Barlogie et al. 1982, Raber et al. 1982, Tanke et al. 1983, Cornelisse et al. 1984). The finding that DNA content of nuclei or whole cells could relatively easy be analyzed with flow cytometry by the use of fluorescent DNA dyes soon led to flow cytometric analysis of DNA content of individual chromosomes in suspension using this technique (Gray et al. 1975b, Stubblefield et al. 1975).

Large chromosomes will bind relatively high amounts of dye compared to the smaller ones. Upon excitation by laser light the emitted fluorescence is a measure for the amount of bound dye and thereby indicative for the chromosome size. The amount of fluorescence obtained from a chromosome suspension measured by flow cytometry can be plotted in a so called univariate flow karyogram. One axis (horizontal) shows the fluorescence intensity and the other (vertical) shows the relative number of chromosomes. Depending on the variation within the chromosome population, chromosomes with identical fluorescence intensities (i.e. with approximately the same length and therefore, with the same amount of dye bound) will show up as unique histogram peaks in the univariate plot. Figure 1.4 shows what the result would be of such a measurement when chromosomes from a rat leukemia cell line are analyzed.

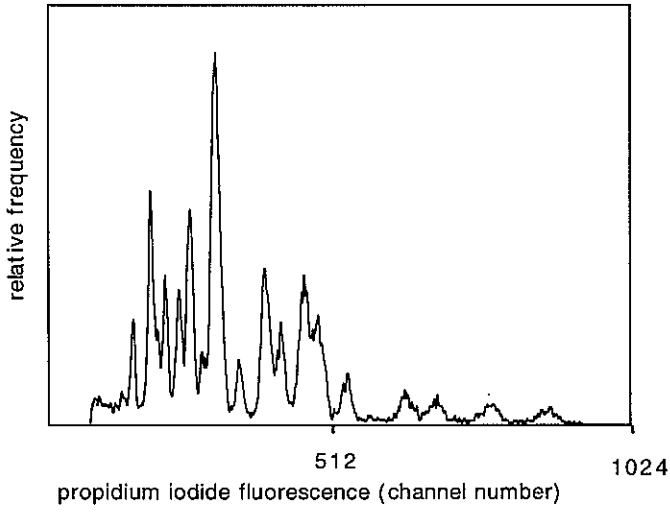

Figure 1.4
Fluorescence distribution of chromosomes from rat leukemia cells (LT-12) stained with the fluorescent dye propidium iodide (PI). The size of each individual chromosome is reflected by the amount of PI fluorescence.

The chromosomes were obtained from the LT-12 cell line. Details concerning the content of the various peaks are discussed in Chapter III.

Several dyes which bind to DNA can be used in flow cytometry. Ethidium bromide (2,7-diamino-9-phenyl-10-ethyl phenanthridinium bromide) (EB) and propidium iodide (3,8-diamino-5-diethylmethylaminopropyl-6-phenyl phenanthridinium diiodide) (PI) intercalate in the DNA without base pair specificity. Because of this property the resulting fluorescence is indicative for the total amount of DNA in the chromosome. For flow karyotyping these dyes are almost exclusively used for univariate single dye measurements.

1.3.1.2 Bivariate flow karyotyping

There are two variables that determine the amount of fluorescent dye that can be bound per chromosome, i.e. the relative length and the base pair ratio of each chromosome. Chromosomes that have corresponding DNA lengths might differ in the base pair ratio and can be discriminated as such by using base pair specific fluorescent dyes.

Hoechst 33258 (2-(20-(4-hydroxyphenyl)-6-benzimidazole)-6-(1-methyl-4-piperazyl)benzimidazole) (Ho) has a high specificity for adenine-thymidine (A-T) base pairs in the DNA. Equivalent to Ho, the dye DAPI (4'-6-bis (2'-imidazolinyl-4H,5H)-2-phenylindole) binds to regions that are rich in A-T base pairs (Latt and Wohlleb 1975, Lin et al. 1977). Absorption and emission spectra of both dyes are very similar. For flow cytometry excitation can be performed with UV laser light (351-364 nm). Emission maxima of both dyes are around 450 nm.

Chromomycin A3 (CA3) and mithramycin, which are antibiotics, bind preferentially to areas that are rich in guanine-cytosine base pairs (Ward et al. 1965). For this non-intercalating binding to the DNA magnesium ions are required. CA3 and mithramycin, which have identical binding properties and fluorescence absorption and excitation spectra, are frequently used as fluorophores in flow cytometry. Excitation of the dyes can be performed at a wavelength of 458 nm and the broad emission spectrum has a peak around 580 nm. In the work described in this thesis CA3 fluorescence was measured using 500 nm or 550 nm long pass filters.

In order to be able to simultaneously measure differences in base-pair ratio employing the above mentioned dyes, at least two different wavelengths are required. By equipping a flow cytometer with two lasers such multi-parameter analyses can be performed (Shapiro et al. 1977). Staining suspensions of chromosomes with A-T and G-C specific dyes like HO and CA3 in combination with dual laser-beam flow cytometry thus enables to obtain information about chromosome size and base pair ratio of each individual chromosome (Figure 1.5).

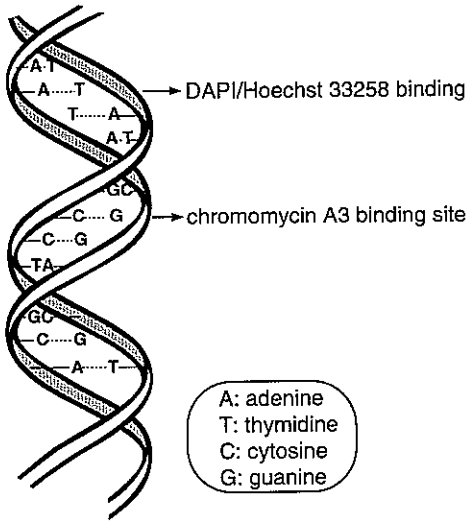

Figure 1.5
Binding of two base pair specific dyes. Hoechst 33258 or DAPI will bind to areas in the chromosome that are A-T rich; chromomycin will bind to areas with relatively high amounts of G-C base pairs

Fluorescence intensities can be plotted in a so called bivariate flow karyogram with CA3 fluorescence on the horizontal axis and Ho or DAPI fluorescence on the vertical axis (Figure 1.6). The distribution of the fluorescence intensities in a bivariate flow karyogram will produce a species specific pattern. Figure 1.6 shows the bivariate distribution of human chromosomes obtained from a healthy male individual. Differences between homologue chromosomes are a known phenomenon from standard cytogenetics. In many cases the differences that appear between homologues give rise to two clusters instead of one, representing a chromosomal pair in the bivariate flow karyogram (indicated as a and b in Figure 1.6). Since there are 22 autosomal chromosome pairs and the individual homologue chromosomes of at least 8 pairs show a considerable degree of variation in size, the resulting karyotype will be almost a unique hallmark for the individual.

Flow karyotyping is a method par excellence to detect small differences in DNA content and composition between the individual chromosomes. The relative positions of the chromosomes can be observed easily while differences down to 1% in DNA content can be measured.

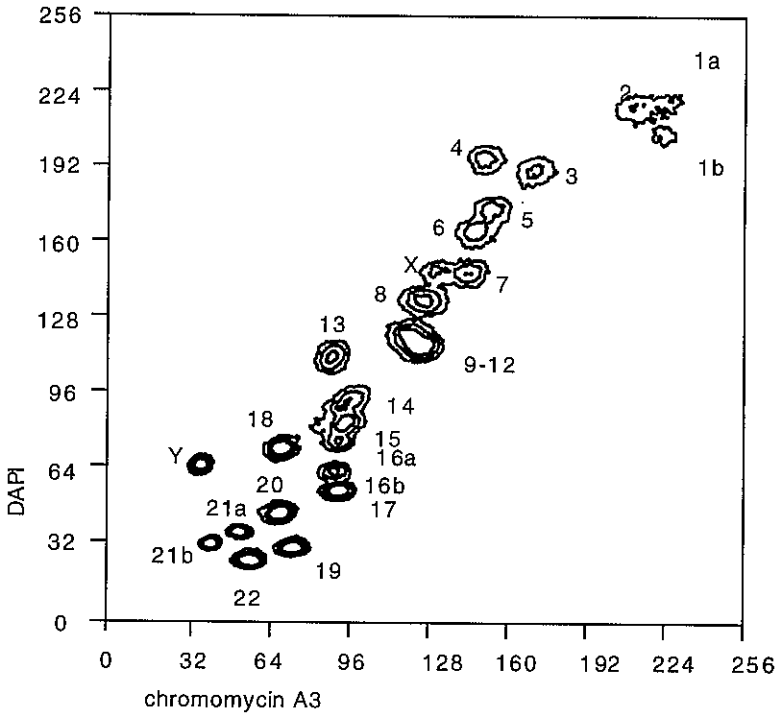

Figure 1.6
Bivariate flow karyogram from a normal male (peripheral blood lymphocytes were stimulated with PHA). Each cluster is indicated by the number of the chromosome that it represents. In several cases individual homologues of chromosome pairs can be seen (as indicated by a and b).

Flow cytometric analysis of chromosomes in suspension has developed during the last decade as a useful tool for various purposes. Analysis of the chromosomal constitution of a given cell type can be performed easily and rapidly (Bartholdi et al. 1984, Arkesteijn et al. 1986, Harris et al. 1987)]. High speed sorters have been developed to facilitate sorting experiments (Peters et al. 1985). After sorting of intact or translocated chromosomes hybridization with specific probes can provide information about the localization of certain genes (Lebo et al. 1984, Collard et al. 1985). Highly purified chromosomal DNA can be obtained for the construction of chromosome specific libraries (Davies et al. 1981, Lalande et al. 1984, Gray et al. 1987). Chromosome painting (see section 'Fluorescence in situ hybridization'; this Chapter) is one of the methods that has recently found widespread applications mainly because of the availability of flow-sorted chromosomes.

In this thesis the applicability of flow karyotyping as a diagnostic tool in clinical leukemia samples is investigated. In cases where the chromosomal aberrations result in a sufficient loss or gain of DNA, their presence is indicated by changes in the

chromosome clustering pattern: one or more clusters appear or disappear in the bivariate flow karyogram resulting in an abnormal pattern. A typical example is the translocation involving chromosomes 9 and 22 in case of CML which can perfectly well be demonstrated by means of flow karyotyping (Arkesteijn et al. 1988).

1.3.2 In situ hybridization

In situ hybridization (ISH) has become a powerful technique to localize chromosome specific nucleic acid sequences in the cell. With this technique nucleic acid sequences can be visualized using radioactive or non-radioactive detection. In particular (non-radioactive) fluorescence in situ hybridization (FISH) has found wide application in various fields of research because of its high spatial resolution and its ability to detect multiple targets. The development of FISH started with the covalent coupling of fluorescent labels directly to RNA (Bauman et al. 1980, Bauman et al. 1981), and evolved in a number of direct or indirect labeling techniques. Visualization of the hybridized probe-target sequences can be performed by the use of various DNA probe modification methods including: 2-acetylaminofluorene (AAF) modification (Landegent et al. 1984, Tchen et al. 1984) mercuration (Hopman et al. 1986b, Hopman et al. 1986a), biotinylation (Langer et al. 1981), and digoxigenin labeling (Herrington et al. 1989a, Herrington et al. 1989b, Martin et al. 1990).

Using FISH, specific nucleic acid sequences could be located on chromosomes and in nuclei (Cremer et al. 1986, Pinkel et al. 1986, Trask 1991, Jenkins et al. 1992 and others). Fluorescent detection of specific alpha satellite repetitive probes enables the recognition of the centromeres on the chromosomes as clearly localized and brightly fluorescent spots in metaphase spreads or in nuclei. This provides the means to enumerate the copy number of chromosomes. The number of centromeres present in the cell is reflected by the number of spots per nucleus. (Figure 1.7)

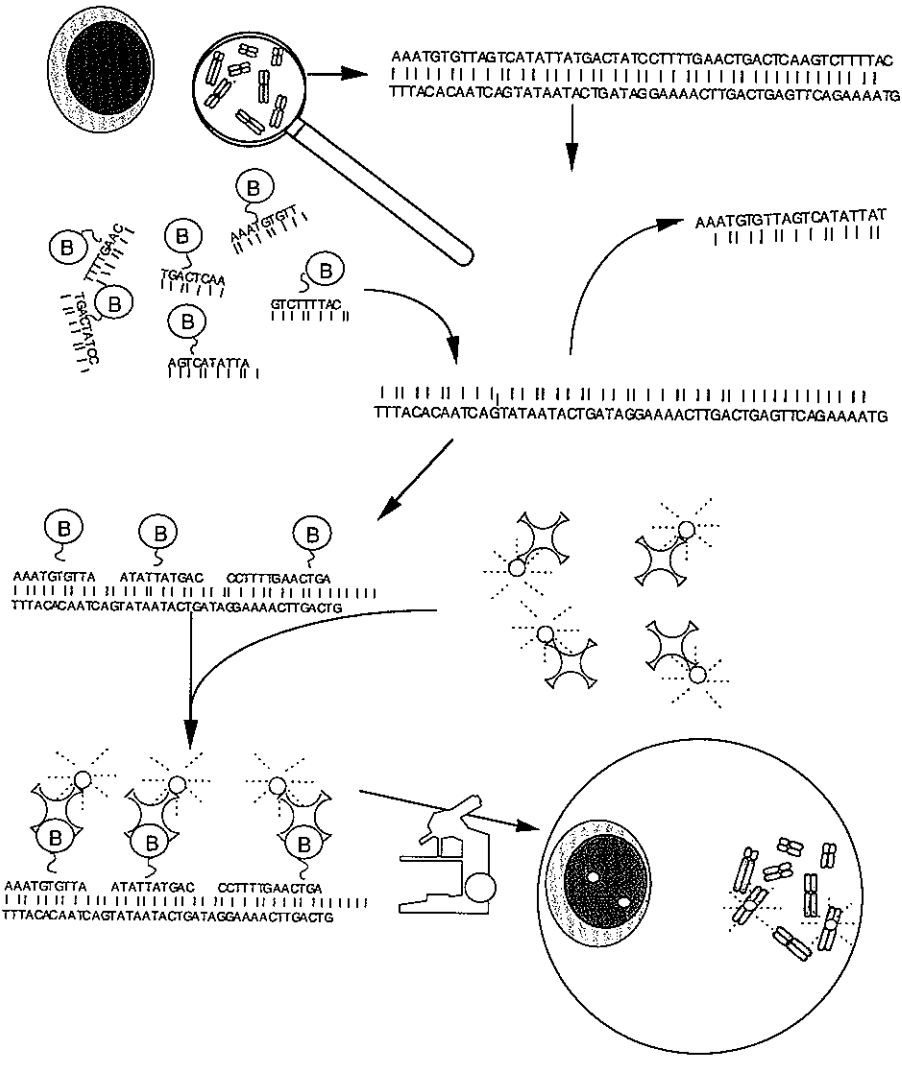

Figure 1.7
Principle of fluorescence in situ hybridization (FISH). DNA in nuclei or chromosomes is denatured without loss of nuclear or chromosome morphology. Hybridization takes place with complementary labeled DNA. The labeled DNA is visualized by binding to fluorescently labeled conjugate. Microscopic inspection reveals the presence of fluorescent spots in nuclei or chromosomes indicating the domains of probe binding. In this figure, B stands for biotin. The labeled probe is detected using strong and specific avidin-biotin binding properties. The avidin can be labeled with fluorescein.

Numerical aberrations have been detected in this way in malignant cells (Cremer et al. 1986, Devilee et al. 1988b, Hopman et al. 1988, Anastasi et al. 1990, Van Dekken et al. 1990b, Van Dekken et al. 1990c, Jenkins et al. 1992, Pagliaro and Stanley 1993).

Interphase FISH has several advantages that make it an attractive alternative for conventional cytogenetic analysis. No short term cultures are required, analysis of the metaphases and recognition of the banded chromosomes is not necessary. Due to the fact that interphase nuclei, that are derived directly from the patient, are studied, selective outgrowth of subpopulations during short term culture is prevented and a representative percentage of aberrant cells in the patient is obtained. The number of nuclei that can be analyzed routinely with FISH is at least tenfold higher than the number of metaphases that is routinely analyzed with conventional cytogenetic analysis. Therefore, FISH allows rapid quantitative analysis and follow-up of patient material from diagnosis through complete remission.

Numerical chromosomal aberrations have been associated with a number of leukemias. An overview is given in Table 1.2.

Table 1.2 Recurring numerical chromosomal aberrations in hematological malignancies

disease	numerical aberration
Chronic myelocytic leukemia (blast crisis)	+8, +19, +Ph
Acute myelocytic leukemia (M1-M7) incl. MDS	-Y, +4, -5, -7, +8, +9, +11, +13, +21, +22
Acute lymphocytic leukemia	-20, +21, 50-60 chromosomes
Adult T-cell leukemia	+3
Chronic lymphocytic leukemia	+12
Non-Hodgkin's lymphoma	-Y, +3, +12, +X
Myeloproliferative disorder	-12, -Y, +8, +9, +21

Data derived from Mitelman et al. 1991, Dewald et al. 1993b, Rowley 1994

In 17% of the AML cases and 1% of the ALL cases a karyotype is found with 41-45 chromosomes. In 22% of the AML cases and 42% of the ALL cases a karyotype is found with a chromosome number higher than 46. In CML in blast crisis numerical chromosomal changes reach up to 70% (Data obtained from Heim and Mitelman 1987, Sandberg 1990) .

Cells can be classified as aberrant and hence assigned to a malignant subpopulation on the basis of the loss or gain of hybridization sites in the cells, reflected by less or more fluorescent spots. The detection of small numbers of cells bearing numerical aberrations using the FISH procedure might be hampered by the fact that the lower detection level is set by the natural occurrence of normal cells which display an aberrant number of spots. This has been shown in normal cell populations from healthy volunteers (Poddighe et al. 1991).

Multiple simultaneous hybridizations have been performed (Nederlof et al. 1989, Nederlof et al. 1990). This approach offers the possibility to reduce the threshold level of FISH, assuming that one hybridization occurs independent from the other, thus

reducing the chance of double false hybridization signals in one cell. In patients who underwent a sex-mismatched allogeneic bone marrow transplantation (SMM allo-BMT) the presence or absence of donor and host cells is reflected by the presence or absence of the corresponding sex chromosomes. With FISH, chimerism has been demonstrated using the sex chromosomes as markers (Durnam et al. 1989, Van Dekken et al. 1989, Przepiorka et al. 1990, Przepiorka et al. 1991, Bernasconi et al. 1993, Dewald et al. 1993a, Wessmann et al. 1993). The presence of X and Y chromosomes in one cell are unambiguously correlated to each other and therefore, this group of patients is the ideal target for chimerism studies with double colour FISH.

Besides numerical aberration, structural aberrations can be observed in interphase cells using fluorescence in situ hybridization (FISH). One of the most frequently applied ways to do so is to employ probes that are specific for chromosome regions adjacent to the chromosomal breakpoint. The probes that are specific for either side of the breakpoint are labeled differently and visualized using two different fluorescent labels. A fluorescence microscope equipped with double band-pass filters allows the simultaneous observation of the fluorescent labels. This enables one to discriminate between nuclei in which all fluorescent spots are at a distance from each other (such cells do not carry a translocation) and nuclei in which two spots with different labels are in such close proximity to each other that a translocation is likely to have occurred. The best example of this is the use of probes specific for the *ABL* and *BCR* regions on chromosomes 9 and 22. Probes were labeled with FITC and Texas red The Philadelphia translocation could be detected in interphase cells by the close proximity of one green and one red fluorescent spot. (Arnoldus et al. 1990, Tkachuk et al. 1990).

An important application of the in situ hybridization technique is the possibility to visualize whole chromosomes. In this procedure a composition of DNA sequences is used that originate from one unique chromosome type. Using this DNA as a probe, chromosomes can be visualized in metaphases and interphases. This technique is usually referred to as 'chromosome painting' (Pinkel et al. 1988). Highly purified chromosomal DNA that can be obtained after flow sorting of chromosomes serves as a source for chromosome painting probes. Such DNA used to be amplified by cloning into bacterial vectors but currently, the polymerase chain reaction (PCR) is used for this purpose. Hybridization of probes generated in this way requires that non-specific chromosomal DNA is blocked before one unique chromosome set can be visualized. This is performed by chromosome in situ suppression (CISS) hybridization (Landegent et al. 1987) and utilizes excess unlabeled repetitive DNA sequences. These are allowed to prehybridize to the labeled non-specific sequences that also comprise for a large part of repetitive elements. The remaining single-stranded labeled elements hybridize specifically to the chromosomes. In situ hybridization with DNA libraries from sorted human chromosomes has improved identification of marker

chromosomes (Cremer et al. 1988a, Cremer et al. 1988b, Lichter et al. 1988, Pinkel et al. 1988, Brothman and Patel 1992).

1.4 Minimal residual disease (MRD)

1.4.1 Definition of MRD

To learn more about the development of leukemia, to be able to monitor its growth in patients and to adapt adequate treatment strategies, the discrimination of leukemic cells from their normal counterparts is crucial. At diagnosis this is generally not a problem. Patients present with symptoms that are the result of a lack of sufficient functional blood cells and show a blood or bone marrow picture with an overload of immature blast-like cells. However, upon treatment the number of leukemic cells will often be reduced to an amount that escapes conventional detection by the light microscope. Leukemia relapse is a major problem after remission-induction chemotherapy or after allogeneic and autologous bone marrow transplantation. Low numbers of leukemic cells survive the treatment and grow out to cause a relapse in a later stage. This situation is referred to as 'minimal residual disease'. The amount of minimal residual leukemic cells that can be detected in a bone marrow sample depends on the method that is used (as reviewed by Hagenbeek, 1992).
Figure 1.8 shows the levels of detection for a number of the currently available techniques.

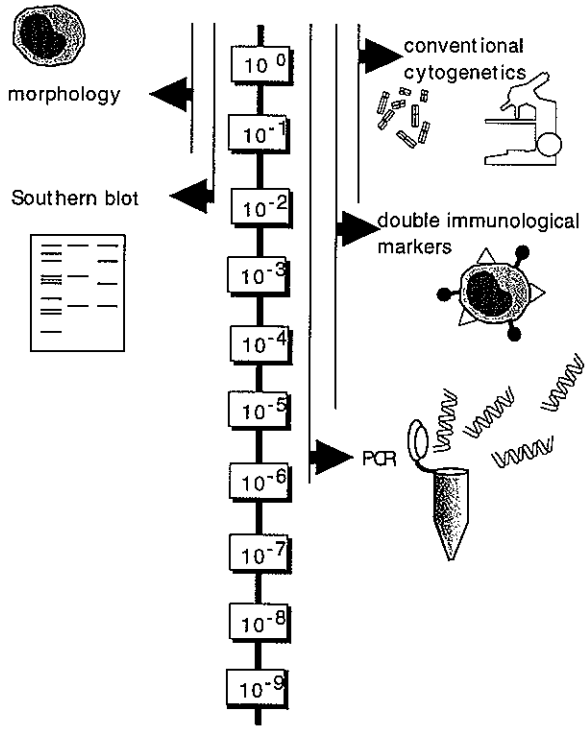

Figure 1.8
Levels of detection of leukemic cells in human leukemia.

1.4.2 Pitfalls in the detection of MRD

New detection methods are aimed at the reduction of the detection level of leukemic cells. Detection of remaining leukemic cells enables one to predict at an early stage whether to proceed or modify treatment strategies and in general to learn more about disease progression at the stage of minimal residual disease. Factors like sample size, technique or lack of homogeneity of cell distribution, determine the limits of MRD detection.

First of all MRD detection is restricted by the sample size. With decreasing numbers of residual leukemic cells in a patient a sample of increasing size has to be taken to be able to detect leukemia. A bone marrow aspiration is a 'sample' from the complete cell compartment of the patient which only allows an estimation of the percentage of leukemic cells in the blood or bone marrow compartment.

Secondly, the type of detection method used determines to a great extent the limit of detection. Besides the sensitivity that can theoretically be reached, the number of

cells that can be processed in one test is a limiting factor. Irrespective of the sensitivity of the technique, the detection level will be 'one in the sample size' when the number of cells that can be processed in one experiment is smaller than the theoretical detection level.

Finally, even in a situation where there is a considerable amount of leukemia regrowth, successful detection of leukemic cells depends on the site where the bone marrow was collected. Focal regrowth of leukemia in bone marrow can cause considerable false negative results in bone marrow aspirations or biopsies. The heterogeneic distribution of MRD was studied in detail in an acute myelocytic leukemia model in the Brown Norway rat (BNML) (Martens et al. 1987, Martens et al. 1990).

1.5 The scope of the thesis and organization of the chapters

The central question underlying the research as described in this thesis was whether the cytogenetic information that is carried by the cell (i.e. the chromosomal aberrations associated with the malignant cells) could contribute to an improvement of the detection of leukemic cells. Methods have been developed to obtain cytogenetic information on a large scale.

Bivariate flow karyotyping was applied to an animal model of leukemia as well as to clinical leukemia samples. This method offers the ability to analyze the chromosomes of a large number of cells in an objective and statistically relevant way. Using the preclinical animal model, the stage for further research was set by demonstrating that flow karyotyping is a useful tool for the recognition of leukemia. In situations where leukemia-associated chromosomal aberrations are found (like it is the case of BNML) leukemia type chromosome patterns can be discriminated from normal flow karyograms. (Chapter III).

In Chapter IV this is further investigated in blood and bone marrow samples obtained from patients with CML. In this chapter it is demonstrated that chromosomal aberrations like the Philadelphia chromosome can be made visible using bivariate flow karyotyping and that quantitative analysis can be performed.

The aspect of specific stimulation of subpopulations of cells is further worked out in Chapter V. Bone marrow or blood from leukemia patients was investigated but, depending on the type of stimulation, bivariate flow karyograms from either healthy or leukemic cells were obtained. This indicates that a careful choice of stimulating factors has to be made. It further implies that a quantitative analysis of the number of leukemic cells in the original sample cannot be made. In this chapter it is shown that supplementary quantitative information can be obtained in the form of FISH on interphase cells from the same samples.

To quantify the number of leukemic cells in the bone marrow in much greater detail, FISH on interphase cells was introduced (Chapter VI). In this study the feasibility of the FISH procedure for the follow-up of leukemia patients was examined. Firstly, the FISH procedure was optimized and the performance of the probes and the background frequencies of "aberrant" peripheral blood cells in normal individuals was determined. Secondly, for the follow-up study, patients with numerical chromosomal aberrations in their leukemic cells, as judged by conventional cytogenetic analysis, were selected. They were followed from the time of diagnosis, through the phase of complete remission (detection of MRD) to relapse, if this occurred. In a third study patients were followed who underwent sex-mismatched allogeneic BMT (SMM allo-BMT) on the basis of the presence or absence of donor- or recipient sex chromosomes to determine the degree of chimerism. For this purpose two probes, that were detected with different fluorescent labels, were used simultaneously.

In an attempt to further reduce the detection level of aberrant cells in samples from patients with leukemia it was investigated whether FISH and flow cytometry could be combined. Chapter VII describes how nuclei were hybridized in suspension and subsequently analyzed by flow cytometry. A double hybridization was performed, which allowed the identification of cells as being leukemic or not. Hybridization combined with the ability to sort populations of hybridized cells allowed to improve the detection level to 1 in 250.000.

Finally, in Chapter VIII the main conclusions of the research in this thesis are described and the possible future applications are discussed.

Chapter II

Materials, methods and technical developments

In this thesis each of the following chapters is preceded by a description of materials and methods. This chapter describes in detail how the most important methodologies were developed and used.

2.1 Chromosome studies by flow karyotyping

Flow karyotyping does not differ from conventional karyotyping with regard to the fact that metaphase chromosomes are required. The amount of chromosomes, however, that is needed to obtain a high resolution flow karyogram is much higher. While conventional cytogenetics require approximately 10 to 30 metaphases to determine a karyotype, a flow karyogram is composed of at least 20,000 chromosomes which resembles an equivalent of approximately 450 mitotic cells.

2.1.1 Cell cycle and proliferation

To obtain and study chromosomes cells have to be actively in cell cycle. Going through the cell cycle step by step, the following stages can be discriminated (Figure 2.1). Shortly after cell division cells are in a G1/G0 state. In this stage of the cell cycle, the cellular machinery is set to produce RNA and proteins. No DNA synthesis occurs. Though the difference between G1 and G0 is morphologically not clearly recognizable, it is generally accepted that cells can only traverse through the cell cycle from G1. In both G1 and G0 the amount of DNA is constant in time and designated as 2n (where n is the haploid amount of DNA). At the end of G1, cells enter the synthesis- or S-phase in which RNA and protein production is reduced to a minimum and DNA synthesis takes place. During the period of S-phase the amount of DNA in the cells is increasing gradually. The S-phase ends when the amount of DNA in the cell has doubled as compared to G1. This situation is called G2. In this stage cells contain 4n DNA and they are ready to divide the DNA into two 2n over the two daughter cells.

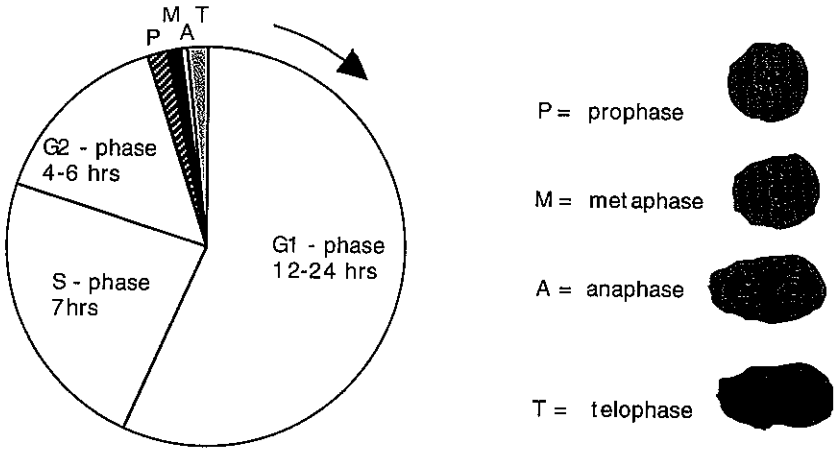

Figure 2.1
The various stages of the cell cycle. Four subsequent stages of the mitotic process are shown. Metaphase is the stage in which chromosomes can be studied.

The cell division starts with disappearance of the nuclear membrane and the condensation of the nuclear DNA into separate chromosomes (referred to as prophase). Microtubuli are being assembled that will finally align the chromosomes at the equatorial plane. This particular stage is called metaphase. The chromosomes consist of two chromatids which are separated and then move towards the opposite poles of the cell (referred to as anaphase). Over the years several models have been proposed that describe the way in which this mechanism takes place (for review see Rieder and Salmon 1994). The two chromatid clusters decondense again while a new nuclear membrane is formed around the two clusters. With the formation of a cell membrane between the two new nuclei the cell cycle is completed. Two daughter cells have been formed, ready to continue in G1 or to leave the cell cycle (temporarily) to enter G0. The duration of the cell cycle varies very considerably from cell type to cell type. Only in a relatively short period of the cell cycle, cells are in metaphase and chromosomes can be studied or isolated. In an average cell cycle, G1 takes 12-24 hours, S-phase 7 hours, G2 4-6 hours. Mitosis takes 1 hour, from which the metaphase is only a part.

To study chromosomes, two requirements must be fulfilled. The first one is to stimulate cells to enter the cell cycle. The second is to accumulate the number of cells in metaphase and extend their period of staying in this state.

2.1.2 Mitotic index

In populations of cells or cell lines that are characterized by a high proliferative index, a high number of cells will continuously pass the mitotic phase in cell cycle. This will result in a high mitotic index (= number of metaphases per 100 nuclei). Leukemic cells in freshly obtained bone marrow samples that were used for flow karyotyping, however, needed to be specifically stimulated to obtain cells in mitosis. Leukemic cells respond to a number of growth factors by proliferation (Griffin et al. 1986, Delwel et al. 1987, Vellenga et al. 1987).

Optimal stimulation of proliferation of leukemic cells depends on subtle differences varying from sample to sample. Since most of the studied cases in this thesis represent newly obtained (diagnostic) material, detailed information about the optimal dosing of the various growth factors was unknown. Therefore it was attempted to find a protocol to which the majority of the leukemic cells would respond by proliferation. For this purpose the leukemias from the myeloid lineage were stimulated by the addition of granulocyte macrophage-colony stimulating factor (GM-CSF) and/or interleukin-3 (IL-3). Recombinant GM-CSF (Behring Werke Ger.) and IL-3 (Biogen, Willemstad, Curacao, Netherlands Antilles) or conditioned media derived from the GCT cell line (DiPersio et al. 1978, DiPersio et al. 1980) were used. GCT cells express IL-1 alpha, IL-1 beta, IL-6, macrophage colony-stimulating factor (M-CSF or CSF-1), GM-CSF and transforming growth factor beta (TGF-beta) as well as both A and B chains of platelet-derived growth factor (PDGF) (Liesveld et al. 1993). It was attempted to stimulate acute lymphocytic leukemia cells with phorbol ester (TPA) or interferon-gamma. Unstimulated and phytohemagglutinin (PHA) stimulated cultures were performed in both myelocytic and lymphocytic leukemias. Furthermore, in a number of cases cell samples were cultured in the presence of a conditioned medium derived from the gibbon T-cell line MLA-144 that produces abundant amounts of IL-2 (Rabin et al. 1981). Cells were grown in alpha modification of minimal essential medium (alpha MEM; Flow Laboratories Irvin, Scotland, UK) at a concentration of 10^6 cells per ml. The medium was supplemented with 2mM L-Glutamine, Penicillin, Streptomycin and 10% fetal calf serum (FCS).

The main objective of the study was to obtain chromosomes in suspension. From the cell suspensions a limited number of additional assays could be performed; i.e. determination of cell recovery after culture and the mitotic index. On the basis of cell counting by eosin exclusion, 10^6 cells per ml were set up in culture flasks (Falcon). The number of living cells was counted after 3 days of culture using eosin exclusion. A cell sample was examined by microscope using a Bürker type hemocytometer. Cells that had not taken up the dye were classified as being alive. The number of living cells was compared to the number of cells that was plated at day zero. The

results, expressed in percentage recovery of living cells after 3 days, are displayed in Table 2.1.

Table 2.1 Recovery of living cells after 3 days of in vitro culture

	AML			ALL			CML		
	n	rec. (%)	stdev	n	rec. (%)	stdev	n	rec. (%)	stdev
GM-CSF	10	**51.0**	30.2						
IL-3+ GM-CSF	39	**79.8**	93.2	5	**72.5**	57.5	14	**59.7**	31.0
GCT-CM (BM)	14	**85.1**	63.6	2	**39.9**	28.1	14	**78.1**	49.1
GCT-CM (PBL)	7	**99.5**	117						
MLA-CM	9	**111.3**	99.6	2	**62.8**	9.5	5	**51.7**	29.3
MLA+GCT	10	**84.8**	84.0				4	**126.8**	118.8
IFN				1	**74.8**	-			
TPA				2	**103.6**	20.2			
IFN +TPA				2	**228.8**	196.9			
PHA	58	**56.5**	47.0	17	**66.8**	30.6	31	**45.6**	31.1
blank	11	**63.6**	43.4	9	**59.4**	41.3	6	**67.8**	42.6

n = number of cases
rec. = recovery
stdev = standard deviation
AML = acute myelocytic leukemia
ALL = acute lymphocytic leukemia
CML = chronic myelocytic leukemia
BM = bone marrow
PBL = peripheral blood lymphocytes

GM-CSF = Granulocyte-Macrophage colony stimulating factor
IL-3 = Interleukin 3
GCT-CM = Giant Cell Tumor conditioned medium
MLA-CM = a Gibbon T-cell line conditioned medium
IFN = Interferon-gamma
TPA = 12-O-tetradecanoylphorbol-13-acetate
PHA = Phytohemagglutinin
blank = no additions

The results given in this Table indicate that at the end of the culture period in almost every case the total number of cells was lower than at the beginning. The loss of cells during culture can be explained by cell death and adherence to the culture bottle. Although the net cell production is often negative, dividing cells are present in most of the cultures as is reflected by the mitotic index after three days (Table 2.2).

Table 2.2 Mitotic index of the various cultures of leukemia samples

	AML			ALL			CML		
	n	mi	stdev	n	mi	stdev	n	mi	stdev
GM-CSF	6	2.0	1.2						
IL-3+ GM-CSF	14	2.0	2.1	5	1.3	1.5	8	3.4	3.3
GCT-CM	14	3.6	3.1				5	6.4	6.6
MLA-CM	9	1.8	1.7	1	0.6	-	1	2.0	-
MLA+GCT	9	3.3	2.8				2	6.3	1.3
MLA-CM+PHA				2	12.0	10.0			
IFN +TPA				1	0	-			
PHA	14	5.1	5.6	5	3.1	4.2	14	4.4	4.3
PHA enriched	6	7.3	3.9						
blank	12	1.6	1.9	3	2.2	2.2	3	1.6	1.2

Abbreviations as in Table 2.1

mi = mitotic index (number of metaphases/ 100 nuclei)

enriched = T-cell rich fraction from the SRBC rosetting assay

On average the specifically stimulated samples had a higher mitotic index than the controls (blank). Stimulation aiming at specific proliferation of leukemic cells resulted in mitotic indices that were highest in AML and CML when GCT-CM was added; for ALL this was the case with the addition of MLA-CM + PHA. With respect to the number of samples that was tested and the observed standard deviations, the data must be interpreted with care. Chromosomal aberrations were found in none of the ALL cases when processed by flow karyotyping. Therefore, the high mitotic index of ALL cells stimulated with MLA-CM + PHA most likely reflects proliferation of remaining normal lymphocytes.

T-cells were depleted from the bone marrow samples by sheep red blood cell (SRBC) rosetting. In some cases cells could be recovered from SRBC rosettes. These were regarded as enriched T-cell fractions. These cells were also stimulated using PHA (indicated in the table as PHA enriched).

2.1.3 Mitotic arrest

The second requirement is to 'catch' as many cells as possible in mitosis by the use of a spindle poison. Treatment of the cells with a mitotic spindle poison will result in a blockade in the assembly of the microtubuli, the main component for the formation of the spindle apparatus. When spindles are not formed, the chromatids cannot be separated and cells remain in metaphase. The chromatin remains condensed and visible as separate chromosomes in the cells. Isolation of the mitotic cells and release

of the chromosomes in suspension for the purpose of flow karyotyping is discussed in detail in following sections.

In this thesis Colcemid or Tubulazole-C were used as spindle poisons in a concentration of 250 ng/ml for 4 or 16 hours. Theoretically a long blockade (16 hours or longer) by a spindle poison will result in an accumulation of a higher number of mitotic cells than a short blockade (2-4 hours). However, prolonged incubation with a spindle poison could lead to the negative side effect of chromatid separation which is particularly unfavorable for flow karyotyping. This will be discussed in section 2.1.7

2.1.4 Mitotic enrichment by gradient centrifugation for flow karyotyping

From cells that do proliferate only poorly, a sufficient number of mitotic cells can only be obtained when the initial number of cells that was set up in culture was high enough. If such cells grow attached to the surface of the culture dish the mitotic cells can be selectively detached by shaking off. Culturing sufficient numbers of cells will yield a sufficient number of almost pure mitotic cells. Problems arise when mitotic cells cannot be obtained selectively from a culture (e.g. cells growing in suspension). Upon lysis of the cells, only the few mitotic cells will deliver chromosomes in suspension, while all cells will produce particles (organelles, membrane fragments or nuclei). This will unfavorably influence the chromosome/debris ratio. Additional steps have to be taken to improve the quality of the final flow karyogram.

To enhance the final concentration of mitotic cells it was investigated whether mitotic cells could be separated from their interphase counterparts on the basis of their density. For this purpose the in vitro growing BNML cell line LT-12 was used. This cell line grows in vitro in suspension, and it yields sufficient numbers of mitotic cells.

LT-12 cells were set up in culture and after 3 days the spindle poison Tubulazole-C (Janssen Pharmaceutics, Beerse, Belgium) was added at a final concentration of 250 ng per ml. The cells were allowed to accumulate in mitosis for an additional 4 hours after which they were harvested and processed for density gradient centrifugation. The desired gradient densities were obtained by dissolving Nycodenz (Nyegaard, Oslo, Norway) in Hanks HEPES Buffered Balanced Salt Solution (HHBBSS) with 1% bovine serum albumin (BSA). The pH was adjusted to 6.8 and the osmolarity was brought to 280 mOsm. All solutions were checked for their correct density on a digital densitometer (DMA 40; Mettler/Paar, Graz, Austria) at 4 C. Discontinuous gradients were composed in 4 ml plastic tubes (Falcon 2058) by layering 1 ml per density on top of each other. The cell suspension was layered on top of this gradient and spun down at 700 G at 4 C for 20 minutes. The various fractions were collected and washed twice with cold HHBBSS (4 C). The mitotic index and the cell number of

the fractions was determined. Mitotic indices indicate that in this in vitro growing cell line it was possible to selectively isolate the mitotic cells from the interphase cells on the basis of their density (Table 2.3).

The first column shows the densities of which the gradient was composed. The second column shows the mitotic index of the cell samples recovered from the various densities. Mitotic LT-12 cells have a lower density as compared to interphase cells. The highest percentage of mitotic cells was found in the fractions obtained from 1.050 and 1.060. From the total cell sample 64% could be recovered from the various fractions. The material recovered from the bottom of the centrifuge tube consisted of debris and dead cells. Proper cell counting could not be performed on this material. A slightl increase in density of 1.062 was employed throughout the rest of the experiments described in this thesis

Table 2.3 Mitotic enrichment of LT-12 cells on a discontinuous density gradient

density	mitotic index (%)	% of total cells
before	12	100
1.050	98	1
1.060	94	9
1.070	10	22
1.080	1	32
bottom	-	-

density: in g/cm^3
before = cells before gradient centrifugation
bottom = cells on the bottom of the tube after gradient centrifugation

An increased mitotic index by density gradient centrifugation is not the only reason for an improvement of the quality of the flow karyogram. Dead cells do not sustain the pressure evoked by the centrifugal force and will sediment on the bottom of the tube together with debris particles. Figure 2.2 shows for the LT-12 cells how the suspension can be monitored before and after centrifugation on a discontinuous density gradient. It shows cells before density gradient centrifugation (a), and after gradient centrifugation on densities 1.062 (b) and 1.080 (c). Panel d shows the material that was recovered from the bottom of the centrifuge tube.

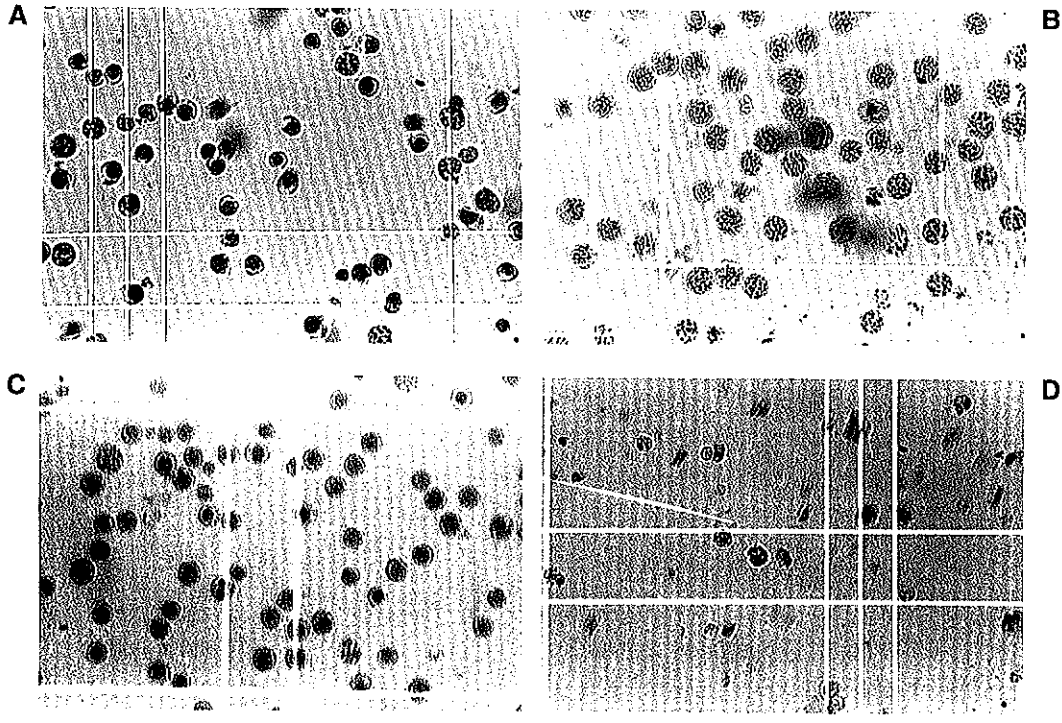

Figure 2.2
Determination of the mitotic index. Cells were recovered from a discontinuous density gradient.
Panel A) Cell sample before gradient
Panel B) Cells recovered from fraction 1.062
Panel C) Cells recovered from fraction 1.080
Panel D) Cells from the bottom to the tube
Cells are stained with Türk's solution and observed in a Bürker type hemocytometer at a magnification of 40X.

The mitotic enrichment of human leukemia cells after gradient centrifugation was determined by counting mitotic cells in each layer of the gradient. Table 2.4 is an extension of Table 2.2 and shows the mitotic index on each density.

Table 2.4 Mitotic index of the various cultures of human leukemia samples at the densities 1.062 and 1.080 g/cm^3 after gradient centrifugation.

		AML			ALL			CML		
	Density	n	mi	stdev	n	mi	stdev	n	mi	stdev
GM-CSF	before	6	**2.0**	1.2						
	1.062	6	**1.8**	1.3						
	1.080	6	**0.4**	0.6						
IL-3+ GM-CSF	before	14	**2.0**	2.1	5	**1.3**	1.5	8	**3.4**	3.3
	1.062	14	**5.1**	5.3	5	**1.5**	2.0	8	**10.8**	16.4
	1.080	14	**0.7**	1.1	5	**1.8**	2.2	8	**2.0**	2.3
GCT-CM	before	14	**3.6**	3.1				5	**6.4**	6.6
	1.062	14	**5.9**	4.0				5	**8.3**	7.0
	1.080	14	**1.3**	4.0				5	**1.0**	1.6
MLA-CM	before	9	**1.8**	1.7	1	**0.6**	-	1	**2.0**	-
	1.062	9	**4.2**	2.7	1	**5.5**	-	1	**11.0**	-
	1.080	9	**1.3**	1.4	1	**1.7**	-	1	**0**	-
MLA-CM+GCT	before	9	**3.3**	2.8				2	**6.3**	1.3
	1.062	9	**5.8**	5.1				2	**48.0**	49.5
	1.080	9	**1.3**	1.7				2	**0.6**	0.9
MLA-CM+PHA	before				2	**12.0**	10			
	1.062				2	**12.1**	12.7			
	1.080				2	**3.1**	1.7			
IFN +TPA	before				1	**0**	-			
	1.062				1	**0**	-			
	1.080				1	**0**	-			
PHA	before	14	**5.1**	5.6	5	**3.1**	4.2	14	**4.4**	4.3
	1.062	9	**4.8**	5.1	5	**2.5**	3.0	14	**4.5**	6.9
	1.080	9	**1.3**	1.7	5	**4.9**	7.0	14	**3.0**	3.5
PHA enriched	before	6	**7.3**	3.9						
	1.062	6	**5.7**	6.2						
	1.080	6	**5.7**	5.1						
blank	before	12	**1.6**	1.9	3	**2.2**	2.2	3	**1.6**	1.2
	1.062	12	**7.2**	16.8	3	**3.8**	3.5	3	**8.1**	13.1
	1.080	12	**1.0**	1.0	3	**0.2**	0.4	3	**1.0**	1.8

abbreviations as in table 2.2
density in g/cm^3

Two densities were chosen (i.e. 1.062 and 1.080) on the basis of the results obtained with the rat leukemia model. It has to be stressed that the various types of human leukemias (and most probably even each individual leukemia) have different densities on which mitotic cells settle. To investigate the optimal density for each

sample would have been too time-consuming and would result in an unfavorable distribution of one limited bone marrow sample over too many density fractions. The rat densities were taken as a general guideline.

2.1.5 Chromosome isolation

Cells in log phase were blocked in mitosis with Tubulazole-C or Colcemid (Calbiochem, San Diego, Ca) as described earlier. The suspensions were washed twice in HHBBSS. From the suspension 25 µl was taken and mixed with 25 µl of water and placed at 37°C. After 5 minutes of swelling, the sample was mixed with 50 µl of Türk's solution and counted in a Bürker type hemocytometer. In this way interphase and mitotic cells could easily be discriminated and the mitotic index determined (Figure 2.2).

To increase the percentage of mitotic cells and removal of dead cells and cell debris, cell suspensions were processed by discontinuous density gradient centrifugation as described above.

The chromosome isolation method described by Bijman (Bijman 1983) was modified to obtain high resolution flow karyograms in clinical samples after the discontinuous density gradient centrifugation. Cells from each separate fraction were centrifuged once more in HHBBSS, the supernatant discarded and the inside of the tube dried as much as possible with a paper tissue. The cell pellets from the fractions were resuspended in chromosome isolation buffer consisting of 20mM NaCl, 8mM $MgCl_2$ and 20mM Tris-HCl pH 7.5 and placed at 37°C for 5 minutes. The swollen cells were lysed by addition of an equal volume of a Triton X-100 solution (0.8% in water) to the chromosome isolation buffer. Subsequently chromosomes were released in suspension by careful shearing through the tip of an Eppendorf pipette. The lysed suspensions were centrifuged for 3 minutes at 150 G. In this way a large fraction of the nuclei could be removed. The supernatant which contains the chromosomes was transferred to a new tube.

Chromosomes were stained at least 2 hours prior to measurements with Propidium Iodide (PI) (10 µg/ml) for univariate measurements or with Hoechst 33258 (Ho) DAPI (5.4 µM) and chromomycin A3 (CA3) (26 µM) for bivariate analysis on a dual-laser beam flow cytometer. In most cases chromosome suspensions were stored at -20°C after the addition of DMSO (10% final concentration). Frozen chromosome suspensions could be stored for weeks, defrosted and refrozen again without visible loss of resolution in the flow karyogram.

2.1.6 Instrumentation for flow cytometry

Uni- and bivariate analyses were performed using the RELACS III, a flow cytometer specially designed and built at the Radiobiological institute of TNO, Rijswijk, The

Netherlands, to measure chromosomes in suspension (Stokdijk et al. 1985, Van den Engh and Stokdijk 1989). The chromosome suspension was introduced into the cuvette by the use of a perfusion pump that was modified to hold the 1 ml syringes that contain the chromosome suspensions. The pump enabled careful control of the rate of injection of the chromosome suspension in between 1.5 to 29.0 µl per minute into the core of the sheath fluid stream.

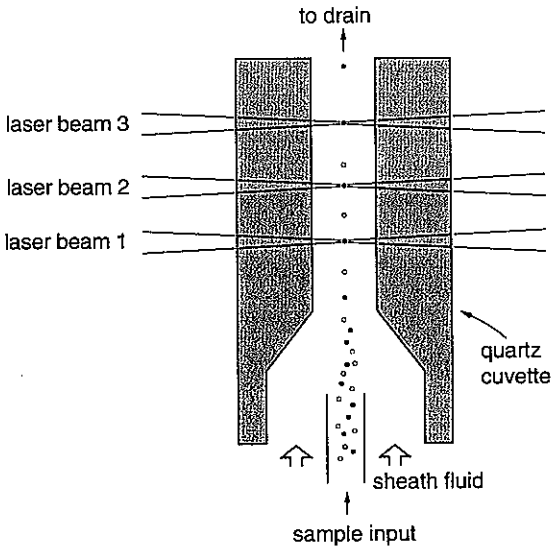

Figure 2. 3
Schematic representation of the RELACS III quartz cuvette. From the three lasers laser 1 and 2 were used for chromosome measurements.

The fluid and sample streams move upwards instead of downwards. Hydrodynamic focusing of the chromosome sample is ensured by the special shape of the quartz cuvette (Figure 2.3). Filtered water (Milli-Q; Millipore, Molsheim, France) was used as sheath fluid. The throughput of the sheath fluid (rate between 8 to 12 ml per minute) was controlled by a peristaltic pump which ensured a minimum of fluctuation and enabled careful and reproducible adjustment so that optimal hydrodynamic focusing of the chromosome suspensions at the laser-interception points was achieved.

The sample was usually run through the flow cytometer at a rate ranging from 20 to 800 chromosomes per second, depending on the concentration of chromosomes in the suspension.

The RELACS III was designed to operate with 1 to 3 lasers (Figure 2.4). For the purpose of bivariate analysis of chromosome suspensions two lasers were directed on the cuvette with two spherical lenses which can be moved independently. The two lasers intersect the cuvette at approximately 200 µm distance from each other. The capacity to move the lens in three dimensions enabled one to steer the laser

beam and to focus it on the cuvette at the measuring point. The lens units create oval shaped spots at the point where the chromosomes are measured. Signals generated at the measuring point in the cuvette are also spatially separated by a set of pinholes behind the cuvette with a diameter of 1 mm. Mirrors are placed at different heights to reflect the light signals of the lasers separately as is shown in Figure 2.4. Up to seven photomultipliers can be used to measure the signals. Due to the special optical design, the RELACS III is a very sensitive flow cytometer with high resolution. Therefore, the machine is very suitable for measurements of chromosomes and determination of flow karyograms. The electronic and data processing parts of the machine are described in detail by Van den Engh and Stokdijk (Van den Engh and Stokdijk 1989).

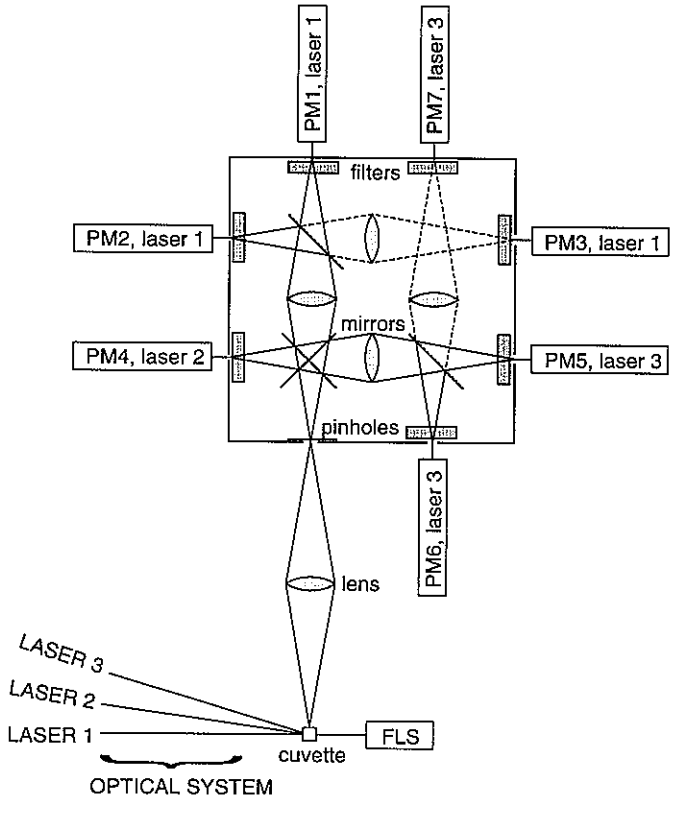

Figure 2.4
Simplified schematic representation of the RELACS III lay-out (top view). For bivariate chromosome measurements two lasers were used.

PM =Photomultiplier
FLS =Forward light scatter detector

PI was excited with an argon ion laser tuned at 488 nm, 500 mW (laser: Coherent Innova 90, Palo Alto, CA, USA). PI fluorescence was measured through two KV 550 filters (Schott, Glaswerke, Mainz, Ger.). Ho or DAPI was excited with an argon ion laser tuned in the UV range (351 nm and 364 nm; laser: Spectra Physics, Series 2000,

Mountain View, CA, USA) at 350 mW laser power. Ho or DAPI fluorescence was measured using two KV 408 filters (Schott). Chromomycin-A3 (CA3) was excited at 458 nm (laser: Coherent Innova 90) at 200 mW. CA3 fluorescence was measured through two 550 nm longpass filters (LL550; Corion Corp., Holliston, Ma., USA).

The fluorescence signal generated by the first laser was used to trigger the electronic pulse processing system. Chromosome measurements were performed by analysis of either pulse heights or pulse integration. Data of approximately 20,000 chromosomes were stored in list mode and subsequently analyzed using ELDAS software (R.R. Jonker and L. Budel; unpublished). For univariate analysis at least 3 parameters were stored (i.e. fluorescence, forward light scatter and time of flight). Bivariate analysis requires that at least 4 parameters are stored. The analysis of relative peak content (i.e. the calculation of the number of events within a cluster) was performed with rectangular windows around the area of interest. Only the events in the window were taken into account ('simple boxing').

2.2 On the presence of separate chromatids in chromosome suspensions

At several occasions during flow karyotyping, a clustering pattern was observed that could not be explained by the presence of chromosomal aberrations. This phenomenon was observed to varying degrees in a number of clinical leukemia specimens. It also appeared to be present in the in vitro growing LT-12 rat leukemia cell line (derived from the BNML) which is employed for preclinical model studies of human acute myelocytic leukemia (Hagenbeek and Van Bekkum 1977, Van Bekkum and Hagenbeek 1977). The BNML as well as the LT-12 cell lines are cytogenetically well defined and the bivariate flow karyogram is thoroughly studied (Arkesteijn et al. 1987). In an attempt to find an explanation, in this section this phenomenon is studied in detail.

2.2.1 Materials and methods

Cell cultures

Cultures of the in vitro established BNML cell line LT-12 were set up at a concentration of 5 x 10^5 cells/ml. Cells were grown in alpha MEM supplemented with penicillin and streptomycin and 5% of FCS. Cell cultures were placed in a humidified incubator which was constantly gassed with 5% CO_2/95% air. In the phase of exponential growth (after 3 to 4 days) cell cultures were treated with Colcemid or Tubulazole-C in various concentrations (ranging from 50 to 500 ng/ml) and incubation times (4 and 16 hours).

Nucleated peripheral blood cells from patients with leukemia were cultured similarly for 4 days at a concentration of 2 x 10^6 cells/ml in the presence of 10% (v/v) of conditioned medium containing GM-CSF.

Preparation of chromosome suspensions for flow karyotyping

Chromosome suspensions were prepared as described in section 2.1.5. In brief, after the mitotic blocking period the mitotic cell fraction was enriched using a discontinuous density gradient as described in section 2.1.4 on all samples. After a swelling period of 10 minutes at 37°C in a buffer containing Mg^{++} ions (8mM), NaCl (20mM) and Tris-HCl (20mM) cells were lysed using an equal volume of Triton X-100 solution (0.8% in water). Chromosomes were stained directly after lysis with Hoechst 33258 (Ho; 5.4 μM) and chromomycin A3 (CA3; 26 μM) at least 2 hours prior to measurements for analysis on a dual-laser beam flow cytometer.

Flow karyotyping

Chromosome suspensions were measured on a dual-laser beam flow cytometer as described in section 2.1.6 The bivariate cluster patterns were compared to the standard average configuration obtained from cell cultures blocked for 4 hours with Tubulazole-C, to detect disappearing or newly arising clusters. Quantitative analysis was performed by calculating the number of events per cluster in each flow karyogram with the aid of ELDAS software by means of 'simple boxing' with square windows.

2.2.2 Results

Figure 2.5 shows the bivariate distribution of human chromosomes from a male CML patient. Mitotic cells were obtained after incubation of the cells for 4 hours with 250 ng/ml Tubulazole-C. As a result of the translocation t(9;22) 2 clusters appear in the karyogram that are not present in karyograms from normal human cells (compare with Figure 1.7). The appearance of a bivariate flow karyogram from CML patients is discussed in detail in Chapter IV.

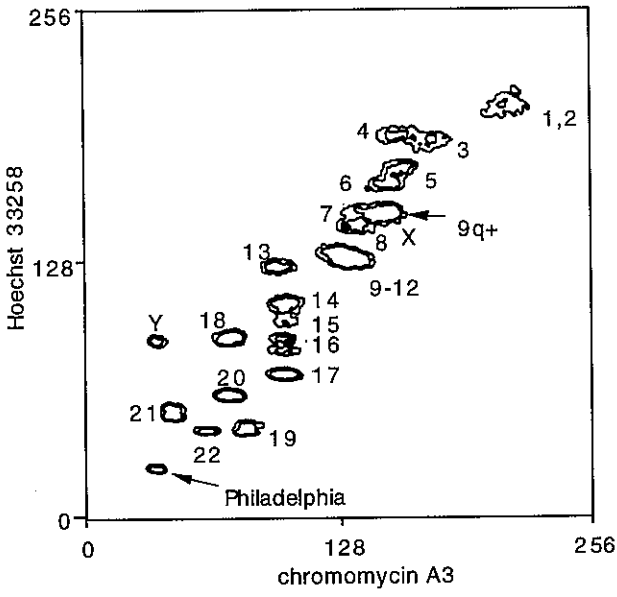

Figure 2.5
Bivariate distribution of chromosomes isolated from peripheral blood cells of a male CML patient. The aberrant clusters caused by the t(9;22) translocation are indicated by arrows.

This bivariate flow karyogram was included for reasons of comparison with the one that is shown in Figure 2.6. In the last figure, the bivariate distribution is shown from chromosomes derived from cultured peripheral blood cells of another CML patient. To obtain chromosomes, the cells from this patient were incubated with 250 ng/ml Tubulazole-C for a prolonged period of time, i.e. 16 hours.

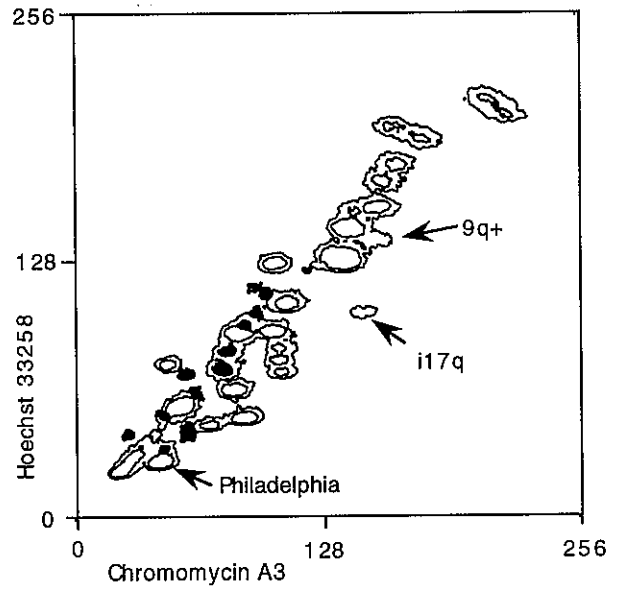

Figure 2.6
Bivariate distribution of chromosomes isolated from peripheral blood cells of a male CML patient. In addition to the known aberrant cluster pattern caused by the t(9;22) translocation and an iso-17q which was also found, a number of aberrant clusters can be observed; indicated in black. To display the aberrant clustering pattern (black) the first contour line was drawn at a lower level than in figure 2.5. Therefore a higher proportion of the debris is visualized at the lower left corner of the karyogram.

The t(9;22) translocation products were observed in their expected position (indicated as Philadelphia and 9q+). Furthermore, as confirmed by conventional cytogenetic analysis a cluster was found that represents an iso-17q. Besides that, clusters appeared in the flow karyogram at unusual places (indicated in black). The relative frequency of these unexpected clusters was determined and appeared to be lower than that of the other chromosomes. The conventional cytogenetic analysis of this patient did not confirm the presence of marker chromosomes that could lead to this unusual clustering pattern.

In Figure 2.7, panel I, a bivariate flow karyogram is shown for the in vitro growing cell line obtained from the BNML. These cells were incubated with Tubulazole-C for 4 hours. To serve as a landmark, some chromosome clusters are indicated as A, B, C, D and E. In panel II a bivariate flow karyogram is shown from the same cell line incubated with Tubulazole-C for 16 hours. The amplifiers were adjusted to show only the small chromosomes. The indications A, B, C and D represent the same clusters as in panel I. Again, in panel II the irregular clustering pattern (in comparison with the regular pattern in panel I) is indicated in solid black. Apparently, the clusters in black (indicated with a, b, c, d and e) contain half the amount of fluorescence and the distribution appears to be similar to the bivariate distribution of the standard pattern as in Figure 2.7 panel I.

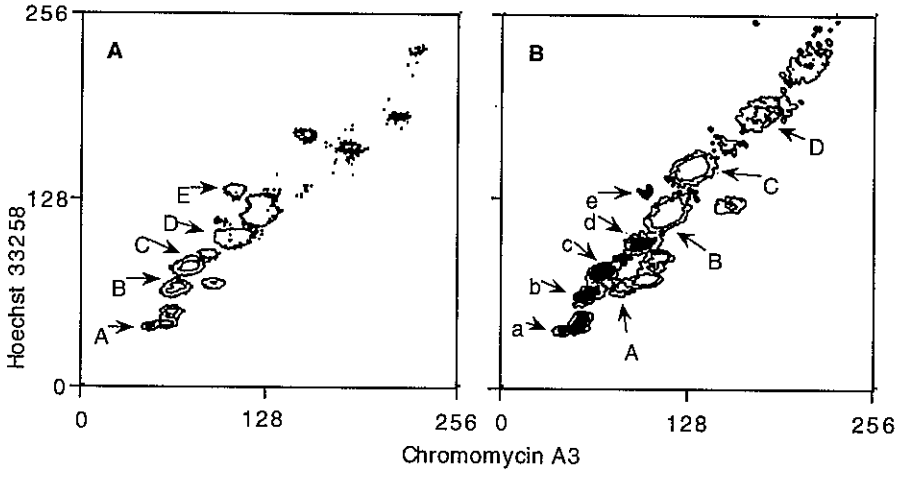

Figure 2.7
Bivariate flow karyograms from the BNML in vitro cell line.
Panel I. Cells incubated with Tubulazole-C for 4 hours .
Panel II. Cells incubated with Tubulazole-C for 16 hours. As compared to panel I, amplifier gains were set approximately twice as high to enhance the small chromosomes. The unexpected clusters indicated in black (a-e), apparently contain half the fluorescence of the flow karyogram of chromosomes. Chromosome clusters A, B, C, and D are corresponding in both panels.

To explain this phenomenon, the contribution of the time of mitotic inhibition as well as the concentration of the spindle poison were investigated. In Table 2.4. the results are given from one representative experiment.

Table 2.4 Relative frequency of unexpected clusters in chromosome suspensions of the LT-12 cell line

	Tubulazole-C		Colcemid	
Incubation time (hrs)	4	16	4	16
Concentration*				
50	nd	nd	10.1%	nd
100	3.5%	12.9%	7.8%	36.2%
250	5.3%	11.6%	24.4%	35.5%
500	8.3%	24.8%	15.4%	42.4%

nd = not determined.
*: concentration in ng/ml; incubation time in hours.

Various concentrations and incubation times of the spindle poisons were tested in the LT-12 cell line. The presence of the aberrant clustering pattern was calculated by counting the number of events found in chromosome cluster E (panel I) and compared to the number of events in the corresponding cluster e (panel II). The presence of events occurring in the background explains the low percentages of events in Table 2.4 when low concentrations of spindle poisons and short incubation times are used. From the table it can be seen that the frequency of aberrant signals increases with both concentration and incubation time. Comparing the effects of Colcemid to those of Tubulazole-C, it seems that at an equal drug concentration the Tubulazole-C effects are less pronounced.

2.2.3 Discussion

Besides the clearly visible Philadelphia chromosome, many additional clusters are found at unexpected positions in the flow karyogram of the CML patient (Figure 2.7, panel II). The pattern formed by these clusters (indicated in black) matches the normal distribution rather well, albeit at half the fluorescence intensity for both fluorescence parameters.

A possible explanation is that the aberrant clustering pattern is caused by the presence of single chromatids in the chromosome suspension, originating from chromosomes that are separated at the centromere. This normally occurs during the anaphase of the mitotic cell division. Routinely the cell cultures were blocked in mitosis for 16 hours. The number of mitotic cells will increase with time finally yielding larger quantities of chromosomes. This prolongation of the incubation time is accompanied by a higher degree of chromosome condensation. Although this would be unacceptable for the microphotography-based cytogenetic analysis (due to

loss of banding resolution) it is not a particular problem for flow karyotyping purposes. However, from the data of the BNML in vitro cell line it can be concluded that longer mitotic blocking periods and an increase in the concentration of the blocking agent go together with an increase in chromatid segregation (Table 2.4). This phenomenon has been observed to different degrees a number of times in human flow karyograms. For example the flow karyogram from the CML patient in Figure 2.5 does not show a chromatid cluster pattern at all. There is no indication that some chromosomes more frequently divide into single chromatids than others. The effect seems to be random, i.e. when single chromatids were observed in the flow karyogram, they always gave rise to a complete "chromatid flow karyogram".

To exclude that electronic or other machine disturbance caused the shadow pattern in the bivariate flow karyograms, samples were run on different machines. The results were similar. Samples with and without aberrant clusters were observed when run shortly after each other

In the literature chromatid segregation in metaphases has been described as "premature centromere division" or "C-anaphase" by several groups (Mehes 1978, Chamla et al. 1980, Mehes and Bajnoczky 1981, Rudd et al. 1983, Gabarron et al. 1986, Madan et al. 1987, Chamla 1988). Whether the phenomenon described in these references is caused by the same underlying process as the presence of chromatids in chromosome suspensions remains unclear. The presence of chromatids in chromosome suspensions was later confirmed using PCR on flow-sorted chromatids (Telenius et al. 1993). A suspension with both single chromatids and chromosomes might very well lead to misinterpretation in the chromosome analysis and it will certainly yield impurities when chromosomes are sorted. To avoid this problem one could either attempt to obtain a 100% pure chromosome suspension or, conversely, induce 100% chromatid separation. The fact that a high mitotic yield due to a long incubation time (16 hrs) leads to chromatid segregation, might make it attractive to induce this phenomenon in 100% of the mitotic cells. In our experiments, however, it was impossible to reach this goal. Suspensions always contained both chromatids and chromosomes or chromosomes alone. Longer incubation times (24 hours) lead to flow karyograms of lesser quality. Forceful shearing of the chromosome suspension had no effect on the generation of extra chromatids and only random chromosome breaks were observed as reflected by an increase in the background debris level (data not shown).

Incubation of rat cell suspensions for 4 hours with Tubulazole-C at a concentration of 100 ng/ml always results in flow karyograms in which no chromatid clusters are observed. The value of 3.5% in this situation (Table 2.4) reflects the background level. However, the presence of chromatids cannot be excluded.

The data in Table 2.4 indicate that chromatid segregation is also influenced by increasing the dose of the spindle poison with a fixed time of inhibition or by

increased duration of the mitotic inhibition with a fixed dose of spindle poison. So far, it can be concluded that it is advisable not to extend the spindle poison blocking period beyond 4 hours. Under such conditions chromatids are not observed with either Colcemid or Tubulazole-C. It has to be stressed, however, that small amounts of single chromatids might still be present which remain unnoticed by regular analysis of the flow karyogram. Their number may be too small to detect them as separate clusters. The presence of small amounts of chromatids might still play a role in sort impurities. For the purpose of probe generation by PCR of sorted chromosome fractions, cross hybridization might occur between probes generated from, for example, sorted chromosome 14 and chromosome 3, or probes generated from probe 20 with 7 and X, since these chromosomes overlap the positions of the chromatid clusters (Figure 2.6).

2.3 Interphase cytogenetics with fluorescence in situ hybridization

In this thesis FISH was used as a procedure to follow-up patients with leukemia. Patients with numerical chromosomal aberrations in their leukemic cells were selected, as judged by conventional cytogenetic analysis that were followed from the time of diagnosis, through the phase of complete remission (detection of "minimal residual disease") to relapse, if this occurred. Furthermore, we followed patients who underwent sex-mismatched allogeneic BMT (SMM allo-BMT) on the basis of the presence or absence of donor- or recipient sex chromosomes to determine the degree of chimerism. All patient studies were performed on bone marrow samples.

2.3.1 Labeling of probe DNA

The probes that are used in this thesis are repetitive sequences specific for mainly alpha satellite DNA sequences on unique chromosome pairs. Probe DNA was inserted into plasmids and used to transform E. coli bacteria that were grown in cultures. The plasmids were isolated and checked for the presence of the correct insert by restriction enzyme digestion and DNA gel electrophoresis. Intact plasmid as well as purified insert were labeled and used as DNA probe. Complete plasmid with insert was labeled with either biotin-16-dUTP or digoxigenin-11-dUTP using the nick-translation procedure (Sambrook et al. 1989). The efficiency of the labeling procedure was checked by spotting samples onto nitrocellulose membranes for the amount of incorporated biotin and by DNA gel electrophoresis for the length of the fragments. Average probe fragment sizes of 200 to 400 base pairs allowed hybridization patterns in which chromosome specific fluorescent spots could be seen in interphase.

2.3.2 Fluorescence in situ hybridization

Cell suspensions were treated with hypotonic buffer (75 mM KCl) for 10 minutes at room temperature. Cells were spun down and fixed with Carnoy s fixative under

continuous vortexing. This procedure was repeated 3 times. Fixed cells were used either directly or were stored in fixative at -20°C for later analysis. After fixation, cells were spotted onto cleaned microscope slides and the area was marked with a diamond tipped pen.

Directly before the hybridization procedure slides were placed in ethanol for at least 60 minutes at room temperature and air dried. Slides were then incubated in 0.1 M HCl/0.1% Tween-20 for 15 minutes to remove matrix and histon proteins and to improve accessibility of the nuclei for the DNA probe.

Denaturation of the target DNA was accomplished by placing the slides in 70% formamide/2x standard saline citrate(SSC) pH 7 at 70°C for 2 minutes followed by dehydration in an ice-cold ethanol series of 70%, 85% and 100%. The hybridization mixture consisted of 1 µg/ml probe DNA, 1 mg/ml sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate and 2 x SSC in 50% formamide at pH 7. The probe was denatured in the hybridization mixture for 10 minutes at 90°C and quenched on ice immediately. Approximately 12 µl of this denatured hybridization mixture was pipetted onto the marked area of the microscope slide. The area was covered with a plastic coverslip and the slide was placed in a humid atmosphere at 37°C. Hybridization was performed for 4 hours. After hybridization slides were washed 3 times for 2 minutes in 2 x SSC followed by a stringent washing in 50% formamide, 2 x SSC. Five minutes at 45°C was used routinely but with probes known for high non-specific binding, stringency was increased by either increase of the temperature, of the formamide concentration or of the time of washing. Slides were washed in 2 x SSC and finally placed in 4 x SSC/ 0.05% Triton X-100 (SSC-T).

Visualization of the biotin-labeled probe binding was accomplished by incubation of the slides with avidin-FITC (av-FITC) conjugate; 5 µg/ml (Vector, Burlingame, CA, USA) in 5% non-fat dry milk in SSC-T with 0.002% sodium-azide, for 20 minutes at 37°C. After this period, cells were washed 3 times for 2 minutes each with SSC-T buffer. In those cases where amplification of the signal was required, slides were incubated with biotin-labeled goat-anti-avidin; 5 µg/ml (Vector) in 5% non-fat dry milk, for 20 minutes at 37°C. After washing, the av-FITC incubation step was repeated. When single hybridizations were performed nuclear DNA was counterstained with propidium-iodide (1 µg/ml) in Slowfade (Molecular Probes, Eugene, OR, USA). When double hybridizations were performed the biotin-labeled probes were detected using Avidin-Texas red (Vector) (red fluorescence) and the digoxigenin labeled probes were detected using anti-digoxigenin-FITC (green fluorescence) while DNA was counterstained with DAPI (1µg/ml).

Figure 2.8 shows what can be expected when cells and/or metaphase spreads are processed for FISH with one chromosome specific probe.

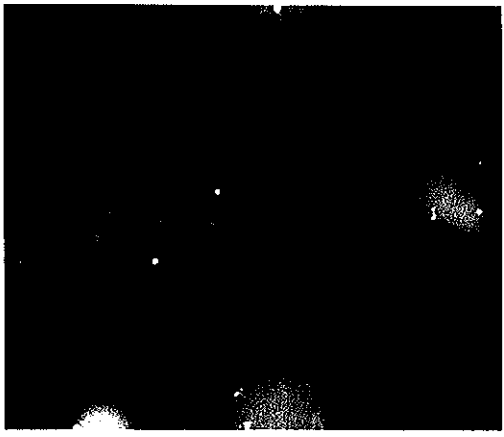

Figure 2.8
FISH on a metaphase slide from a healthy individual. Hybridization with the chromosome 12 specific DNA probe shows two fluorescent signals in metaphase and interphase cells.

In samples obtained from healthy individuals a number of cells can be observed in which the hybridization pattern does not reflect the diploid number of chromosomes that can be expected in these cells. The phenomenon of false positive and negative hybridization is discussed in detail in Chapter VI.

Chapter III

Bivariate flow karyotyping of acute myelocytic leukemia in the BNML rat model

Parts of this work appeared as a publication
G.J.A. Arkesteijn, A.C.M. Martens, R.R. Jonker, A. Hagemeijer and A. Hagenbeek
Cytometry 8:618-624 (1987)

3.1 Abstract

Univariate as well as bivariate flow karyotyping has been performed on chromosome suspensions obtained from the Brown Norway myelocytic leukemia (BNML); a rat model for human acute myelocytic leukemia (AML). Flow karyograms were obtained from both the in vivo transplantable parent line and from an in vitro established cell line. Density gradient centrifugation performed on cells arrested in mitosis resulted in an enrichment of mitotic cells. Furthermore with this procedure leukemic and non-leukemic cells could be separated. Univariate analysis with propidium iodide (PI) as a DNA stain revealed the position of the several tumor-specific marker chromosomes in the in vitro cell line. Estimations of the peak position of the various chromosomes was done by comparing the univariate flow karyogram with a computer simulated karyogram from the BNML that was derived from the mean length of the individual chromosomes in conventionally prepared metaphase slides. By comparing the bivariate flow karyogram of the in vivo BNML cells with the flow karyogram of normal BN cells it was clearly demonstrated which peaks are involved in the altered chromosomal pattern of the BNML. No differences were found between the flow karyograms of the in vitro and the ex vivo-derived chromosome suspensions in this rat leukemia model.

3.2 Introduction

Cytogenetic analysis is one of the most important tools in the diagnosis of leukemia. For a large number of leukemias as well as other hematological disorders, tumor-associated chromosomal translocations are a major characteristic (Rowley 1985). However, on a routine basis standard cytogenetics allow only a limited number of metaphases to be studied due to the time-consuming process of chromosome analysis by microscope. Though this method results in highly detailed analysis of chromosomal translocations, flow karyotyping may offer the advantage of screening up to thousand of chromosomes per second in an objective, quantitative way.

With single laser flow cytometry, characteristic chromosomal abnormalities have been detected in a variety of cell lines among which a Chinese hamster cell line (Gray et al. 1975a, Carrano et al. 1978), a human colon carcinoma cell line (Gray et al. 1984), a rat rhabdomyosarcoma cell line (Kooi et al. 1984), several human melanoma cell lines (Arkesteijn et al. 1986) several Burkitt's lymphoma cell lines and a chronic myelocytic leukemia cell line (Wirschubsky et al. 1983). Van den Engh et al. (Van den Engh et al. 1985) reported on the isolation and uni- and bivariate measurements of chromosomes from amniotic cell cultures.

This paper describes the validity of flow karyotyping for the purpose of detecting chromosomal translocations in acute leukemia. The study was performed in the Brown Norway acute myelocytic leukemia (BNML), a rat model that is being employed as a model for human acute myelocytic leukemia (AML) (Hagenbeek and Van

Bekkum 1977, Van Bekkum and Hagenbeek 1977). Previous studies revealed several leukemia associated chromosomal markers in the BNML. Both ex vivo leukemic cells and cells from an in vitro established leukemic cell line (Lacaze et al. 1983, Lanotte et al. 1984) derived from the parent BNML line were analyzed.

3.3 Materials and methods

Experimental animals

The ex vivo experiments were performed with the inbred Brown-Norway (BN/Bi) rat strain produced in the Rijswijk colony. Male rats between 13 and 16 weeks of age were used (mean body weight: 240 g).

The rat leukemia model (BNML)

The BN acute myelocytic leukemia (BNML), which was induced with 9,10-dimethyl 1,2-benzanthracene in a female Brown Norway rat, shows striking similarities with human AML. The leukemia proved to be transplantable by intravenous transfer of leukemic cells within the BN rat strain. Some of its major characteristics are:

a) Slow growth rate.
b) Severe suppression of normal hemopoiesis due to an absolute numerical decrease in the number of hemopoietic stem cells (CFU-S).
c) The presence of clonogenic leukemic cells (in vivo: LCFU-S, in vitro: clonogenic assay).
d) Response to chemotherapy as in human AML.

An additional advantage of this model is that normal stem cells (CFU-S) and leukemic clonogenic cells (LCFU-S) can be selectively discriminated by modified spleen colony assays (Hagenbeek and Van Bekkum 1977, Van Bekkum and Hagenbeek 1977).

Conventional karyotyping

Cytogenetic studies were performed on bone marrow cells of a leukemic male rat transplanted with BNML cells. The bone marrow cells were cultured for 24 hours and standard harvesting procedures were used. The chromosomes were identified using R-banding with acridine orange and G-banding after trypsin denaturation and Giemsa staining. Recommendations for standard nomenclature were followed (Committee for a standardized karyotype of rattus norvegicus 1973, Levan 1974).

Cell cultures

Cultures of the in vitro established BNML cell line were set up in a concentration 5 x 10^5 cells per ml. Cells were grown in Alpha Modification of Eagles Medium

supplemented with penicillin and streptomycin and 5% of fetal calf serum (FCS). Cell cultures were placed in a humidified incubator that was constantly gassed with 5% CO_2/95% air. In the phase of logarithmic growth (after 3 days) cell cultures were treated with Colcemid (Calbiochem, San Diego, Ca) (0.05 μ/ml final concentration) for 4 to 5 hours.

Lymphocyte cultures from a normal BN spleen suspension were set up in a concentration of 2 x 10^6 cells/ml. Lymphocytes were cultured in Alpha medium supplemented with 10% FCS, 1% PHA, penicillin and streptomycin. On the third day of culture Colcemid was added (0.05 μg, final concentration) for the last 4 hours. Other culture conditions were as mentioned above.

Ex vivo leukemic cells

Male leukemic BNML rats were injected intravenously with colchicin (100 μg/kg) 4 hours prior to either spleen or bone marrow collection. Rats were in a stage where a majority of the cells in these organs is leukemic (day 14-16 after intravenous transfer of 10^7 BNML cells) (Hagenbeek and Van Bekkum 1977, Van Bekkum and Hagenbeek 1977). Single cell suspensions were made from spleen and bone marrow as described before (Martens and Hagenbeek 1985).

Discontinuous density gradients

After collection cells were washed twice in Hanks HEPES Buffered Balanced Salt Solution (HHBBSS). The discontinuous gradient centrifugation method was slightly modified as described by Trask (Trask 1985).The suspensions were layered on top of a four stage gradient and spun down as described below.

The desired gradient densities were obtained by dissolving Nycodenz (Nyegaard, Oslo, Norway) in HHBBSS with 1% Bovine Serum Albumin (BSA). The pH was adjusted to 6.8 and the osmolarity was brought to 280 mOsm. All solutions were checked for their correct density on a DMA 40 digital densitometer (Mettler/Paar, Graz, Austria) at 4°C. Discontinuous gradients were made by layering 2 ml per density on top of each other in 10 ml siliconized glass tubes. The cells were spun down in a table top centrifuge for 20 minutes at 1800 rpm (700 G) at a temperature of 4°C.

Preparation of chromosome suspensions for flow karyotyping

After centrifugation the cells at the interfaces of the various fractions were collected and washed twice in HHBBSS at 4°C. The chromosome isolation procedure was a modification of the method described by Bijman (Bijman 1983). The cell pellets from the fractions were resuspended in chromosome isolation buffer consisting of 20mM NaCl, 8mM $MgCl_2$ and 20mM Tris-HCl pH 7.5 at 37°C. After 10 minutes samples were taken from these suspensions of swollen cells and mixed 1 to 2 with Turk's

solution containing acetic acid (1%) and crystal violet (0.1% w/v) in water. Samples were counted in a Bürker type hemocytometer. Mitotic cells could easily be discriminated from interphase cells and therefore the mitotic index as well as total cell content could be determined simultaneously. The swollen cells in the chromosome isolation buffer were lysed by addition of an equal volume of a Triton-x-100 solution in water (0.4% final concentration). Subsequently chromosomes were released in suspension by careful shearing through a 23 gauge needle.

Chromosomes were stained at least 2 hours prior to measurements with Propidium Iodide (PI) (10 µg/ml) for univariate measurements or with Hoechst 33258 (Ho) (5.4 mM) and chromomycin A3 (CA3) (26 mM) for bivariate analysis on a dual-laser beam flow cytometer.

Chromosome measurements

Measurements of the chromosome suspensions were performed on a dual-laser beam flow cytometer. This cytometer was designed and built in the Radiobiological Institute TNO in Rijswijk (Stokdijk et al. 1985, Van den Engh and Stokdijk 1989). Propidium iodide staining was measured with an argon ion laser tuned at 488 nm, 500 mW (laser: Coherent Innova 90, Palo Alto, CA). PI fluorescence was measured through two KV 550 filters (Schott, Glaswerke, Mainz, Ger.). Hoechst (Ho) was excited with an argon ion laser tuned in the UV range (351 nm and 364 nm; laser: Spectra Physics, Series 2000, Mountain View, CA) at 350 mW laser power. Ho fluorescence was measured using two KV 408 filters (Schott Glaswerke, Mainz, Ger.). CA3 was excited at 458 nm (laser: Coherent Innova 90, Palo Alto, CA) at 200 mW. CA3 fluorescence was measured through two 500 nm longpass filters (LL500; Corion Corp., Holliston, Ma., USA). Data of approximately 20,000 chromosomes were stored in list mode and subsequently analyzed using the ELDAS software package (R.R. Jonker and L. Budel; unpublished).

Peak assignment

Since PI fluorescence intensity has a linear relationship with the DNA content, assignment of the peaks in the PI stained flow karyogram to the respective chromosomes could be achieved on the basis of the length of the chromosomes. From a number of metaphases (n=20) the relative length of the individual chromosomes was determined for the normal BN female rat as well as for the BNML (A. Th. van der Velde and J. C. Klein, personal communication). On the basis of these data, flow karyograms were simulated by computer with a coefficient of variation (CV) of 2% assuming Gaussian distribution. Comparing the PI flow karyograms from the leukemic cells with the computer simulation it appeared to be possible to identify the peak position of the larger chromosomes and of the chromosomal markers.

3.4 Results

Conventional cytogenetic studies

Cytogenetic studies revealed a few metaphases with normal male karyotype 42,XY of host origin and a majority of abnormal metaphases of leukemic origin: 41,XX,-9,1p+,2p+,8q+,12q-,20q+ (Figure 3.1).

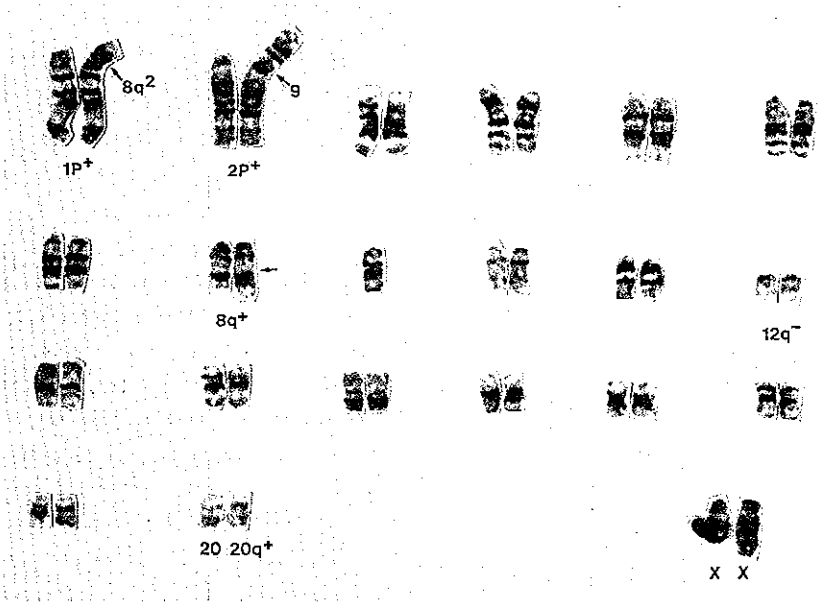

Figure 3.1
G-banded metaphase chromosomes from the BNML originating from leukemic bone marrow.

The XX gonosomal configuration is in accordance with the female sex of the animal in which the leukemia was originally induced and is an independent indication that the abnormal karyotype is of leukemic origin.

The markers were interpreted as follows:

2p+:	translocation of the major part of chromosome 9 onto the short arm of chromosome 2 in an almost Robertsonian translocation.
1p+:	translocation of the distal half of chromosome 8 onto the short arm of chromosome 1.
8q+:	translocation of an unidentified chromosomal segment distally to band 8q2.
12q-, and 20q+:	reciprocal translocation (12q;20q).

All 20 metaphases analyzed showed a very consistent abnormal karyotype: all markers were found and there was no evidence for clonal progression or side lines. The same major markers had already been observed previously in earlier passages of

the cell line (A. Th. van der Velde and J. C. Klein, personal communication). This indicates a stable type of aneuploidy in this leukemia. This is in contrast with the observations in solid tumors where karyotype instability is the rule.

Flow karyotyping

a. The in vitro BNML cell line

Enrichment of mitotic cells derived from the in vitro cell line employing gradient centrifugation is shown in Table 3.1. It is clear that in the homogeneous leukemic cell population in culture, metaphase cells can be separated from interphase cells on the basis of their density.

Table 3.1 Enrichment of BNML ex vivo and in vitro mitotic cells on a discontinuous density gradient

	Mitotic Index on density fraction (g/cm^3)						
	Before	1.050	1.060	1.070	1.075	1.080	1.100
BNML, ex vivo	1.1	0	-	2.4	-	1.5	-
	4.7	-	-	7.0	16.2	7.4	2.6
	3.1	-	-	-	6.5	-	-
	3.8	-	-	-	12.6	-	-
mean	3.2	0	-	4.7	11.7	4.5	2.6
BNML in vitro	8.2	100*	92.6*	1.6	-	3.4	-
	2.8	-	77.0	-	-	-	-
	4.5	-	92.0	-	-	-	-
	14.0	-	83.0	-	-	1.2	-
	8.3	-	43.0	1.6	-	-	-
mean	7.6	100	77.5	1.6	-	2.3	-

\- = not determined

* = The majority of mitotic cells was recovered in the 1.060 fraction; 8.1 x 105 cells versus 3.4 x 10^3 cells in the 1.050 fraction

Mitotic cells from the cell line appear to have a density in between 1.050 and 1.060 g/cm^3. Interphase cells however have a density higher than 1.060. Dead cells appear on the bottom of the centrifuge tube. Employing gradient centrifugation the average mitotic index could be increased from 7.6 to 77.5%. PI stained chromosomes obtained in this way yielded a univariate flow karyogram as presented in Figure 3.2.

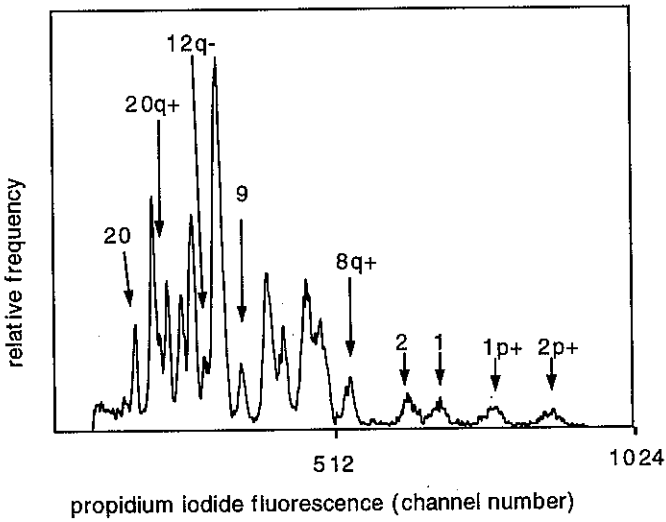

Figure 3.2
Univariate flow karyogram (PI stained) of the BNML in vitro cell line. Numbers indicate the expected chromosome positions.

To assign the various chromosomes to the distinct peaks, the flow karyogram was compared with the results from the computer simulated histograms from normal BN and BNML. The simulations were produced on the basis of length measurements of individual chromosomes derived from 20 metaphase spreads. On the horizontal axis the relative length of the chromosomes is plotted. The histogram is created by assuming a gaussian distribution around each chromosome position. The resulting simulated histograms (Figure 3.3) show that there are several distinct differences between the normal BN female and the BNML chromosomes.

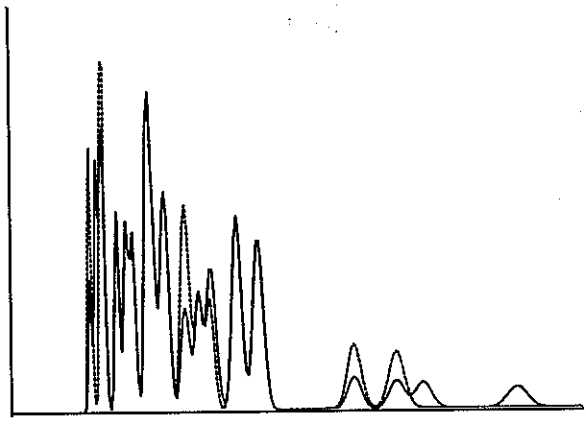

Figure 3.3
Computer simulated flow karyogram of the BNML (solid line) and the normal BN female (dotted line). Simulation was based on the mean relative length measurements of the chromosomes on standard metaphase microscope slides.

The most outstanding difference is the appearance of two additional peaks on the right side of the BNML simulation. The same pattern of peaks can be seen in the

univariate flow karyogram after PI staining where two peaks appear on the far right of the flow karyogram, which is the result of the presence of two large translocation products. One is a near Robertsonian translocation between chromosomes 2 and 9 (2p+, Figure 3.1) as described above. This marker is the largest in the whole karyogram and therefore the peak appears on the far right of the flow karyogram. The other is the translocation between chromosomes 1 and 8 (1p+) (Figure 3.1) and its peak is appearing in between 2p+ and chromosome 1. The lengths of the other leukemia markers fall within the range of the normal chromosomes. Their positions in the PI stained flow karyogram are as indicated by arrows in Figure 3.2.

Bivariate measurements of the chromosomal suspensions of the in vitro BNML cell line resulted in a flow karyogram (Figure 3.4 A) in which 14 distinct spots can be seen.

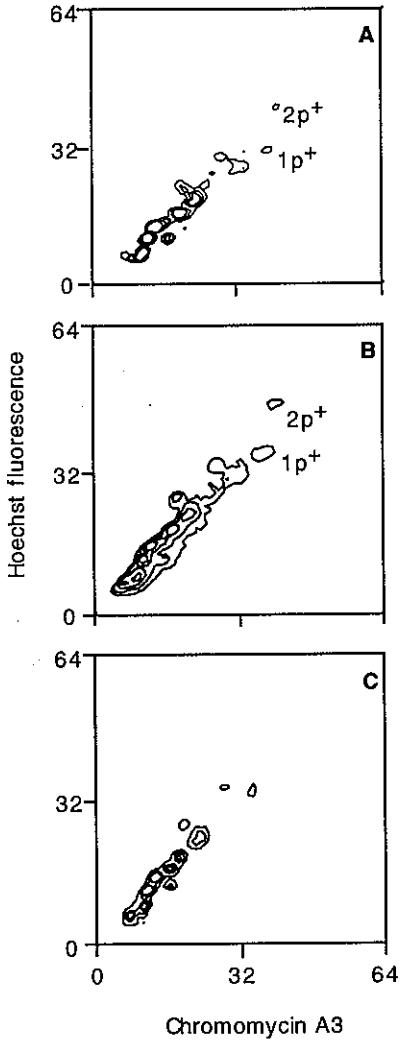

Figure 3.4
Contour plot from the bivariate flow karyogram of the in vitro BNML cell line (A), ex vivo BNML cells (B) and PHA stimulated lymphocytes of the normal BN female (C).

Although correlation of peak position and length of the chromosomes is more complicated in this case, it is obvious however that the two spots on the upper right part of the bivariate flow karyogram correspond with the two large translocation products 1p+ and 2p+. A three-dimensional representation of this bivariate flow karyogram (Figure 3.5A) demonstrates the differences that occur when compared to the normal karyogram obtained from PHA stimulated lymphocytes from a BN female (Figure 3.5B). The position of the marker chromosomes are as indicated in Figure 3.5A.

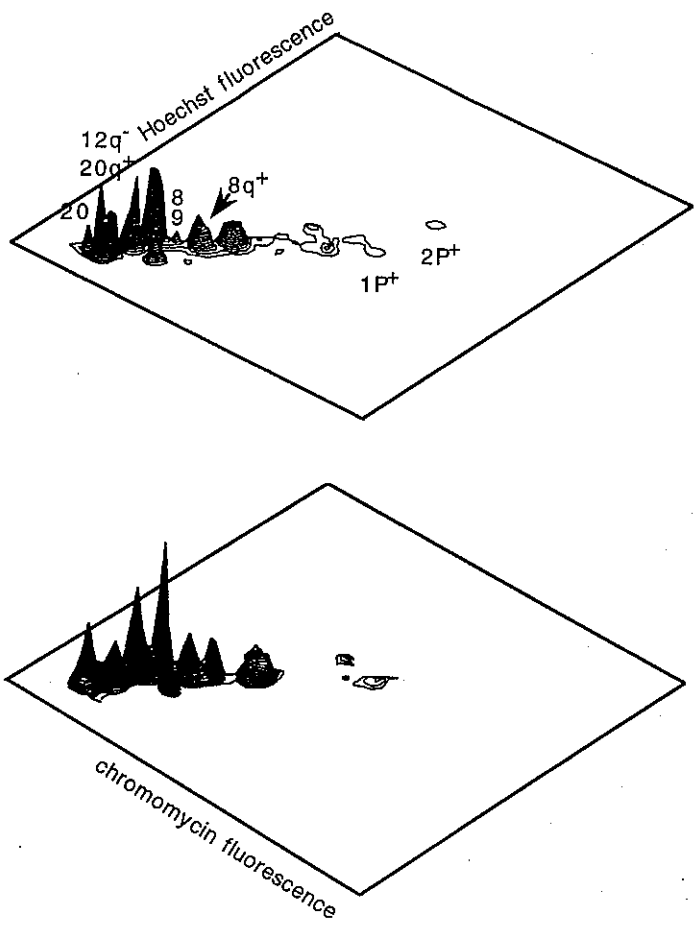

Figure 3.5
Three-dimensional representation of the bivariate flow karyogram of the in vitro BNML cell line (A) and normal PHA stimulated female BN lymphocytes (B). Arrows indicate the area where the differences due to chromosomal changes in the BNML cell line can be observed.

b. Ex vivo normal and leukemic cells

Gradient centrifugation on the ex vivo BNML cells resulted in a mitotic enrichment to a much lesser extend as compared with the in vitro cell line (Table 3.1). Sex chromosomes provided evidence whether the chromosomes were of recipient or leukemic origin. Bivariate flow karyotypes of the several fractions indicated that in the fractions with the lower density (1.070 g/cm^3) the majority of the mitotic cells is of recipient origin; the Y chromosome can be seen in these flow karyograms as a separate cluster (Figure 3.6).

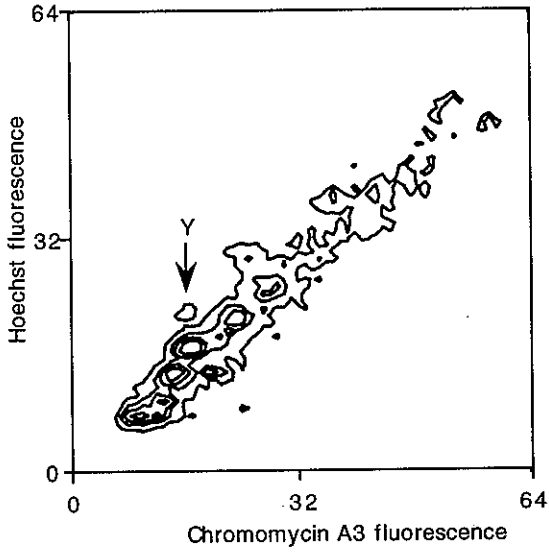

Figure 3.6
Contour plot from the bivariate flow karyogram of a density of 1.070 g/cm^3 containing predominantly recipient normal male BN cells originating from the same gradient as in Figure 3.4 B.

In the higher density fractions (1.080 g/cm^3) the majority of mitotic cells is of leukemic nature which is demonstrated by a more pronounced appearance of the marker chromosomes 1P+ and 2P+ and the disappearance of the Y chromosome (Figure 3.4B). Comparing the BNML flow karyogram with the one derived from the normal BN female (Figure 3.4C) demonstrates several differences which are apparently due to the altered chromosomal pattern in the BNML leukemia. The most outstanding difference is the presence of the two large marker chromosomes in the ex vivo bivariate flow karyogram. Again as was the case for the univariate PI karyogram, the smaller marker chromosomes fall within the area of the normal chromosomes. Their position can best be determined by quantitative analysis of the several clusters, and visualized as demonstrated for the in vitro cell line in Figure 3.5. No differences were found between the flow karyograms from the BNML in vitro cell line and the fresh ex vivo BNML cells.

3.5 Discussion

In this study it was demonstrated that it is possible to identify leukemia in a rat model for AML by tracing its leukemia-specific chromosomal pattern using uni- and bivariate flow karyotyping. Ex vivo leukemic cells as well as cells from an in vitro cell line that was derived from this BNML were studied. So far the several clusters in the flow karyogram were not identified by means of chromosome sorting. Instead, univariate flow karyotyping with PI as a DNA stain was performed (Figure 3.2). Fluorescence intensity is correlated with the amount of DNA which is an indirect measure for the length of the chromosomes. Fluorescence intensity was compared to length measurements of the conventionally prepared karyotype. This was done with the aid of a computer simulated BNML karyogram that was created on the basis of

these length measurements (Figure 3.3). It was noticed that, although the positions of the larger chromosomes and the chromosomal markers were evident, there was not a complete match up for the simulation and the real PI measurement. The large chromosomes altered slightly in position when compared to the simulation whereas for the lower channel numbers only clusters of chromosomes could be discriminated that fitted to some extent the simulated model. An explanation might be that there is not the complete linear relationship between PI fluorescence and relative length, that might be expected on a theoretical basis. Furthermore differences in length between the chromosomes might not have been sufficient to measure them accurately in the conventional karyogram. . Another explanation might be that differences in condensation state of the chromosomes were not taken in consideration, allowing different amounts of PI stain to bind to the DNA of the chromosome than might be expected on the basis of their length. This means that only an estimated position can be given for the smaller marker chromosomes. That the compactnes of the DNA (condensation state of the chromosome) can be of influence on dye binding capacity has been discussed earlier (section 1.3.1.1; this thesis). Conventional cytogenetics revealed the presence of the marker chromosomes in 100% of the cells for this in vitro cell line. Theoretically this means that the areas of the peaks for the chromosomal markers 1p+ and 2p+ should equal that for chromosomes 1 and 2. Experimental data indicate however that the total number of events that occur within the 1p+ and 2p+ area is only 72% of the amount that is found in the 1 and 2 area. This phenomenon often occurs in the area of the larger chromosomes. Chromosome breakage due to the isolation procedure will most frequently occur in the larger chromosomes and will lead to under-representation of the larger chromosome peaks. No or less forceful shearing however results in clumps of chromosome clusters that are not released from the metaphase cells (Van den Engh et al. 1985). In this particular model two of the specific markers are very large. Recently we were able to detect tumor specific chromosomal markers in human clinical samples of chronic myelocytic leukemia (Arkesteijn et al. 1988). In contrast to this model the marker chromosomes in these patients are very small (Philadelphia chromosome) and therefore chromosome breakage in this particular marker plays only a very minor role. Procedures are now being developed in order to minimize the disruptive effects of the isolation procedure.

When dual-beam measurement was employed using Ho 33258 and CA3 more clusters of chromosomes were visible (Figure 3.5) and therefore this method gives a better insight in the changes that appear in the region of the smaller chromosomes. Comparison of the leukemic and normal bivariate flow karyogram (Figure 3.5) demonstrates which chromosome peaks are involved in the leukemic translocations. Together with the information obtained from the computer simulation it was possible to assign the various altered peaks in the BNML bivariate flow karyogram to the various chromosomal markers. The position of the two large marker chromosomes 1p+ and 2p+ in the BNML flow karyogram is obvious. As can

be seen in Figure 3.5A the marker 8q+ gives rise to an extra shoulder as indicated by the arrow. The peak for chromosomes 8 and 9 is reduced markedly. This can be explained by the fact that for both chromosome 8 and 9 one of the two homologues is involved in a translocation. The translocations 12q- and 20q+ together give rise to one additional peak as indicated in Figure 3.5A. The reduction of the peak on the far lower left of the BNML flow karyogram is due to the fact that one of the two homologues of chromosome 20 is involved in a reciprocal translocation with chromosome 12 and therefore disappears from this position.

When a density gradient was employed to enrich for mitotic cells, especially for the in vitro BNML cell line, good enrichment was obtained. The mean mitotic index of 7.6% for the unfractionated material increased to 77.5% for the fraction with the highest yield (Table 3.1). For the ex vivo material enrichment was significantly less (from 3.2% to 11.7%). This might be due to the fact that cell suspensions from both spleen and bone marrow are more heterogeneous with overlapping density distributions of normal and leukemic cells. In fact, fresh ex vivo BNML cells show a significant variation in density as reported before (Hagenbeek et al. 1981). Despite the low mitotic enrichment, gradient centrifugation on these samples was performed in order to attempt to separate leukemic and non-leukemic mitotic cells and subsequently measure them separately. Furthermore dead cells and other debris that might affect the quality of the flow karyogram is eliminated in this way. However, with this procedure a selection is made for the leukemic cells. This, together with the fact that only mitotic leukemic cells are being analyzed, rules out the possibility of giving an accurate figure for the percentage of cells that contain the leukemic markers and leaves one with a yes or no answer. This method of flow karyotyping has led to studies of stages of leukemia growth, with the key question being at what stage of the disease leukemia specific markers can be detected. This might open the way for similar investigations in human acute leukemia in the phase of complete remission.

Chapter IV

Bivariate flow karyotyping in human Philadelphia-positive chronic myelocytic leukemia

This work appeared as a publication
G.J.A. Arkesteijn, A.C.M. Martens and A. Hagenbeek
Blood 72:282-286 (1988)

4.1 Abstract

Chromosome analysis on clinical leukemia material was done by means of flow cytometry (flow karyotyping) to investigate the applicability of this technique in the detection of leukemia-associated abnormalities. Flow karyotyping was performed on blood and bone marrow samples from 8 patients with chronic myelocytic leukemia (CML) after a culture period of four days and arresting the cells in metaphase during the last 16 hours. Discontinuous density gradient centrifugation proved to be essential for removing debris and dead cells from the cell suspensions. By this procedure the mitotic index increase ranged from 2 to 80 times initial values. Chromosomes were isolated and stained with two base pair-specific fluorochromes, i.e., chromomycin A3 and Hoechst 33258, and run through a specially designed dual-laser beam flow cytometer. Generally, 20,000 chromosomes or more were measured per sample. The data were computer-stored in list mode. Besides the clear detection of the specific Philadelphia chromosome, trisomies and other additional chromosomal aberrations (like an i(17q)) were visualized. Quantitative analysis revealed the percentage of subclones containing a certain chromosomal anomaly. Conventional cytogenetic analysis confirmed these findings. In seven of eight cases, CML could be diagnosed on the basis of the presence of a Philadelphia chromosome in the flow karyogram. In one of these seven, the conventional cytogenetic analysis was unknown at that time. The remaining six all matched the standard cytogenetics. The one failure out of eight could be attributed to the specific stimulating conditions in the culture. Although it is impossible by this technique to determine the position of the breakpoint, the involved chromosomes in the translocation event could be identified. In some cases, low percentages of aberrations could not be detected. This study shows that CML can be diagnosed on the basis of flow karyotypic results. Additional chromosomal aberrations can be detected provided that changes in the amount of DNA per chromosome have occurred. Exact quantification of the composition of subclones in the case of mosaicism appear difficult.

4.2 Introduction

Cytogenetic analysis is one of the most important tools in the diagnosis of leukemia. For a large number of leukemias as well as other hematological disorders, tumor-associated chromosomal anomalies are a major characteristic (Rowley 1985). Conventional cytogenetic banding techniques result in a highly detailed analysis of the chromosomal constitution of a metaphase and the exact location of the chromosomal rearrangements (Yunis 1981). However, on a routine basis, conventional cytogenetics allow only a limited number of metaphases to be studied due to the time-consuming process of chromosome analysis by microscope. By means of bivariate flow karyotyping, it is possible to analyze chromosomes in suspension after staining them with two DNA base pair-specific fluorochromes (Langlois et al. 1982, Van den Engh et al. 1985). This suspension is run through a flow cytometer that is equipped with two lasers tuned to the wavelengths suitable to excite the fluorochromes. The

resulting amount of fluorescence is correlated to the amount of dye bound and is, therefore, a measure for the absolute amount of adenosine-thymidine (A-T) and guanine-cytosine (G-C) base pairs for each chromosome. The combination of both yields the ratio G-C/A-T, which allows determination of the position of a chromosome in the flow karyogram. In this way, flow karyotyping offers the advantage of screening up to 1,000 chromosomes/s in an objective, quantitative way.

This technique allows the rapid determination of karyotypes of many cell types from several species. With single-laser flow cytometry, aberrant chromosomal patterns have been detected in a variety of cell lines, among which are the Chinese hamster cell lines(Gray et al. 1975a, Carrano et al. 1978), a human colon carcinoma cell line (Gray et al. 1984), several melanoma cell lines (Arkesteijn et al. 1986) and Burkitt's lymphoma cell lines (Wirschubsky et al. 1983). Only a few reports deal with flow karyotyping for clinical diagnostic purposes. Van den Engh et al. (Van den Engh et al. 1985) reported on high-resolution bivariate flow karyograms derived from amniotic cell cultures. In principle, this method opens the way for using flow karyotyping as a tool in antenatal diagnosis. In this way abnormalities like trisomy for chromosome 21 (Down's syndrome) have been detected by flow cytometry (Trask 1985).

The present report deals with the investigation of flow karyotyping as a diagnostic tool for human chronic myelocytic leukemia (CML). The cytogenetic characteristic of CML is the presence of the Philadelphia chromosome (Ph^1) in the leukemic cells in more than 90% of the patients (Nowell and Hungerford 1960, Rowley 1973) as a result of the reciprocal translocation t(9;22)(q34;q11) (Watt and Page 1978, Sandberg 1980). Ph^1 as well as additional chromosomal aberrations (+Ph^1, +8, i(17q)) that are often associated with the transition to a blast crisis in this disease (Sonta and Sandberg 1978, Van den Berghe et al. 1978) could be detected in this way.

4.3 Materials and methods

Patients

The patients, two males and six females, suffered from CML. Conventional cytogenetic analysis showed the presence of a Ph^1 in all of the cases; in four cases, additional chromosomal abnormalities were observed. The patients ranged in age from 21 to 64 years. At the time of blood or bone marrow sampling, patients had not been receiving treatment for at least seven days. Five patients were in the chronic phase of the disease.

Sample treatment

Approximately 10 ml. peripheral blood or 5 ml. bone marrow was collected in sterile heparinized flask and kept at 4 C during the complete procedure of cell separation. Erythrocytes were eliminated by centrifuging the samples for 15 minutes at 1,800 rpm

(700 G) in a table-top centrifuge on a layer of lymphocyte separation medium (Litton Bionetics, Kensington, UK). Interface cells were collected and washed twice in Hanks' HEPES buffered balanced salt solution (HHBBSS) and diluted to the appropriate concentration for culturing.

Culture conditions

The cells were cultured in an Alpha modification of Eagles medium (Flow Laboratories, Irvine, UK) supplemented with glutamine and antibiotics at a final concentration of 10^6 cells/ml in culture medium. Cells were cultured in the presence of either 1% phytohemagglutinin (PHA, Wellcome Reagents, Dartford, UK) or with 10% conditioned medium (GTC, GIBCO, Paisley, UK) containing the hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were cultured for four days at 37 C in a humidified atmosphere of 5% CO_2/95% air. During the last 16 hours, Colcemid was added (Calbiochem, San Diego) (0.05 mg/ml final concentration) to accumulate cells in mitosis. The Colcemid treated cells were washed twice with HHBBSS. The suspensions were layered on top of a discontinuous density gradient and spun down as described in the next section.

Discontinuous density gradient

The discontinuous gradient centrifugation method as described by Trask (1985)(Trask 1985) was slightly modified. The desired gradient densities were obtained by dissolving Nycodenz (Nyegaard, Oslo, N) in HHBBSS with 1% bovine serum albumin. The pH was adjusted to 6.8, and the osmolarity was brought to 280 mOsm. All solutions were checked for their correct density on a digital densitometer (DMA 40 Mettler/Paar, Graz, Austria) at 4 C. Discontinuous gradients were composed by layering 2 ml of each selected density on top of each other in 10 ml siliconized glass tubes. The cells were centrifuged for 20 minutes at 1800 rpm (700 G) at a temperature of 4 C.

Preparation of chromosome suspensions for flow karyotyping

Cells were collected from the various gradient fractions and washed twice in cold HHBBSS at 4 C. The cell pellets were resuspended in chromosome isolation buffer consisting of 20 mM NaCl, 8 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5 at 37 C. After 10 minutes samples were taken from these suspensions of swollen cells and mixed with Türk's solution containing acetic acid (1%) and crystal violet (0.1% wt/vol.) in water. Samples were counted in a Bürker-type hemocytometer. Mitotic cells could easily be discriminated from interphase cells, and therefore, the mitotic index as well as the total cell content could be determined simultaneously. After swelling in the chromosome isolation buffer for ten minutes, the cells were lysed by the addition of an equal volume of Triton-X-100 solution in water (0.4% final concentration), which resulted in an immediate release of the chromosomes. Subsequently, the suspensions were sheared carefully through the tip of an Eppendorf pipette.

Chromosomes were stained directly afterwards with Hoechst 33258 (Ho) (5.4 mM) and Chromomycin A3 (CA3) (26 mM) at least two hours prior to measurement on a dual-laser beam flow cytometer.

Flow karyotyping

Chromosomes were measured on a dual-laser beam flow cytometer (RELACS-III) designed and built at the Radiobiological Institute TNO in Rijswijk (G.J. van den Engh and W. Stokdijk, manuscript in preparation). Ho was excited with an argon ion laser tuned into the UV range (351 and 364 nm; Spectra Physics series 2000, Mountain View, CA) at 350-mW laser power. Ho fluorescence was measured through two KV 408 filters (Schott Glaswerke, Mainz, Ger.). CA_3 was excited with an argon ion laser tuned at 458 nm (Coherent Innova 90, Palo Alto, CA) at 200 mW. CA_3 fluorescence was measured through a 550-nm longpass filter (LL550 Corion Corp., Holliston, MA). Measurements were performed with the CA_3 fluorescence as a trigger signal. Data of approximately 20,000 chromosomes were stored in list mode and subsequently analyzed by using the ELDAS software package (R.R. Jonker and L. Budel, manuscript in preparation). The bivariate cluster pattern was compared with the average standard configuration to detect disappearing or newly arising clusters. Quantitative analysis was performed by computing the total number of events within each cluster with the aid of the ELDAS software package, thus revealing information about the relative frequency of the various chromosomes.

4.4 Results

Gradient centrifugation

Gradient centrifugation after the four days culture period proved to be necessary to obtain flow karyograms with low debris background. The elimination of dead cells by means of gradient centrifugation resulted in a reduction of debris particles in the chromosome suspension. This proved to be essential for the maintenance of good-quality chromosomes in the suspension. In this way, karyograms could be obtained from suspensions with a mitotic index as low as 4% and evaluated. All of the presented cases were processed for gradient centrifugation. This resulted in a two- to 80-fold increase in the mitotic index in the cell suspension that was layered at the density of 1.060.

Flow karyotyping

In Figure 4.1, a flow karyogram is presented without any aberration (patient 1). Each contour line is drawn at selected percentages of the total amount of events to show optimal cluster separation. The position of the chromosomes in the flow karyogram are well determined by sorting of the clusters and identification by banding (Gray et al. 1979, Yu et al. 1981).

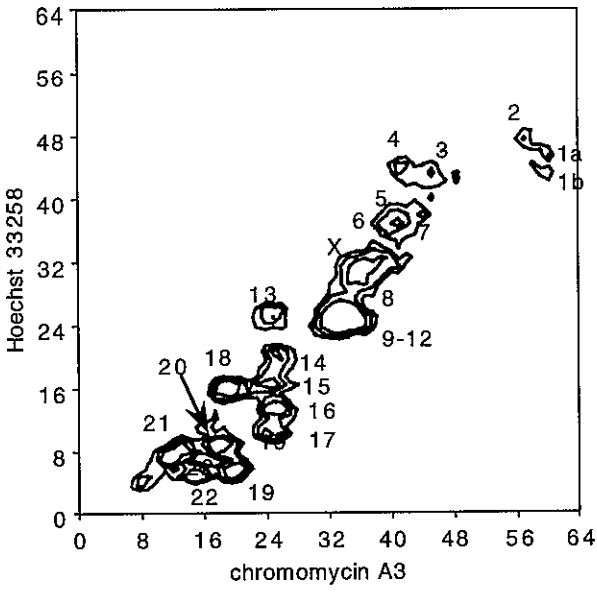

Figure 4.1
Contour plot from the chromosomal distribution of a bivariate flow karyogram from a female CML patient in the chronic phase (patient 1). The karyogram was obtained from peripheral blood cells that were stimulated with PHA. It shows the normal distribution of chromosomes without the specific CML translocation. Contour lines were drawn at selected levels to show optimal chromosome cluster separation.

This flow karyogram is derived from peripheral blood lymphocytes from a female CML patient in which conventional cytogenetic techniques revealed the presence of a Ph^1 in 100% of the metaphases. The nucleated cells were cultured for four days in the presence of PHA. The fact that no chromosomal aberrations were detected in flow means that there is at least a normal subpopulation of cells (most likely lymphocytes) that responds to the PHA stimulus. This patient is listed in the Table as patient 1.

Table 4.1. Comparison of Flow Karyotyping and Conventional Cytogenetics

patient no.	C/A	sex	Source	Culture conditions	Aberrations in flow	Incidence (%)	Conventional cytogenetics	Incidence
1	C	F	PB	PHA	No aberrations	100	46,xxt(9;22)	100
2	C	F	PB	PHA	Ph^1	100	Unknown	
3	A	F	BM	PHA	Ph^1, +8, +17, +19	100	46,xx,t(9;22)	47
							47,xx,t(9;22), +19	6
							49,xx,t(9;22), +8, +17, +19	41
							50,xx,t(9;22), +8, +17, +19, +19	3
							47,xx,9p-,q+, 22q- ,+8,i(17q)	3
4	C	F	PB	GCT-CM	Ph^1	100	46,xx,t(9;22)	100
5	C	F	PB	GCT-CM	Ph^1, -8, -9/12, -19	100	46,xx,t(9;22), 8q+, 12q- , 19q-	100
6	A	M	PB	GCT-CM	Ph^1	100	46,xy,t(9;22)	100
7	C	F	BM	GCT-CM	Ph^1, +16/14, +8	100	46,xx,t(9;10;22)	100
					-17	60	46,xx,t(9;10;22),-2,-17	one cell
8	A	M	PB	GCT-CM	Ph^1, $+Ph^1$, +8, +8	100	46,xy,t(9;22)	one cell
					i(17q), -17	60	47,xy,+8,t(9;22),i(17q)	15
							49,xy,t(9;22),+8, +8,i(17q), +22q-	80

Abbreviations: C, chronic phase; A, Accelerated phase; PB, peripheral blood; BM, bone marrow.

In Figs 4.2 A and B, a flow karyogram is presented from a female CML patient with the typical CML-specific translocation (i.e. t(9;22) that resulted in a Ph^1 as the only aberration (patient 4). In this case peripheral blood cells were cultured in the presence of GCT-CM. The specific Ph^1 was present in 100% of the analyzed material.

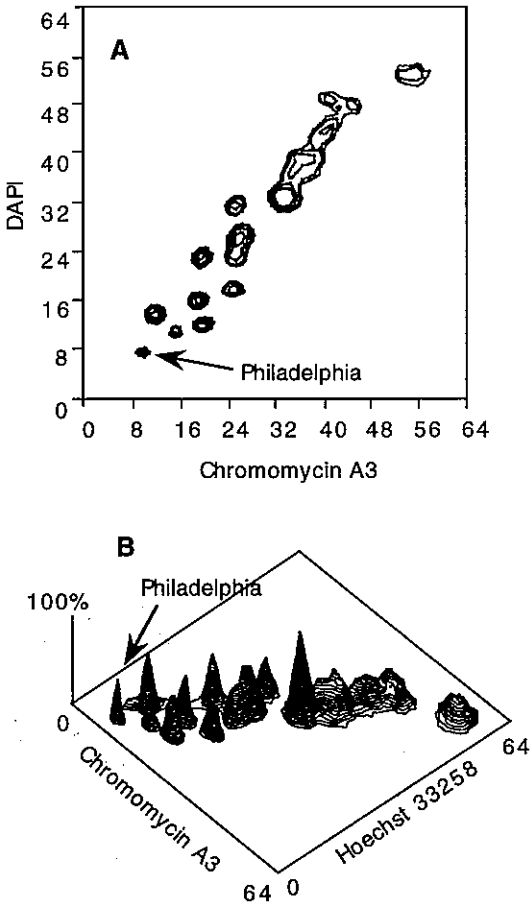

Figure 4.2
Chromosomal distribution of a bivariate flow karyogram from a female CML patient in chronic phase (patient 4). White blood cells were stimulated with GCT-CM. Contour plot (A) and three-dimensional representation (B) display a Ph^1 chromosome as the only aberration in this karyogram.

Figure 4.3 shows the flow karyogram of the bone marrow from a female CML patient (patient nr. 3) after stimulation in culture with PHA. To focus on the small chromosomes, this area (i.e. from Ph^1 to 9 to 12) is expanded, and the larger chromosomes are out of range at the top right-hand. The position of the Ph^1 is indicated by an arrow in Figure 4.3.

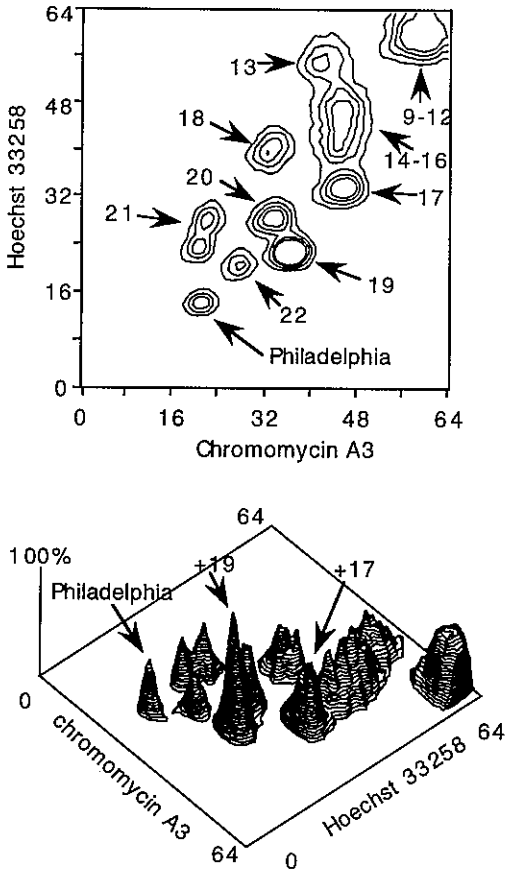

Figure 4.3
Chromosomal distribution of a bivariate flow karyogram from a female patient in accelerated phase (patient 3). Amplifier gains were adjusted so that only the small chromosomes are displayed. The flow karyogram was obtained from bone marrow cells that were stimulated with PHA. Contour plot (A) and three-dimensional representation (B) shows the Ph^1 as well as a trisomy for chromosomes 19 and 17.

This patient is listed in Table 4.1 as patient 3. In this three-dimensional representation of the flow karyogram, the peak heights differ among various chromosomes. Quantitative analysis reveals that the content of the peaks containing chromosome 22 and the Ph^1 are reduced by half compared with the others. In this particular case, chromosome peaks 17 and 19 appear to contain 1.5 times as many events as expected, thus indicating the presence of a trisomy for these chromosomes. This same patient appeared to have a trisomy for chromosome 8 as well (see Table 4.1). In addition, the two homologues for chromosome 21 can be seen separated from each other in this karyogram.

The results from all of the patients analyzed thus far are listed in Table 4.1. In all patients except for no. 1, the presence of the Ph^1 found with flow karyotyping was in accordance with the conventional cytogenetic findings. In some of the cases the percentage of a particular chromosomal aberration found in flow differs from the conventional cytogenetic technique (patients 3, 7, 8), and in some cases very low percentages of aberrant chromosomes were not detected (3%; patients 3, 7). In patient

5 the aberrant chromosomes 8, 12, and 19 were not detected. However, the involvement of these chromosomes was noticed by the disappearance of one of the homologues from the normal cluster position indicated as -8, -9/12 and -19.

In patient 7 the complex translocation t(9;10;22) could not be identified as such. Conventional karyotyping analysis revealed that the 9q+ was larger as a result of this complex translocation. Chromosome 10 was reduced in size and contains the part that is usually attached to chromosome 9q+. The size of the Ph^1 was comparable to a Ph^1 in a regular-case CML. In flow, the Ph^1 was detected at its expected position. The cluster of chromosomes 14 to 16 contained one chromosome too many. This is most probably the contribution of the small chromosome 10. The enlarged 9q+ could not be detected as such with flow karyotyping. However, an extra chromosome on the position of chromosome 8 was observed. that might be the contribution of the enlarged chromosome 9q+.

Although not explicitly indicated for all of the patients in Table 4.1, the cluster for the normal chromosome positions was reduced by half in all of the cases when one of the homologues was involved in an alteration that resulted in DNA loss or gain.

4.5 Discussion

In this study it is demonstrated that flow karyotyping can be performed on clinical material. Chromosomal aberrations occurring in clinical samples of CML patients can be detected by bivariate flow cytometry.

Gradient centrifugation resulted in chromosome suspensions with an increased mitotic index. The growing fraction, mainly blast cells containing the mitotic cells, have a lower density than do differentiated cells. Therefore, by eliminating cells without mitotic capacity, the mitotic index can be increased between two and 80 times. In ideal cases (cell lines) it is even possible to obtain up to 100% mitotic cells from mixed suspensions by density separation (Arkesteijn et al. 1987).

In seven of eight cases, the Ph^1, which is the major cytogenetic hallmark of leukemic cells of CML patients, was detected by flow karyotyping. Also, additional chromosomal changes could be detected. Although, besides the Ph^1, the second translocation product (i.e., the 9q+) should theoretically be seen as a separate cluster, its close proximity to chromosome 8 does not always allow clear separation from this chromosome. In the cases presented, the presence of 9q+ could only be demonstrated as a small shoulder or even only by quantitative means.

These studies were done in a double-blind manner. As can be seen in Table 4.1, patient 1 appeared to be without a Ph^1 in the flow karyogram (Fig 4.1), whereas conventional cytogenetics revealed this marker in all of the metaphases examined.

As already mentioned, this means that at least a normal cell population, apparently T lymphocytes, is responding to the mitogenic stimulus and possibly overgrowing the leukemic cells. CML is generally regarded as a stem cell disorder (Fialkow et al. 1977). However, involvement of T-lymphocytes is sparse and sometimes contradictory. Some investigators found that T-lymphocyte metaphases and PHA-responding cells in the chronic phase of CML contain the typical chromosomal markers (Shabtai et al. 1980, Itani and Hoshino 1982, Nitta et al. 1985, Jonas et al. 1992) although many did not find the Ph^1 in all of these cells (Fialkow et al. 1978, Löwenberg et al. 1980, Bagnara et al. 1981, Swart et al. 1981, Kearney et al. 1982, Nitta et al. 1985, Bartram et al. 1987, Jonas et al. 1992). However, peripheral blood cells containing the leukemia-specific chromosomal markers can, in some cases, be stimulated with PHA as is demonstrated in cases 2 and 3. The stimulation with both PHA and GM-CSF should therefore be performed separately in each patient. If the PHA stimulus results in a normal karyogram, it can serve as a perfect internal control for the leukemia karyogram obtained from the GM-CSF-stimulated culture of the same patient. Recently we have demonstrated that stimulation with either PHA or GM-CSF in a CML patient in relapse after a sex-mismatched bone marrow transplantation results in two completely different flow karyograms, i.e., one being of healthy male donor origin (indicated by the Y chromosome) and one being of leukemic (Ph^1) female origin (manuscript in preparation).

In all of the cases analyzed, the percentage of abnormal chromosomes differ from the conventional cytogenetic analysis. It should be stressed that with the conventional cytogenetic technique the samples are routinely cultured for 24 or 48 hours without any stimulation. Our culture system of four days may have selectively favored the growth of certain subpopulations of leukemic cells. Another source of selection might have been introduced by using gradient centrifugation by selecting a certain light-density fraction of cells from which the mitotic fraction is obtained to compose the flow karyogram. Finally the number of cells analyzed per sample might be a cause for discrepancy. Conventional karyotypes are deduced from 15 to 35 metaphase spreads at the most, whereas one flow karyogram is composed of an average of 20,000 chromosomes corresponding to an average of 500 mitotic cell equivalents.

From this study we conclude that flow karyotyping can be performed on clinical CML material. Under selected culture conditions and with mitotic indexes above 4% without debris-causing particles, flow karyotyping will result in detailed cytogenetic information. However, the sensitivity of this new method in detecting small subpopulations of abnormal chromosomes is still a matter of concern. Therefore at present, flow karyotyping should be classified as a useful source of information in addition to conventional karyotyping. Since repeated cytogenetic analyses are quite time-consuming and expensive, flow karyotyping might be an acceptable alternative for monitoring the karyotype of a CML patient in chronic phase to detect new chromosomal abnormalities that often precede blast crisis. In addition, one could use

flow karyotyping to monitor the fraction of metaphases that have the Ph^1 in patients who undergo aggressive chemotherapy or interferon therapy during chronic phase, either of which may in some cases suppress the Ph^1 clone.

Acknowledgments

The authors wish to thank Dr. A. Hagemeijer (Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam) for performing conventional cytogenetic analyses, Dr. J.W.M. Visser and his department for technical assistance on the RELACS III, Dr. R. Jonker for software support and Carla Ophorst for expert technical assistance.

Chapter V

Clinical applications of flow karyotyping in myelocytic leukemia by stimulation of different subpopulations of cells in blood and bone marrow samples

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G.J.A. Arkesteijn, H van Dekken, A.C.M. Martens and A. Hagenbeek.
Cytometry 11:196-201 (1990)

5.1 Abstract

Examples are presented in which normal as well as abnormal chromosome distributions could be obtained from the same individual by means of bivariate flow karyotyping. Selective stimulation of T-lymphocytes obtained by E-rosetting from the blood of a patient with acute myelocytic leukemia resulted in a normal flow karyogram. The specific stimulation of myelocytic leukemia cells with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) yielded flow karyograms displaying the leukemia-associated chromosome abnormalities. The resulting flow karyograms could be used to discriminate between homologue differences, which appear normally in virtually every individual, and leukemia-associated chromosomal aberrations. In the case of a female chronic myelocytic leukemia patient who received bone marrow from an HLA-identical male donor, specific stimulation of various subsets of cells enabled to discriminate between leukemic host cells and non-leukemic donor cells. Both the leukemia specific translocations and sex chromosomes were used as markers to analyze the flow karyograms obtained from the same sample.

5.2 Introduction

Flow karyotyping is a well-established method of analyzing chromosomes in suspension by means of a flow cytometer, resulting in either uni- or bivariate flow karyograms. In these karyograms virtually all human chromosomes can be discriminated on the basis of their size and base pair ratio (Langlois et al. 1982, Van den Engh et al. 1985, Arkesteijn et al. 1986, Harris et al. 1986, Arkesteijn et al. 1987, Harris et al. 1987, Arkesteijn et al. 1988, Van den Engh et al. 1988). The resulting chromosome clustering pattern is characteristic for the species from which the chromosomes are derived. Whenever a change in position of one of the chromosome clusters occurs due to an event through which the DNA content is altered (i.e. a translocation, deletion or amplification) this will show in the flow karyogram by a newly appearing peak or cluster and/or a quantitative reduction of the chromosome clusters that are involved in this event (Arkesteijn et al. 1988). Whether two chromosomes can be seen as separate clusters mainly depends on the quality of the flow karyogram. The coefficient of variation that is usually obtained in bivariate flow karyograms from leukemia patient material allows the detection of separate chromosomes that differ approximately 2% in DNA content.

Within each species, provided it is not inbred, the flow karyogram will display differences in the DNA content between the homologues of certain chromosomes. This phenomenon can be observed very well in uni- and bivariate flow karyograms. For man the largest variations occur in the chromosomes 1, 9, 16, and Y although in a bivariate flow karyogram almost every chromosome pair shows some degree of disparity.

When the difference between two homologues is large enough this will show in the bivariate flow karyogram as two separate clusters. Such homologue differences will contribute to the variation in the average standard position of the chromosome clusters in the bivariate flow karyogram (Gray et al. 1988). By means of family flow karyotyping it is possible to trace the origin of the homologue. Such investigations may shed light on inheritable diseases. (Harris et al. 1987, Van den Engh personal communications).

Whenever two clusters are observed for one chromosome in cases where altered cluster positions can be expected, like in the case of leukemia, additional analysis focusing on homologue differences is essential to identify small aberrations. The only feasible way to discriminate homologue differences from leukemia-specific chromosomal aberrations is by comparing this flow karyogram with the flow karyograms from first line relatives or, as is demonstrated in this study, with the flow karyograms of normal cells from the same patient.

Leukemia cells can be stimulated to proliferate in vitro by the addition of specific growth factors to the culture medium (Griffin et al. 1986, Delwel et al. 1987, Vellenga et al. 1987). We therefore made use of the possibility to specifically stimulate either the myelocytic leukemia cells with GM-CSF and IL3 or the normal T-cells with phytohemagglutinin (PHA). The flow karyogram from the PHA stimulated cells will indicate the normal homologue positions. Comparison of this with the flow karyogram from the GM-CSF and IL3 cultured cells will exclude homologue differences from leukemia associated chromosome aberrations.

Quantitative cluster analysis reveals which of the chromosomes are involved in a translocation, amplification or deletion. In this paper an example is given of a patient with acute myelocytic leukemia (AML) characterized by the loss of the Y chromosome as well as other rearrangements. Fluorescence in situ hybridization (FISH) with a Y specific DNA probe confirmed the loss of the Y chromosome in this particular patient. The procedure, which aims at specific stimulation of both normal and leukemic cells from blood or bone marrow also enables to determine the emergence or the persistence of relapsing leukemia and state of chimerism in (sex-mismatched) bone marrow transplant recipients. Here we report on a female patient with chronic myelocytic leukemia (CML) who received a bone marrow transplant from a male donor. At relapse, four years after transplantation, two cell populations could be distinguished by means of flow karyotyping; one of normal donor origin (indicated by the presence of the Y-chromosome) and one of leukemic origin (indicated by the presence of the Philadelphia chromosome and the absence of the Y-chromosome).

5.3 Materials and methods

Patients

A male patient (patient a) was diagnosed with acute myelocytic leukemia (AML; FAB classification M1). He was treated with daunorubicin and cytosine arabinoside. Blood samples were taken for flow karyotyping and FISH.

A female patient (patient b) who was diagnosed with chronic myelocytic leukemia (CML) was transplanted during the chronic phase with bone marrow from her HLA-identical brother. Four years after transplantation she showed clinical signs of an imminent relapse. At this time blood and bone marrow samples were taken for flow karyotyping and FISH.

Sample treatment

Either blood or bone marrow was collected in sterile heparinized flasks. After collection and during the whole process of cell separation the samples were kept at 4 C. Cells were isolated as described previously (Arkesteijn et al. 1988). In brief, nucleated cells were collected by layering either blood or bone marrow on lymphocyte separation medium (LSM; Organon, Durham, NC) and spun down in a table top centrifuge for 20 minutes at 700 G. Interphase cells were collected and washed twice in Hanks HEPES Buffered Balanced Salt Solution (HHBBSS). Only in the case of the male patient, in addition to this, the resulting cell suspension was mixed with 2-aminoethyl isothiouronium boride (AET)-treated sheep red blood cells (SRBC). This suspension was layered on LSM and spun down for a second time at 700 G for 20 minutes. The cell fraction originating from the AET-SRBC rosettes in the pellet was called the T-cell enriched fraction, the cell fraction originating from the interphase layer was called T-cell-depleted. Prior to culturing of the T-cell enriched fraction, the AET-SRBC were lysed by treating them for a short period with a buffer consisting of NH_4Cl 155 mM, EDTA 0.1 mM, and $NaHCO_3$ 11.9 mM and washed twice in HHBBSS.

Cell cultures

Cells were cultured in Alpha modified Eagle's medium. T-cells were cultured in the presence 10% fetal calf serum (in case of patient a, only the T-cell-enriched fraction; in case of patient b, a part of the total cell suspension). These cells were stimulated with 1% PHA.

Leukemic cells were stimulated with human recombinant GM-CSF (patient a and b,10 U/ml) and human recombinant IL3 (only patient a, 10 ng/ml). In the case of patient a, factors were added to only the T-cell-depleted fraction, and in patient b a part of the total cell suspension. IL3 was obtained from Gist-Brocades NV Delft NL, GM-CSF was obtained from Biogen SA, Willemstad NL-Antilles. After four days in culture at 37 C, 100% humidity and 5% CO_2, 250 ng/ml Tubulazole-C (Janssen Life

Science Products, Beerse, Belgium) was added during the last 4 hours of culture to accumulate cells in mitosis.

Chromosome isolation

Chromosomes were isolated as described previously (Arkesteijn et al. 1988) with some slight modifications. Cells were spun down on a discontinuous density gradient (Nycodenz, Nyegaard, Oslo, N.) containing 1% BSA in 4 ml plastic tubes at 4 C with a speed of 700 G The density of the upper layer was 1.062 g/cm^3 and the bottom layer was 1.080 g/cm^3. Mitotic T-cells were mainly found on the 1.080 layer whereas the mitotic leukemic cells layered mostly on top of 1.062. Dead cells and debris were found in the bottom fraction of the tube. The cells from the various densities were washed twice in cold HHBBSS. The washed cells were resuspended in prewarmed (37 C) swelling buffer (20 mM NaCl, 8mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5). After 2 - 5 minutes at 37 C the cells were lysed by the addition of an equal volume of Triton-X-100 (0.8% in water). The suspensions were then rapidly cooled in melting ice. The chromosomes were stained with Chromomycin A3 (CA3; 26 ng/ml) and DAPI (5.4 ng/ml, patient a) or CA3 and Hoechst 33258 (Ho; 5.4 ng/ml, patient b). Chromosome suspensions were either measured on the same day or stored at -20 C after the addition of dimethyl sulfoxide (DMSO; 10% final concentration). Frozen chromosome suspensions could be stored for weeks, defrosted, and refrozen again without any visible loss of resolution in the flow karyogram.

Flow karyotyping

Chromosomes were measured on the RELACS III, a dual-beam flow cytometer especially designed and built at the Radiobiological Institute TNO for the measurement of chromosomes in suspension (Van den Engh and Stokdijk 1989). CA 3 was excited with a laser tuned at 458 nm at 250 mW (Coherent Innova 90, Palo Alto, CA). Ho and DAPI were excited in the UV range (351 and 364 nm) at 350 mW laser power (Spectra Physics series 2000, Mountain View, CA). CA3 fluorescence was measured through a 550 nm longpass filter (LL550 Corion Corp., Holliston, MA). The fluorescence from Ho or DAPI was measured through two KV 408 filters (Schott Glaswerke, Mainz, Ger.). The CA3 fluorescence was used as a trigger signal. Data were stored in listmode on a computer (Hewlet Packard, series HP 9000). Further analysis was performed using the ELDAS software package (Jonker et al. 1987). Flow karyotyping as well as conventional cytogenetic analysis was done in a double blind fashion.

Fluorescence in situ hybridization (FISH)

In situ hybridization was performed on nuclei obtained from patient a and b after the first LSM treatments (i.e., after elimination of erythrocytes and granulocytes). Cells were fixed in methanol/acetic acid (3 : 1) and dropped on glass slides. The in situ hybridization and fluorescent detection protocol was described previously (Pinkel et al. 1986, Van Dekken et al. 1988). The human Y-specific repeat pY2.45 was obtained

commercially (Amersham Int., Amersham, UK). This satellite DNA probe hybridizes to the large block of heterochromatin found on the long arm of the Y chromosome (Cooke et al. 1982). Complete plasmid DNA was labeled with biotin-11-dUTP using the BRL-nick translation kit (BRL, Gaithersburg, MD) according to the manufacturers directions. The hybridized probe was detected using avidin-FITC. Nuclei were counterstained with propidium iodide (PI; Sigma, St. Louis, MO) to allow simultaneous observation of total DNA and hybridized probe. The DNA stain was used at a concentration of 1 µg/ml in an antifade solution containing p-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) to preserve the fluorescein fluorescence during microscopy (Johnson and Nogueira Araujo 1981).

5.4 Results

The flow karyograms from the AML patient (a) are shown in Figure 5.1. The differences between the karyogram derived from the GM-CSF/IL3- and the PHA-stimulated cells are indicated by solid arrows, the (homologue) clusters that appear in both karyograms are indicated by open arrows. The left panel shows the flow karyogram that was obtained from the T-cell-depleted fraction of nucleated peripheral blood cells from this male patient. The cells were stimulated in vitro with GM-CSF and IL-3.

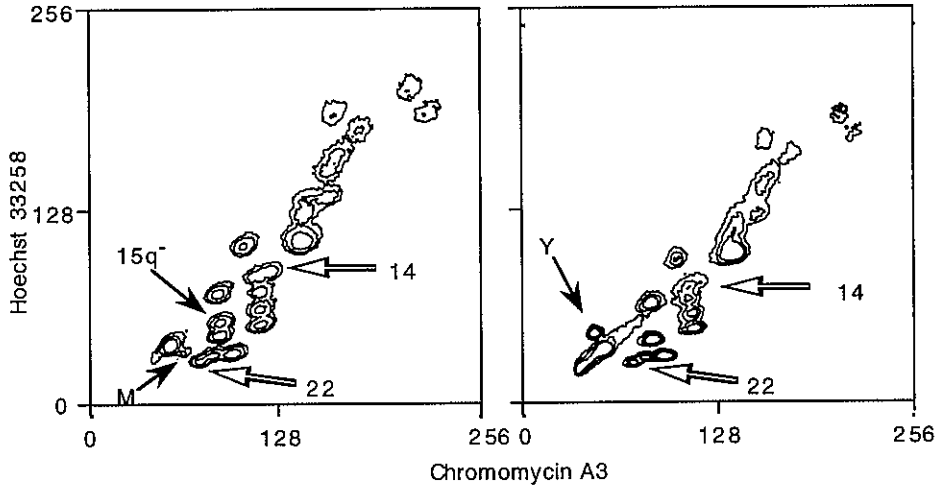

Figure 5.1
Bivariate flow karyograms from the same blood sample of an acute myelocytic leukemia patient (patient a).
Left panel: T-cell-depleted fraction stimulated with GM-CSF and IL3.
Right panel: T-cell enriched fraction (SRBC) stimulated with PHA.
Open arrows point to similarities in both flow karyograms, solid arrows point to differences between leukemia and normal cell types.
A limited amount of contour lines was drawn at levels that showed most optimal cluster separation.

A number of abnormalities where found in this flow karyogram i.e., the absence of the Y chromosome, an extra cluster closely above chromosome cluster 20 (indicated as 15q-) and a cluster close to chromosome 21 (indicated as M). Quantitative analysis of the chromosome clusters revealed the absence of one of the homologues in the cluster for chromosome 15. As can be seen in this karyogram a few clusters might be pointed out as being either chromosome abnormalities or homologue differences (arrows) i.e., two clusters on the position of chromosome 22 and the separation in two clusters for chromosome 14. The flow karyogram derived from the PHA-stimulated T-cell-enriched fraction in the right panel clearly shows the presence of the Y chromosome in the normal cells. Furthermore the clusters indicated by M and 15q- close to chromosome 20 and 21 are absent in the right panel, whereas the double clusters for chromosome 22 and 14 are present in both flow karyograms. Therefore, the latter two relate to homologue differences.

The (reciprocal) exchange of DNA, resulting in a linear shift in the number of channels to a new position in the flow karyogram, is inversely correlated to the channel shift for the second cluster in the translocation event. Therefore the aberrant cluster pattern cannot be explained by a translocation between chromosomes 15 an Y. In such a case a second new cluster would have appeared. No such cluster can be found in this karyogram. These observations strongly suggest that the Y chromosome is missing and that a part of one of the homologues of chromosome 15 is deleted. Calculations of the decrease in channel number of this new cluster compared to the original position of the #15 chromosomes indicates that 25% of the DNA is deleted. On the basis of quantitative analysis in the flow karyogram it was found at least 80% of the metaphases contained two of these partially deleted chromosomes while the remaining 20% contained one copy. The origin of the cluster indicated as M which was present in 20% of the metaphase cells analyzed by flow karyotyping remained unclear.

The loss of the Y chromosome was confirmed by the use of in situ hybridization with a Y specific probe to nuclei from unstimulated peripheral blood cells. Y chromosomes could be detected in only 5% of the nuclei of peripheral blood cells (Figure 5.2).

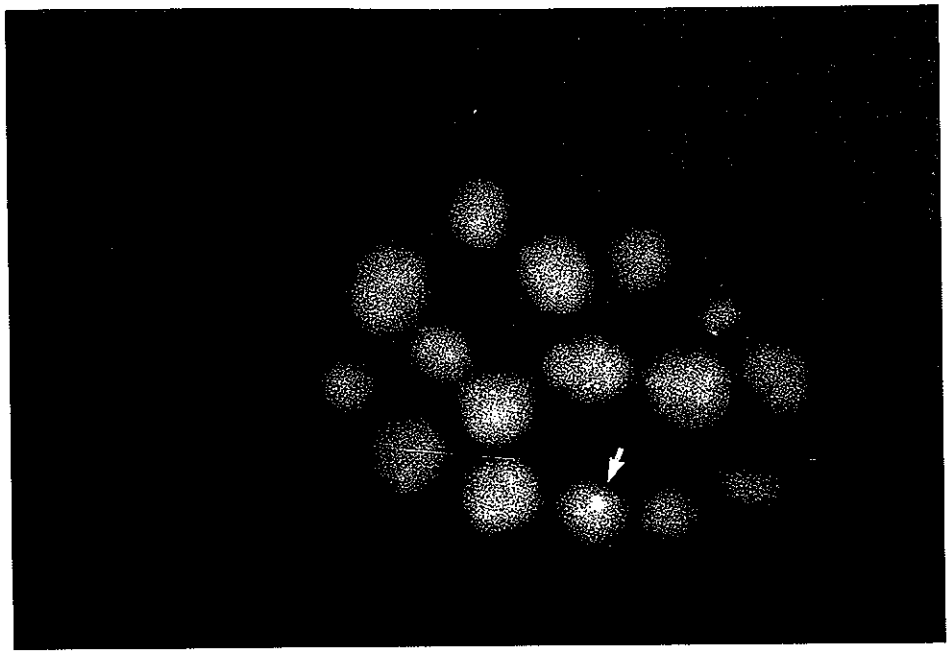

Figure 5.2
Photomicrograph, showing fluorescence in situ hybridization with a Y specific DNA probe on whole nuclei from patient a. Only a small fraction (5%) bears the Y chromosome as is visualized by a clear white spot (arrow). Nuclei are counterstained with propidium iodide (grey). Magnification 40 X objective

These results match the conventional cytogenetic analysis of this patient very well. In 83% of the metaphases the loss of the Y chromosome was noticed and a large deletion was found for the q arm of chromosome 15. In the conventional cytogenetic analysis which was derived from 31 metaphase spreads, no marker chromosome was found that could explain for the cluster that was indicated by M in the flow karyogram.

The flow karyogram from the sex-mismatched female bone marrow transplanted patient (b) is shown in Figure 5.3. In the right panel the normal flow karyogram from the male donor origin can be seen after stimulation of the cells with PHA, as can be recognized by the Y chromosome. In the left panel the Philadelphia chromosome can be seen as indicated. The Y chromosome is absent in this panel.

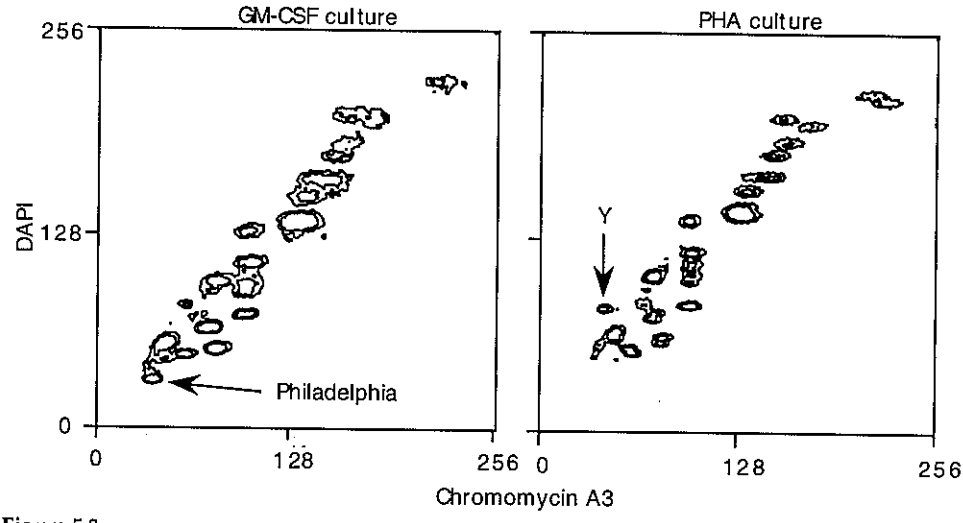

Figure 5.3
Bivariate flow karyograms from the same blood sample of a female CML patient four years after a sex-mismatched bone marrow transplantation.
Left panel: GM-CSF simulated cells.
Right panel: PHA stimulated cells.
The leukemia- and donor derived chromosomes are indicated by arrows.
A limited amount of contour lines was drawn at levels that showed most optimal cluster separation.

In situ hybridization to mononuclear peripheral blood cells of this patient revealed the presence of a Y chromosome in 68% of the cells (Figure 5.4)

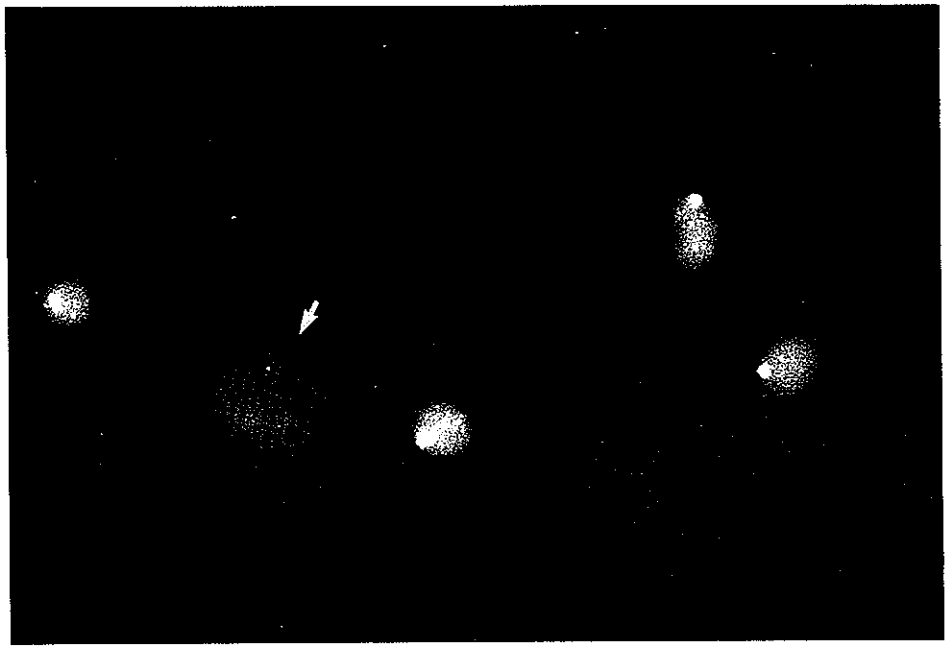

Figure 5.4
Fluorescence in situ hybridization with a Y specific DNA probe on whole nuclei from patient b. Among the Y-bearing cells of donor type, the presence of host derived leukemic cells (arrow) is evident.

5.5 Discussion

As is demonstrated by this study, leukemia-specific chromosomal aberrations can be detected by flow karyotyping very effectively.

For the analysis of flow karyograms in which small deviations of the expected chromosome position might occur as a result of amplifications, deletions and small translocations, it is very important to have a reference to exclude the possibility that variations in the positions of chromosome clusters in flow karyograms are caused by homologue differences. The flow karyogram from normal cells of a patient can serve as an internal reference for the discrimination between tumor-associated aberrations and homologue differences in this individual; in case of leukemia: the normal T-lymphocytes. The approach followed in this study was to collect the normal T-cells from the blood sample of leukemia patients. Separate culturing and specific stimulation of the T-cell-depleted and the T-cell-enriched fraction and subsequent flow karyotyping revealed whether differences appeared between the two flow karyograms (indicating the presence of leukemic cells). As can be seen in the case of patient a, the presence of leukemia associated aberrations (among which the loss of the Y chromosome) is only noticed in the T-cell-depleted and GM-CSF and IL3

stimulated fraction whereas the presence of the Y chromosome indicates that only normal cells were stimulated in the T-cell enriched fraction. In this way leukemia-associated chromosomal aberrations can be identified.

The possibility of stimulating different subsets of cells also provides a means of examining the chimeric state in (sex-mismatched) bone marrow transplanted patients. Again the difference in chromosomal constitution serves as an indicator for the type of cells that were stimulated and produced the flow karyogram. In the case of patient b, it is apparent that the leukemic cells of recipient origin start to take over while there still remains a fraction of normal donor derived T-lymphocytes. In this case the presence of the Philadelphia chromosome is evident; no homologue of chromosome 22 will appear in this region.

As demonstrated in this study the success of flow karyotyping of leukemia specimens mainly depends on the type of stimulating agent that is used for the outgrowth in vitro of a specific subpopulation in the sample. The specific outgrowth of a certain cell type does not allow to draw conclusions from the flow karyogram about the percentage of a certain type of aberration present in the specimen. It allows one to give a yes or no answer about the presence or absence of a certain subpopulation. Further, flow karyotyping gives insight in the type of aberration that is present. On the basis of this information in situ hybridization on nuclei with selected probes can confirm the aberrations and determine the real percentage of the leukemic fraction in the original sample without the bias of in vitro culturing.

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Chapter VI

Follow-up study of leukemia patients after chemotherapy or sex-mismatched bone marrow transplantation using fluorescence in situ hybridization with chromosome specific repetitive DNA probes

Part of this work is submitted for publication
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6.1 Abstract

The purpose of this study was to explore the applicability of fluorescence in situ hybridization (FISH) with chromosome specific repetitive DNA probes for long term follow-up of leukemia patients and of patients that received a sex-mismatched bone marrow transplantation. Patients with myelodysplasia or acute leukemia whose leukemic cells carried numerical chromosomal aberrations were selected and followed with alpha satellite DNA probes that specifically hybridize to one chromosome type. The lower level of detection for aberrant cells is set by the naturally occurring background of cells with numerically aberrant appearance in normal populations of blood and bone marrow cells from healthy individuals. This background was found to range between 0.3-2 percent for the probes used. Although FISH data before and after chemotherapy were generally in agreement with morphological findings discrepancies were noticed in specific cases. These could be explained by the presence of cytogenetically distinct sub-clones that behave differently during treatment, the presence of fully differentiated leukemic cells, changes in the chromosomal constitution (clonal relapse) or misinterpretation of the initial karyotype.

Follow-up of patients that received a sex-mismatched allogeneic bone marrow transplantation, using simultaneous hybridizations with DNA probes specific for the X or for the Y chromosome, demonstrated that some patients, although in complete remission for several years, carried low numbers of recipient cells during the entire period of follow-up. In two cases a cytogenetic relapse was observed by the reappearance of high percentages of recipient type cells.

FISH was found to be a valuable tool for detection of leukemic cells during longer periods of time, with an approximate lower detection limit of 0.3-2 percent. It offers the possibility to rapidly monitor the efficacy of chemotherapy in leukemia and can be used for follow-up after treatment or to assess chimerism after sex-mismatched bone marrow transplantation.

6.2 Introduction

The ability to monitor leukemia patients during chemotherapy treatment with or without subsequent bone marrow transplantation for the presence of residual leukemic cells depends on the characteristics of the leukemic cells and the choice of the detection method. Parameters have to be chosen that allow discrimination of the leukemic cells from their normal counterparts. At the cytogenetic level a considerable fraction of the leukemia cases can be discriminated on the basis of numerical chromosomal aberrations (gain or loss of one or more chromosomes). Numerical aberrations occur in approximately 54% of the patients with acute myelocytic leukemia (AML). In 30% of children and 5% of adults with acute

lymphocytic leukemia (ALL) a hyperdiploid karyotype is found. A near-haploid karyotype is found in 1% of the ALL cases. In chronic myelocytic leukemia (CML) in blast crisis numerical chromosomal changes reach up to 70% (Heim and Mitelman 1987, Sandberg 1990). Although routine cytogenetic analysis provides detailed information on the complete karyogram, it only allows the analysis of a limited number of cells from the dividing fraction in a bone marrow sample and its use is therefore restricted to situations where high numbers of aberrant cells are present (e.g. at diagnosis or at relapse).

In situ hybridization (ISH) has become a powerful technique to localize chromosome specific nucleic acid sequences in the cell (Cremer et al. 1986, Pinkel et al. 1986, Trask 1991, Jenkins et al. 1992). Fluorescent detection of specific alpha satellite repetitive probes in combination with ISH (FISH) enables the recognition of the centromeres on the chromosomes as clearly localized and brightly fluorescent spots in metaphase spreads or in nuclei. This provides a means to enumerate the copy number of chromosomes. The number of centromeres present in the cell will be reflected by the number of fluorescent spots per nucleus. Numerical aberrations have been detected in this way in malignant cells(Cremer et al. 1986, Devilee et al. 1988b, Hopman et al. 1988, Anastasi et al. 1990, Van Dekken et al. 1990b, Jenkins et al. 1992, Pagliaro and Stanley 1993). The detection of small numbers of malignant cells bearing numerical aberrations using the FISH procedure might be hampered by the fact that in peripheral blood cells from healthy individuals small numbers of cells display an aberrant number of spots. This has been shown in normal cell populations from healthy volunteers (Poddighe et al. 1991).

In patients who underwent a sex-mismatched allogeneic bone marrow transplantation (SMM allo-BMT) the presence or absence of donor or host cells is reflected by the presence or absence of the corresponding sex chromosomes. With FISH, chimerism has been demonstrated using the sex chromosomes as markers (Durnam et al. 1989, Van Dekken et al. 1989, Arkesteijn et al. 1990, Przepiorka et al. 1990, Przepiorka et al. 1991, Bernasconi et al. 1993, Dewald et al. 1993a, Wessmann et al. 1993). The presence of one X chromosome is always correlated with a Y chromosome in a male cell and with a second X chromosome in a female cell. Therefore this group of patients is the ideal target for chimerism studies with double colour FISH in which two chromosomes can be detected simultaneously in one cell using two different probes and two different fluorescent dyes.

In this study we have examined the feasibility of the FISH procedure for the follow-up of leukemia patients. Firstly, we optimized the FISH procedure and determined in normal individuals the performance of the probes and the background frequencies of peripheral blood cells with aberrant numbers of spots. Secondly, for our follow-up study we selected patients with numerical chromosomal aberrations in their leukemic cells as judged by conventional cytogenetic analysis and followed them from the time of diagnosis, through the phase of complete remission

(detection of "minimal residual disease") till relapse, if this occurred. In a third study we followed patients who underwent sex-mismatched allogeneic BMT (SMM allo-BMT) with double colour FISH on the basis of the presence or absence of donor- or recipient sex chromosomes to determine the degree of chimerism. All patient studies were performed on bone marrow samples.

6.3 Materials and Methods

Peripheral blood and bone marrow samples

Peripheral blood from a healthy male volunteer was collected in a heparinized vacuum tube. Bone marrow from leukemia patients was collected in sterile heparinized flasks. After collection and during the process of cell separation the samples were kept at 4 C. The red cells were lysed by adding excess buffer, consisting of 155 mM NH_4Cl , 0.1 mM EDTA , and 11.9 mM $NaHCO_3$, to the blood or bone marrow samples. After centrifugation, the remaining nucleated cells were washed twice in Hanks HEPES Buffered Balanced Salt Solution.

Labeling of probe DNA

Complete plasmid with insert was labeled with either biotin-16-dUTP or digoxigenin-11-dUTP using the nick-translation procedure (Sambrook et al. 1989). Probes used in this study are listed in Table 6.1 (Cooke 1979, Willard et al. 1983, Devilee et al. 1986, Donlon et al. 1986, McDermid et al. 1986, Waye and Willard 1986, Moyzis et al. 1987, Waye et al. 1987a, Waye et al. 1987b, Devilee et al. 1988a, Greig et al. 1989, Waye and Willard 1989, Looijenga et al. 1990). After nick translation the labeled products were tested for the amount of incorporated biotin using a direct spot-blot method and for the size of the probe fragments by alkaline gel electrophoresis and blotting. Average probe fragment sizes ranged between 200 and 400 base pairs.

Fluorescence in situ hybridization

Cell suspensions prepared as described above were treated with hypotonic buffer (75 mM KCl) for 10 minutes at room temperature. Cells were spun down and fixed with Carnoy s fixative under continuous vortexing. Fixation was repeated 3 times. Fixed cells were used either directly or were stored in fixative at -20 C for later analysis. After fixation cells were spotted onto cleaned microscope slides and the area was marked with a diamond tipped pen.

Directly before the hybridization procedure slides were placed in ethanol for at least 60 minutes at room temperature and air dried. Slides were then incubated in 0.1 M HCl/0.1% Tween-20 for 15 minutes to remove matrix and histon proteins and to improve accessibility of the nuclei for the DNA probe.

Denaturation of the target DNA was accomplished by placing the slides in 70% formamide/2 x SSC pH 7 at 70°C for 2 minutes followed by dehydration in an ice-cold ethanol series of 70%, 85% and 100%. The hybridization mixture consisted of 1 μg/ml probe DNA, 1 mg/ml sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate and 2 x SSC in 50% formamide at pH 7. The probe was denatured in the hybridization mixture for 10 minutes at 90°C and quenched on ice immediately. Approximately 12 μl of this denatured hybridization mixture was pipetted onto the marked area of the microscope slide. The area was covered with a plastic coverslip and the slide was placed in a humid atmosphere at 37°C. Hybridization was performed for 4 hours. After hybridization slides were washed 3 times for 2 minutes in 2 x SSC followed by a stringent washing in 50% formamide, 2 x SSC. Five minutes at 45°C was used routinely but with probes known for high non-specific binding, stringency was increased by either increase of the temperature, formamide concentration or time of washing. Slides were washed in 2 x SSC and finally placed in 4 x SSC/ 0.05% Triton-x-100(SSC-T).

Visualization of the biotin-labeled probe binding was accomplished by incubation of the slides with avidin-FITC (av-FITC) conjugate; 5 μg/ml (Vector, Burlingame, CA, USA) in 5% non-fat dry milk in SSC-T with 0.002% sodium-azide, for 20 minutes at 37°C. After this period cells were washed 3 times for 2 minutes each with SSC-T buffer. In those cases where amplification of the signal was required, slides were incubated with biotin-labeled goat-anti-avidin; 5 μg/ml (Vector, Burlingame, Ca, USA) in 5% non-fat dry milk, for 20 minutes at 37°C. After washing the av-FITC incubation step was repeated. When single hybridizations were performed, nuclear DNA was counter stained with propidium-iodide (1 μg/ml) in Slowfade (Molecular Probes, Eugene, OR, USA). When double hybridizations were performed the biotin-labeled probes were detected using Avidin-Texas red (Vector, Burlingame, CA, USA) and the digoxigenin labeled probes were detected using anti-digoxigenin-FITC (green fluorescence) while DNA was counter stained with DAPI (1μg/ml).

Scoring of fluorescent spots.

A Zeiss Axioskop-20 microscope was used. Screening of the slides was performed with 63 x objective lens. Every nucleus in the field of view was taken into account. Per slide 300 to 500 nuclei were scored. When the fluorescent signals did not allow a classification to a group with a discrete number of spots the nuclei were scored as "1 or 2", or "split spots" (i.e. fluorescent spots were seen that seemed to be split in two, thus appearing as two smaller spots close together each with half the fluorescence intensity of a single spot). In Table 6.1 the scoring is given in this detailed way. In the following Tables only the percentage of cells with clear-cut fluorescent signals in leukemia or donor/host type of cells is shown. This explains why in Table 6.4 the sum of the percentage host and donor cells is not exactly 100. Per patient each subsequent sample was scored by the same observer.

6.4 Results

Probe performance.

In Table 6.1 the results of a number of probes that were used for FISH are listed. Analysis of peripheral blood cells from a healthy male volunteer revealed that with the FISH method a small percentage of cells appeared to have aberrant numbers of spots. This was the case for all probes tested. For 7 out of 13 autosome specific probes and for the 2 sex chromosome specific probes the frequency of expected numbers of spots per cell exceeds 95%, 3 probes score in-between 94% and 95% and the remaining 3 probes score lower. The probe for chromosome 20 performs slightly lower than 90%. Rarely, nuclei were observed with no spots at all. Observations that are difficult to interpret (i.e. 1 or 2 spots or split spots) are listed in columns 7 and 8 respectively. The percentage of cells missing one fluorescent spot ranges from 5.6 (probe 20) to 0.2 (Yprobe) and the percentage of cells that had one additional fluorescent spot ranges from 2.1 (probe 8) to 0.3 (probes 10 and 12). The presence of nuclei with an aberrant number of fluorescent spots in normal cell populations implies a lower detection level for the analysis of leukemia samples.

Table 6.1 Probe performance on healthy male volunteer blood cells as observed by fluorescence in situ hybridization (in % of cells)

			Frequency of fluorescent spots per nucleus					
	probe							
chromosome	name	reference*	0	1	2	3	1 or 2	split
1	PUC1.77	22		1.4	95.1	0.8	1.9	0.8
3	pa3.5	23	0.8	1.9	96.1	1.2		
7	p7t1	24	0.3	0.3	96.7	0.6	0.9	1.2
8	D8Z2	25		0.9	93.5	2.1	1.5	2.0
9	pHUR98	26	0.3	1.8	94.3	0.9	1.5	1.2
10	D10Z1	27		0.3	97.0	0.3	0.9	1.5
11	pLC11A	28		1.1	96.0	0.8	0.8	1.3
12	pα12H8	29	0.3	0.8	95.4	0.3	1.6	1.6
16	pSE16	30		2.3	92.2	0.6	2.3	2.6
17	p17H8	31		2.1	95.2	0.6	1.5	0.6
18	L1.84	32		1.4	94.9	0.6	1.1	2.0
20	p3.4	33		5.6	89.7	1.3	1.3	2.1
22	p22/1:2.1	23		0.6	95.3	0.9	1.6	1.6
X	pBAMX5	34	1.0	98.6	0.4			
Y	pY2.45	Amersham	0.2	99.4	0.4			

Split spots are defined as two smaller spots close together, each with approximately half the fluorescence intensity of a single spot.

350 nuclei were scored per probe.

*: refers to the article in which the probe is described.

The findings on the peripheral blood samples were taken as a guideline for probe performance but not used to determine the ultimate lower detection level for each probe in bone marrow samples.

Leukemia follow-up

In Table 6.2 the results are given from 8 patients with acute leukemia under therapy. For each patient, the diagnosis and the time point after diagnosis at which bone marrow samples were taken are indicated in the second and third column. In the fourth column the results from conventional cytogenetic analysis are indicated of which only numerical aberrations are given that were found at the specific time points. When no aberrant cells were found, the normal karyotype was listed. The fifth column shows the percentage of blast cells in the bone marrow smear found by cytology. Column 6 indicates the probe that is used. Column 7 shows the percentage of aberrant cells as judged by the number of fluorescent spots per nucleus.

In general, FISH data as well as morphology data show a sharp decrease in the percentage of aberrant cells or blasts after the first course of chemotherapy. Once in remission, in 4 cases (patients 1, 3, 5 and 8), values of aberrant cells established by FISH remained around the levels of those observed in the normal cells from the healthy volunteer (Table 6.1). In three cases (patients 2, 6 and 7) a relapse was observed as judged by the increasing percentage of nuclei with numerical aberrations. Conventional cytogenetic analysis was performed only occasionally at the follow-up time points. In two cases (patient 2: 36 months after diagnosis and patient 3: 5 months after diagnosis) patients were morphologically close to a complete remission. In both cases 100% normal karyotypes were found in the bone marrow whereas FISH showed 3.3% and 0.9% of aberrant cells respectively. Despite the high percentage of blasts in the bone marrow sample from patient 6 at 12 months after diagnosis, the conventional cytogenetic analysis does not indicate the presence of a -Y.

In several cases the percentage of aberrant cells as observed by FISH did not correspond with the percentage of blasts as found by morphology. In patient 4 the percentage of aberrant cells that was found by FISH was much higher than the percentage of blasts found by morphology.

Table 6.2 Follow up of bone marrow samples from leukemia patients with numerical aberrations employing FISH with chromosome specific probes

Patient	Diagnosis (FAB)	time point (months)	Cytogenetic aberration[1]	% Blasts (cytology)	Probe used for FISH	% Numerical aberration (FISH)[2]	
1	AML-M1	0	+10 (100%)	90.0	10	59.3	
		1	-	15.8		1.3	
		2.5	-	14.4		0.6	
		4	-	4.2		0.6	
		5	-	3.0		0.6	
		5.5	-	3.6		0.6	
		9	-	19.6		0.6	
		9.5	-	8.0		0.6	
2	AML-M1	0	+11 (100%)	62.0	11	-	
		36	46, XY (100%)	10.4		3.3	
		38	-	2.6		3.1	
		40.5	-	-		2.9	
		49	-	62.4		25.2	
3	AML-M2	0	+8 (100%)	54.0	8	-	
		5	46, XX (100%)	6.2		0.9	
		6	-	5.0		0.6	
		11	-	4.6		0.9	
		16	-	0.8		0.6	
		21	-	3.2		0.3	
4	AML-M2	0		43.6	Y	97.5	
		1.5	-	4.2		10.8	
		3	-	4.6		1.0	
		4	-	3.8		5.3	
		4.5	-	3.0		3.4	
5	AML-M2	0	minus Y (100%)	81.5	Y	-	
		4	-	78.8		88.8	
		5	-	5.0		3.3	
		6.5	-	3.8		0.9	
		8.5	-	0.6		1.1	
		9.5	-	1.8		0	
		13.5	-	2.8		0.2	
		15	-	1.4		0.6	
6	AML-M4	0	minus Y (14%)	42.0	Y	-	
		3	-	2.6		0.2	
		5	-	4.0		0.3	
		12	46, XY 100%	80.0		95.3	
						#7	#17
7	RAEB	0	(64%), -17(76%	8.3	7 and 17	-	-
		17	-	17.7		67.8	4.9
		18.5	-	0.4		3.5	5.4

Table 6.2 continued

		19	-	dry tap		17.3	2.5
		20	-	>> 5		16.7	1.8
8	AML-M2	0	+8 (100%)	35.4	8	37.3	
		1.5	-	4.6		1.1	
		3	-	2.8		0.0	

1: diagnosed by conventional cytogenetics
2: 300-500 nuclei were scored per sample
3: bone marrow biopsy
-: not determined

The percentage of cells characterized by -Y at 4 and 4.5 months after diagnosis was 5.3 and 3.4 respectively, which is well above the lower detection level of this probe. Blast cell frequencies smaller than 5% indicate morphological complete remission at these timepoints. Proof of imminent relapse was not obtained since this patient died because of a pneumomitis infection shortly after the last bone marrow sample was taken. In patient 7, 67.8% of the cells carry a monosomy for chromosome 7 at 17 months after initial diagnosis while at this stage 17.7% of blast cells are found in the bone marrow. Chemotherapy was given and after an initial decrease in numerically aberrant cell numbers, an increase is observed at 19-20 months that concurs with the rise of the percentage of blast cells in the bone marrow as judged by bone marrow biopsy. In contrast to the observations with the chromosome 7 specific probe, FISH in this patient with a probe specific for chromosome 17, which was reported to be a second numerical aberration, showed low numbers of aberrant cells at any time point and no increase at the time of relapse.

In patient 1, a 90% blast cell count was observed in the bone marrow at diagnosis. This was much higher than the percentage of cells with a trisomy for chromosome 10 found by FISH (59.3%). After the first course of chemotherapy the percentage of cells with a trisomy 10 as observed by FISH dropped to a level close to that of the threshold that can be expected for the chromosome 10 probe. Yet, only a partial remission was achieved (15.8% blasts by morphology). From this time point on the low number of aberrant cells as determined by FISH remained unaltered. The second treatment course did not further reduce the number of blasts in the bone marrow (14.4%). A complete remission was achieved after the third course of chemotherapy. Nine months after diagnosis an increase in blasts was observed (19.6%) while the analysis with FISH remained negative.

Sex-mismatched allo-BMT.

The sex-mismatched bone marrow transplanted patients have been followed using double hybridization with the sex chromosomes as indicators for the presence of host cells in the bone marrow. In control experiments, in which 400 nuclei were scored using double hybridization on normal peripheral blood cells from healthy volunteers, nuclei with a pattern of fluorescent spots that would suggest the opposite

sex were not found, implying a higher sensitivity due to the absence of double false signals (Table 6.3).

Table 6.3 Double hybridization with X and Y chromosome specific probes on healthy donor blood cells (male; % of cells)

	spots per nucleus X probe			
Y probe	0	1	2	split
0	-	0.6	-	-
1	0.3	96.1	0.3	1.4
2	-	-	-	-
split	-	0.6	-	0.8

400 nuclei were scored

The results are given in Table 6.4. The first column shows the diagnosis and the sex-mismatch (male to female or female to male). Column 2 shows the time after transplantation at which bone marrow samples were taken. Columns 3 and 4 show the conventional cytogenetic data, column 5 shows the percentage of host cells and column 6 shows the percentage of donor cells found by FISH. In the double hybridization experiments recipient derived cells were found in almost every sample.

Table 6.4 Bone marrow follow up of sex-mismatched allo-BMT patients with FISH using double hybridization with X and Y probes

	months after	conventional cytogenetics		FISH	
patient	allo BMT	% host cells	% donor cells	% host cells	% donor cells
patient 1	12	0	100	0.7	98.1
AML-M2	15.5	0	100	0.4	98.9
female to male	18.5	-	-	0.0	97.4
patient 2	32	-	-	0.0	98.6
ALL	37	-	-	0.0	97.4
female to male	45.5	0	100	0.0	98.0
	58	0	100	0.0	98.0
patient 3	1	0	100	0.4	97.8
AML	7	0	100	0.7	98.1
female to male	9.5	0	100	0.2	98.0
	12.5	0	100	1.2	97.9
	20	0	100	0.0	97.6
	31	15	85	24.4	75.6

Table 6. 4 continued

patient 4	-1.3	100	0	99.4	0.0
CML-Ph+	25	0	100	2.1	95.8
male to female	38	65.5	34.5	55.1	43.9
	51	91	9	97.0	2.4
patient 5	1.5	0	100	0.0	100
AML-M3	6	0	100	0.0	100
male to female	9	0	100	0.0	99.3
	12	0	100	0.0	100
	18	0	100	0.2	98.8
	24	0	100	0.0	99.2
patient 6	6	0	100	0.0	97.4
CML-Ph+	13	0	100	3.3	95.0
male to female	18	0	100	3.0	95.4
	24	0	100	2.9	96.6
patient 7	-1	100	0	96.2	0.0
CML-Ph+	4	47	53	33.5	62.0
male to female	5	-	-	38.1	60.2
	5.5	-	-	43.9	51.6
	6	-	-	47.3	48.5
	6.5	50	50	48.1	46.9

T-cell depletion of the graft was performed in all patients

Patients 4 and 7: first samples were taken shortly before BMT

400-500 nuclei were scored

- : not determined

Two cases (patients 2 and 5) were completely reconstituted by hematopoietic cells from the donor (full chimerism). In patient 2 no host cells were observed in the bone marrow up to 58 months after transplantation while in patient 5 only one host derived cell was found 18 months after BMT. The other cases showed the continuous presence of host cells in their bone marrow. In two of those a leukemia relapse was observed after transplantation (patients 3 and 4). Patient 4 clearly showed an increase in host derived cells in the bone marrow detected by FISH, while at the same time conventional cytogenetic analysis showed a recurrence of Philadelphia chromosome positive cells. Though apparently in cytogenetic relapse, to date the morphological findings do not indicate a hematological relapse. In patient 7 who received a matched unrelated donor transplantation, high percentages of host cells were present 4 months after transplantation. So far, Philadelphia chromosome positive cells have not reappeared.

6.5 Discussion

FISH has several advantages that makes it an attractive method in addition to conventional cytogenetic analysis. No short term cultures are required, analysis of the metaphases and recognition of the banded chromosomes is not necessary and due to the fact that interphase nuclei which are derived directly from the patient are studied, selective outgrowth of subpopulations during short term culture is prevented and a representative percentage of aberrant cells in the patient is obtained. The number of nuclei that can be analyzed routinely with FISH is at least tenfold higher than the number of metaphases that is routinely analyzed with conventional cytogenetic analysis. Therefore FISH allows rapid quantitative analysis and follow up of patient material from diagnosis through complete remission.

As demonstrated in this study, in the blood from the healthy volunteer a small number of cells can be found with an aberrant number of chromosomes when analyzed with FISH. For some probes low numbers of such cells are found (e.g. probes specific for chromosomes 10, X, Y) For other probes this frequency is higher (e.g. the probe that is specific for chromosome 20). When the number of spots per nucleus is lower than expected, this might be due to poor penetration of the probe, incomplete denaturation or loss of target DNA during the hybridization procedure. Furthermore, overlapping signals that cannot be distinguished in two-dimensional microscopic analysis might contribute to this phenomenon. A higher number of spots per nucleus might be due to non-specific probe binding to other centromeres and subsequent incomplete stringent washing. On the other hand some cells actually might contain aberrant copy numbers for particular chromosomes. Incomplete or rearranged karyotypes in healthy individuals have been observed (Prieur et al. 1988, Kuffel et al. 1991) and the older age of the individual seems to play a role in this phenomenon. Especially in fully differentiated cells the presence or absence of chromosomes will not be detrimental for vital cellular functions provided that relevant genes for those particular functions are not involved. For the detection of leukemia, the percentage of cells with aberrant numbers of spots in bone marrow from patients has to be significantly different from the control values. Different tests have been evaluated (Kibbelaar et al. 1993) in which controls as well as artificial aberrant cell mixtures were made up of the same cell sample. Since a proper control for studies on bone marrow from hematological malignancies is not available (i.e. normal bone marrow cells from the same individual), we considered the values found in nuclei from peripheral blood of a healthy volunteer as a guideline (Table 6.1). With respect to these values the background level for the detection of aberrant cells is reached at a range of 0.3% to 2% (probe 20 not included) depending on the probe that is used and the type of aberration that has to be detected. In this respect our control studies do not greatly deviate from what others have published (Poddighe et al. 1991, Chen et al. 1992) indicating an appropriate FISH methodology.

In the patient studies, the data obtained by FISH were compared to the morphological criteria that are routinely used to determine the remission status of the leukemia patient. In general the FISH data were in agreement with data obtained by cytology. Nevertheless, some patients had higher (patients 1 and 4) others lower (patient 7) numbers of aberrant cells as determined by FISH. The presence of a higher number of aberrant cells can only be explained by the fact that differentiated, non-blast like cells, carry the numerical aberration that is detected by FISH. A lower number of aberrant cells can occur when the conventional cytogenetic analysis indicates a specific numerical aberration while the target region for the probe is not involved and therefore still present in the nucleus as a diploid set. Also subclones with specific numerical aberrations can selectively be eliminated during chemotherapy when they are more sensitive for the treatment. The following cases from our studies further illustrate these points.

In patient 1 the percentage of cells carrying a trisomy for chromosome 10 as determined by FISH is almost twice as low as the percentage of blasts found in the diagnostic marrow aspiration. This observation strongly suggests the presence of at least two sub-clones in the patient, one of which carrying a trisomy for chromosome 10. Nevertheless conventional cytogenetic analysis resulted in a +10 in all the analyzed metaphases. Since conventional cytogenetic analysis allows only the analysis of a small number of cells and since only the dividing fraction can be studied, the non-trisomy containing subpopulation of the leukemic cells might have remained unnoticed. In subsequent marrow studies the percentage of cells with a trisomy 10 as detemined with FISH dropped and remained at a level close to the background values while the morphological data revealed presence of blast cells at several stages during the follow-up period. Apparently the trisomy 10 subpopulation was more sensitive to the chemotherapy employed and was therefore eradicated.

The diagnosis sample of patient 4, with a loss of the Y chromosome in all leukemic cells at diagnosis, shows a twofold higher amount of aberrant cells according to the FISH method as compared to cytology. Also at 1.5 months after diagnosis, when the patient had reached complete remission (4.2% blasts in the bone marrow), 10.8% of the bone marrow cells showed a loss of the Y chromosome. The only explanation for the discrepancy between the number of -Y cells by FISH and the number of blasts in the bone marrow is that differentiated leukemic cells are present. The question whether these cells are fully differentiated or still maintain leukemic potential remains unsolved.

Patient 7, characterized by a monosomy 7 and 17, showed a similar phenomenon as observed in patient 4. When a probe specific for chromosome 7 was used 17 months after diagnosis, the number of aberrant cells found with FISH was higher than the amount of blasts in the bone marrow at that timepoint. It is striking that the fluctuation in the frequency of cells carrying a monosomy 17 neither correlated with

the fluctuations in the percentage of blast cells nor with the percentage of cells with a -7 found by FISH at the same time points. In the leukemic cells of this particular patient a complex karyotype was found including a marker chromosome and the loss of one chromosome 17. Apparently, the centromere region of chromosome 17 was present in the marker and therefore in situ hybridization on interphase nuclei, applying the probe for the alpha satellite DNA from chromosome 17, resulted in two fluorescent spots.

Since in SMM allo-BMT patients the chromosomal constitution of each individual bone marrow cell is either donor or recipient type this offers the possibility to investigate in much greater detail the presence of recipient cells in these patients using the sex chromosomes as markers. Theoretically, after double hybridization with two different probes, the threshold level for double false hybridizations (i.e. cells appearing to be from the opposite sex) is reduced to the product of the chance of false positive or negative hybridizations for each of the individual probes. Threshold detection levels of 0.2% for the Y probe and 0.4% for the X probe results in a chance of 1/500 x 1/250 = 1/125,000 that both probes give a false signal in the same nucleus. In healthy donor material the presence of cells showing a hybridization pattern of the opposite sex for both probes was not found (Table 6.3). The method of scoring by microscope allows at the most the scoring of 1000 cells on a routine basis. For methods with thresholds reaching 1/125,000 other techniques are required. For this purpose the combination of FISH with flow cytometry employing intact nuclei is currently under investigation. This will allow the analysis of higher cell numbers provided that clear-cut discrimination can be achieved between the positive and negative populations.

The SMM allo-BMT patients that were followed could be divided into two categories. Those that had a complete hematopoietic reconstitution of donor bone marrow and those in which recipient type cells could be found. Two SMM allo-BMT patients showed a hematopoietic reconstitution that was (almost) completely of the donor type (patients 2 and 5). To date those patients are in complete remission. Four patients showed long lasting persistence of low levels of recipient cells in the bone marrow (patients 1, 3, 4 and 6) in percentages up to 3.3%. Two of these patients (patients 3 and 4) have developed a relapse. Although relatively high levels of recipient cells were continuously present in patient 7 no hematological signs of relapse were observed.

In the literature controversy exists with regards to the relationship between mixed chimerism and relapse (Singer et al. 1983, Schattenberg et al. 1989, Bernasconi et al. 1993, Roux et al. 1993, Schattenberg et al. 1993). That there is a correlation between mixed chimerism and relapse rate is suggestive but remains unproven by this study. Low numbers of host cells may indicate the presence of leukemic cells that are in a dormant state and therefore do not lead immediately to leukemia. On the other hand the host cells

might be derived from a small population of the normal hematopoietic system that survived marrow ablative therapy (mixed chimerism). At longer time intervals further normal host hematopoietic reconstitution must be considered. Finally the host cells may not be from the hematopoietic system at all but may have concurrently been collected together with the bone marrow aspiration. Stromal cells are not involved in the hematopoietic reconstitution after BMT and are therefore of donor origin (Athanasou et al. 1990). Since only the sex chromosomes are monitored more parameters should be taken into account like immunophenotyping, cell sorting, and subsequent in situ hybridization or DNA/RNA analysis, to elucidate the nature of these cells .

From the present study it can be concluded that FISH is a valuable tool for the sequential analysis of bone marrow samples from leukemia patients whose leukemia cells carry numerical chromosomal aberrations. The speed and ease with which FISH can be performed gives the procedure an advantage over conventional cytogenetic analysis and makes it an attractive technique for the assessment of treatment efficacy. The FISH method is in particular valuable on top of morphological criteria in those leukemias where no other specific characteristics are available to distinguish leukemic cells from the normal marrow cells.

Several points of consideration in detecting "minimal residual disease" have to be made with respect to the interpretation of the results. FISH data may not correlate with the actual disease progression as observed by morphology because of the possible presence of cytogenetically different sub-clones that might behave differently under chemotherapy. Furthermore, with the choice of the DNA probes, conventional cytogenetic data must be interpreted with care. Complex karyotypes might indicate the absence of chromosomes although the region for which the probe is specific is still present (e.g. in a translocation product or a marker chromosome).

FISH as it is used in this study will depend largely on the conventional cytogenetic analysis for the proper choice of probes. Conventional cytogenetic analysis gives highly detailed information from a small number of cells. FISH on the other hand provides less complex information from large numbers of cells. Both methods should therefore be used to supplement each other.

Acknowledgments

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Chapter VII

Hybridization of nuclei in suspension with chromosome specific DNA probes for the application of flow cytometric detection of chimerism in sex-mismatched bone marrow transplanted patients and for the detection of leukemia after sorting

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7.1 Summary

Flow cytometry was used to measure fluorescence intensity of nuclei that were subjected to fluorescence in situ hybridization in suspension with chromosome specific DNA probes. Paraformaldehyde-fixed nuclei were protein digested with trypsin and hybridized simultaneously with a biotin- and DIG-labeled chromosome specific centromere probe. A number of probes was tested in the suspension hybridizations. The method applied, yielded fluorescent hybridization signals that allow discrimination between Y chromosome positive and negative nuclei when analyzed by flow cytometry. The method is especially suited for analysis of bone marrow cells derived from patients who have received a sex-mismatched allogeneic bone marrow transplantation. Male leukemic cells with a trisomy for chromosome 8 were mixed with normal female cells and simultaneously hybridized in suspension with a DIG-labeled probe specific for chromosome 8 and the biotin-labeled Y chromosome probe. Y chromosome positive or negative nuclei were sorted onto microscope slides and subsequently classified as being leukemic or not by fluorescence microscopy, on the basis of the presence of a trisomy for chromosome 8. A 120-fold enrichment could be achieved when 300 Y positive nuclei were sorted from a mixture originally containing 0.5% leukemic cells. Given the specificity of the flow cytometry and FISH procedure, the combination of the two methods can reach a lower detection level of 1 per 250.000

7.2 Introduction

Fluorescence in situ hybridization (FISH) with chromosome specific repetitive DNA probes can be used to identify numerical chromosome aberrations in cells (Devilee et al. 1988b, Hopman et al. 1988, Van Dekken et al. 1990b, Jenkins et al. 1992). In contrast to conventional cytogenetics, where metaphases are required, this interphase cytogenetic technique allows rapid analysis of a great number of cells.

The lower level of detection of FISH has been reported in literature to range from 1% to 5%, depending on the probe and the type of aberration (Poddighe et al. 1991, Chen et al. 1992). This level is set by the occurrence of nuclei with an aberrant number of spots in samples from healthy individuals. Further reduction of the level of detection would require additional independent tumor associated parameters together with the possibility to quantitatively enrich for specific subpopulations and the possibility to analyze a higher number of cells.

The combination of FISH and flow sorting would fulfill these requirements. A large number of cells can be measured in flow cytometry and sorting provides a means to selectively enrich for suspected cells. Hybridization of cells or nuclei in suspension is a prerequisite. FISH on cells or nuclei in suspension has been used for the study of the nuclear architecture by confocal laser scan microscopy (Trask et al. 1988, Van Dekken et

al. 1990d, van Dekken et al. 1990, Hulspas and Bauman 1992, Van Dekken and Hulspas 1993) but found only limited use for magnetic separation of chromosomes (Dudin et al. 1987, Dudin et al. 1988) and measurements by flow cytometry (Trask et al. 1985, Trask et al. 1988, Van Dekken et al. 1990a). In situ hybridization of cells or nuclei in suspension followed by flow cytometry can only be performed provided that the nuclei remain intact after hybridization. Because fixation of nuclei and the stringency of the in situ hybridization procedure negatively influence each other, a balance must be found between these procedures. Nuclei might be well hybridized but not suited for adequate flow cytometric measurement because positive and negative populations cannot be discriminated due to an increased coefficient of variation or vice versa. In this study, a procedure is described by which nuclei retain enough of their shape to allow in situ hybridization in suspension and subsequent flow cytometric analysis of the hybridization signal.

It was found that the difference in fluorescence signal between nuclei with and nuclei without a Y chromosome is large enough to discriminate between positive and negative clusters when nuclei were hybridized with a biotin-labeled Y chromosome specific probe that was detected with Streptavidin-phycoerythrin (PE). Low numbers of host cells, that are frequently observed in the bone marrow in SMM-BMT patients, need to be further classified to be either normal host cells that survived marrow ablative therapy or residual leukemic cells. The technique described here, allows the study of chimerism in leukemia patients who received a sex-mismatched allogeneic bone marrow transplantation (SMM-BMT). With the combination of FISH and flow sorting, nuclei can be characterized as being leukemic or not. A more than a 1000-fold improvement of the lower detection level for residual leukemic cells is achieved compared to the sensitivity of the separate methods.

7.3 Materials and methods

Preparation of the nuclei

Bone marrow samples were diluted two to four times their volume with Hanks HEPES Buffered Balanced Salt Solution (HHBBSS), layered on top of 15 ml lymphocyte separation medium (LSM; Organon, Durham, NC) with a density of 1.077 g/cm^3 and centrifuged at 700 G during 20 minutes. The nucleated cells on the interface layer were collected and washed twice with HHBBSS and finally resuspended in buffer consisting of 20mM NaCl, 8mM $MgCl_2$ and 20mM Tris-HCl pH 7.5. After 5 minutes of incubation at 37 C the cells were lysed by addition of an equal volume of a Triton-x-100 solution (0.8% in water). Subsequently nuclei were released in suspension by careful shearing through the tip of an Eppendorf pipette.

In situ hybridization in suspension.

The nuclei were washed once more in the same buffer, fixed by adding an equal volume of paraformaldehyde solution (4% in PBS) for 16 hours at 4°C, centrifuged and resuspended in PBS afterwards. Approximately 10^6 fixed nuclei were centrifuged, treated by protein digestion with trypsin (0.25% in PBS) for 10 minutes at room temperature, washed with PBS and subsequently resuspended in hybridization mixture consisting of 1 mg/ml sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate and 2 x SSC in 50% formamide. Denaturation of the nuclei was accomplished by incubating at 85°C for 20 minutes under continuous shaking (Eppendorf Thermomixer, Hamburg, Germany). Biotin and the DIG-labeled chromosome specific probes (400 ng each) were denatured simultaneously in hybridization mixture for 10 minutes at 95°C. The denatured probes were added to the nuclei and allowed to hybridize for 4 hours at 37°C under continuous shaking. Probes that were used in the various experiments were repetitive DNA sequences specific for chromosome 1(Cooke 1979), 3(Waye and Willard 1989), 7(Waye et al. 1987b), 8(Donlon et al. 1986), 11(Waye et al. 1987a), 17(Waye and Willard 1986), 20(Waye and Willard 1989), X(Willard et al. 1983) and Y (Amersham, Buckinghamshire, UK.).

After hybridization nuclei were washed in 2 x SSC, centrifuged and resuspended in 50% formamide/2 x SSC at room temperature for 5 minutes. After washing in 2 x SSC, nuclei were resuspended in 4 x SSC with 0.05% triton (SSC-T), containing 5% non-fat dry milk and 10 µg/ml streptavidin-PE conjugate (Vector, Burlingame, Ca) for 1 hour at 37°C. After this period nuclei were washed once with SSC-T and resuspended in PBS. Finally nuclear DNA was stained with DAPI (Sigma, St. Louis, Mi) at 1 µM final concentration. For visual inspection prior to flow cytometry, a small sample was stained with avidin-FITC (Vector, Burlingame, Ca) and propidium iodide instead and analyzed by fluorescence microscopy. After sorting on slides nuclei were allowed to attach to the glass surface by drying. Slides were then placed in EtOH for at least 1 hour. When necessary, a stringent wash was performed to remove aspecific binding of the DIG-labeled probe. Anti-DIG-FITC conjugate (Boehringer, Mannheim, Germany) was used to visualize the DIG-labeled probe hybridization.

In situ hybridization on slides.

The procedure for in situ hybridization was a modified procedure originally described by Pinkel et al. (Pinkel et al. 1986). In brief, cells were fixed with methanol/acetic acid, spotted on clean microscope slides and placed in ethanol for one hour. Probes were labeled with biotin by nick translation. Probe and target DNA were denatured and allowed to hybridize for 4 hours. Hybridization was visualized with avidin-FITC. A Zeiss Axioskop-20 microscope was used to screen the slides with a 63 x objective lens. Every nucleus in the field of view was taken into account. Per slide 300 to 500 nuclei were scored.

Flow cytometry

Nuclei were run through a flow cytometer equipped with two lasers (FACS-Vantage, Becton Dickinson, San Jose, Ca). The system was triggered on the forward light scatter signal. PE was excited by 488 nm argon ion laser light at 100 mW laser power (laser: Coherent Innova 90, Palo Alto, Ca). PE fluorescence was measured through a 575/26 bandpass filter and logarithmically amplified. DAPI was excited with an argon ion laser tuned in the UV range (351 nm and 364 nm; laser: Spectra Physics, Series 2000, Mountain View, Ca) at 350 mW laser power. DAPI fluorescence was measured using a KV 408 filter (Schott Glaswerke, Mainz, Germany).

Mixtures were processed in ascending order to minimize the risk of contamination of nuclei from previous samples remaining in the tubing system. After each sample the system was flushed with bleach (10% in water). Data of approximately 20,000 nuclei were stored and subsequently analyzed using the Lysys-II program (Becton Dickinson, San Jose, Ca). In all sorting experiments only clusters representing the G1/G0 nuclei were sorted.

7.4 Results

When diploid cells from healthy individuals were analyzed with FISH, a small percentage of cells was found showing an aberrant chromosome number. Therefore FISH in suspension was compared to FISH on slides with the key question being: are the results obtained by FISH in suspension comparable to the results as obtained by FISH on slides? Nine probes were hybridized on peripheral blood cells from a healthy male individual according to both methods. The number of signals per cell was scored by fluorescence microscopy (Table 7.1). Columns 2 through 11 show the distribution of the number of fluorescent spots for the probe that were used. For each probe tested, few nuclei deviate from the expected diploid number of chromosomes either after hybridization in suspension or after hybridization on slides. Comparing the groups with the expected number of diploid spots (i.e.: Table 7.1; columns 6 and 7 for the autosomes and columns 4 and 5 for the sex chromosomes), it appears that in suspension hybridization results in a significantly higher number of diploid cells (paired t-test).

When nuclei that were hybridized in suspension were analyzed on the flow cytometer it appeared difficult to reproducibly find fluorescence differences between nuclei with 1 and 2 or higher numbers of hybridization targets. In contrast, analysis by flow cytometry of the nuclei hybridized in suspension with the Y probe consistently resulted in signal differences between Y chromosome positive and Y chromosome negative nuclei that showed up as clearly separated clusters in the bivariate dot plot (Figure 7.1). To test the sensitivity of the flow cytometric measurements artificial mixtures were made ranging from 0.1% to 99.9% male in female cells. Nuclei were isolated and hybridized in suspension with a biotin-labeled

Y chromosome specific probe and a DIG-labeled X chromosome specific probe and stained with streptavidin-PE to measure specific Y probe binding (Figure 7.1).

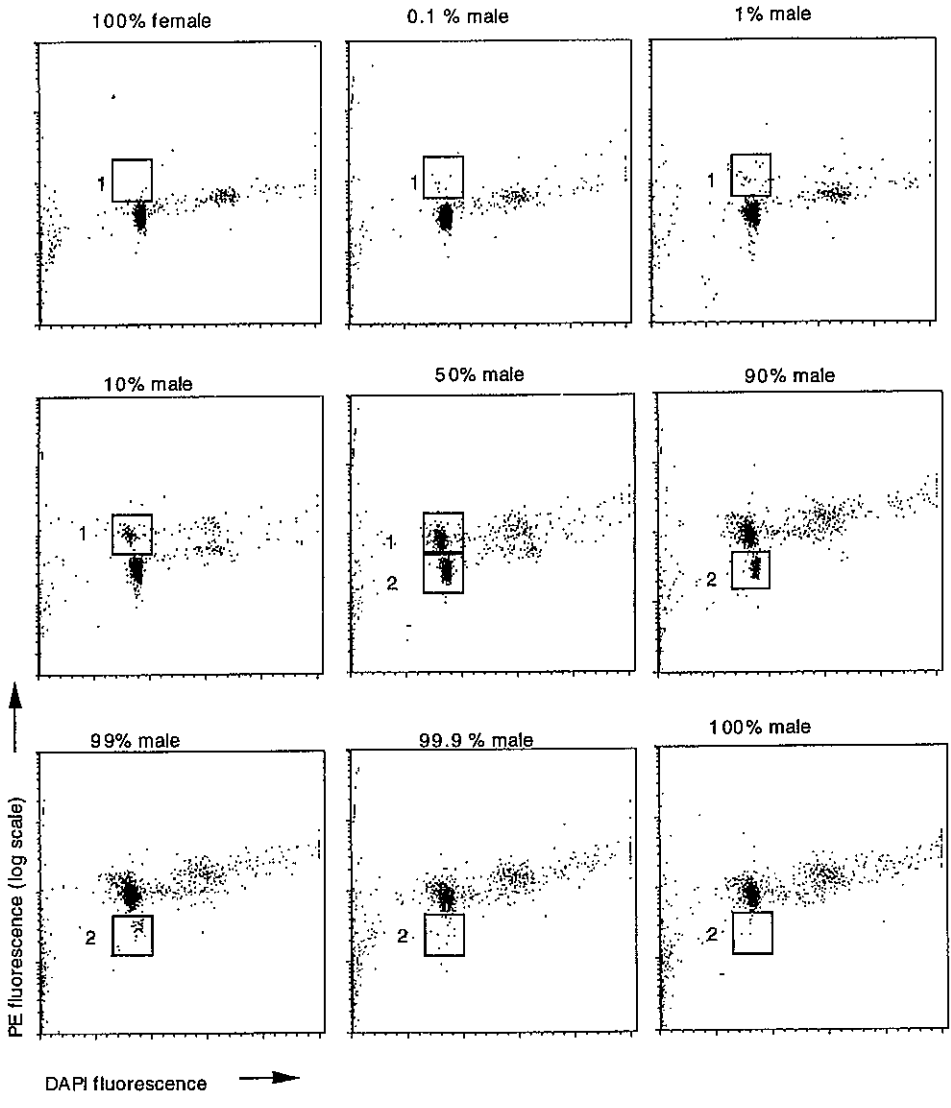

Figure 7.1
Bivariate dot plot of mixtures of male and female nuclei hybridized in suspension with biotin-labeled Y and DIG-labeled X chromosome specific DNA probes. Y probe binding is detected by streptavidin-PE. The dot plots are displayed with DAPI fluorescence (DNA content) on the horizontal axis and PE fluorescence (probe binding) on the vertical axis (logarithmic scale). Sort windows for Y positive and Y negative clusters are indicated as 1 and 2 respectively. The clusters in the area where windows 1 and 2 are set, represent the G1/G0 nuclei. The clusters at twice the DAPI fluorescence represent G2 nuclei and doublets of G1/G0 nuclei.

The percentage of male and female cells that was determined on the basis of flow cytometric analysis of the clusters is given in columns 2 and 3 from Table 7.2. In the mixtures ranging from 1% to 99% male cells, separate clusters could be identified (Figure 7.1). Nuclei were sorted from the regions of interest (indicated as "1" or "2" in Figure 7.1) directly onto microscope slides. The X probe binding was visualized by staining with anti-DIG-FITC conjugate and the nuclei were scored for the presence of one or two fluorescent spots and classified as male or female. Results are shown in Table 7.2.

Table 7.2 Recovery of male or female cells after in situ hybridization in suspension (X and Y probe), flow sorting on the basis of Y probe binding followed by microscopical analysis for chromosome X specific probe binding.

Male cells in mixture	Events inY positive window	Events inY negative window	Male cells (1 spot for the X probe) in Y positive window	Female cells (2 spots for the X probe) in Y negative window
0	0.3	99.7	0.7	-
0.1	0.5	99.5	23.2	-
1	1.8	98.2	72.4	-
10	9.8	90.2	94.7	-
50	38.5	61.5	96.0	96.4
90	83.3	16.7	-	98.4
99	97.6	2.4	-	89.1
99.9	99.3	0.7	-	54.9
100	99.5	0.5	-	1.0

all figures in percentages

\- = not determined

From the 50% samples, nuclei could be recovered with a high purity (more than 90%). With decreasing percentages of mixed cells the percentage of nuclei that was found back after sorting gradually dropped. Analysis of the sorted fractions of the lowest percentages of mixed cells showed that the amount of nuclei remains far above the lower detection level of FISH-on-slides alone. For the mixture containing 0.1% male cells sorting of the Y positive population resulted in 23.1% nuclei with one fluorescent spot for the X probe. In the reversed setting (0.1% female cells and sorting of the Y negative population) 54.9% of the nuclei were found with 2 fluorescent spots for the X probe. In the female control samples, 0.3% of the events were found in the Y probe positive area. After sorting of these nuclei it appeared that 0.7% had 1 spot for the X probe. In the male control samples 0.5% of all the events appear in the Y negative area, while 1% of these nuclei contained 2 fluorescent spots for the X probe.

Bone marrow cells were obtained from a female leukemia patient who received a SMM-BMT. Based on morphological criteria this patient was in a leukemia relapse. To investigate the applicability of FISH in suspension and analysis on the flow cytometer in such patients, the sex difference was used to discriminate between cell populations from host or donor origin. Analysis by flow cytometry resulted in two distinct clusters; a Y positive cell population with high PE fluorescence and a Y negative cell population (Figure 7.2).

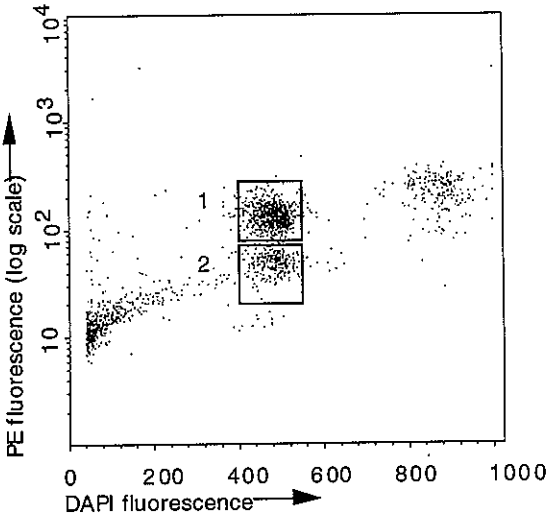

Figure 7.2
Nuclei originating from a female leukemia patient who had received a sex-mismatched bone marrow transplantation and who developed a leukemia relapse. Nuclei were hybridized in suspension with biotin-labeled Y chromosome specific DNA probe. Fluorescence detection of the Y probe binding and axes are as in Figure 7.1. The ratio of Y positive versus Y negative cells was determined by analysis of the number of events in windows 1 and 2.

In the Y positive window (region 1 in Figure 7.2) 10753 events were present and in the Y negative window (region 2 in Figure 7.2) 3793 events . Thus, 26% of the nucleated cells lacked the Y chromosome and were probably of host origin. Residual non-leukemic host cells, however, can be expected in chimeras. This requires an additional leukemia marker to classify these cells as leukemic or not. Therefore, it was investigated whether leukemic cells could be identified after sorting on the basis of the presence of leukemia associated numerical chromosomal aberrations. For this purpose marrow cells from a healthy female were mixed with marrow cells from a male leukemia patient in ratios ranging from 0.5 to 50%. According to conventional cytogenetic analysis, performed on 35 metaphases, 57% of the cells from the bone marrow of the male leukemia patient contained a trisomy for chromosome 8 and an additional 3% carried a tetrasomy 8. With conventional FISH on slides, 70.4% was found to contain three or more spots for chromosome 8 (300 cells were analyzed).

The mixtures were hybridized in suspension with a biotin-labeled Y chromosome specific probe and a DIG-labeled chromosome 8 specific probe and stained with streptavidin-PE to measure specific Y probe binding (Figure 7.3).

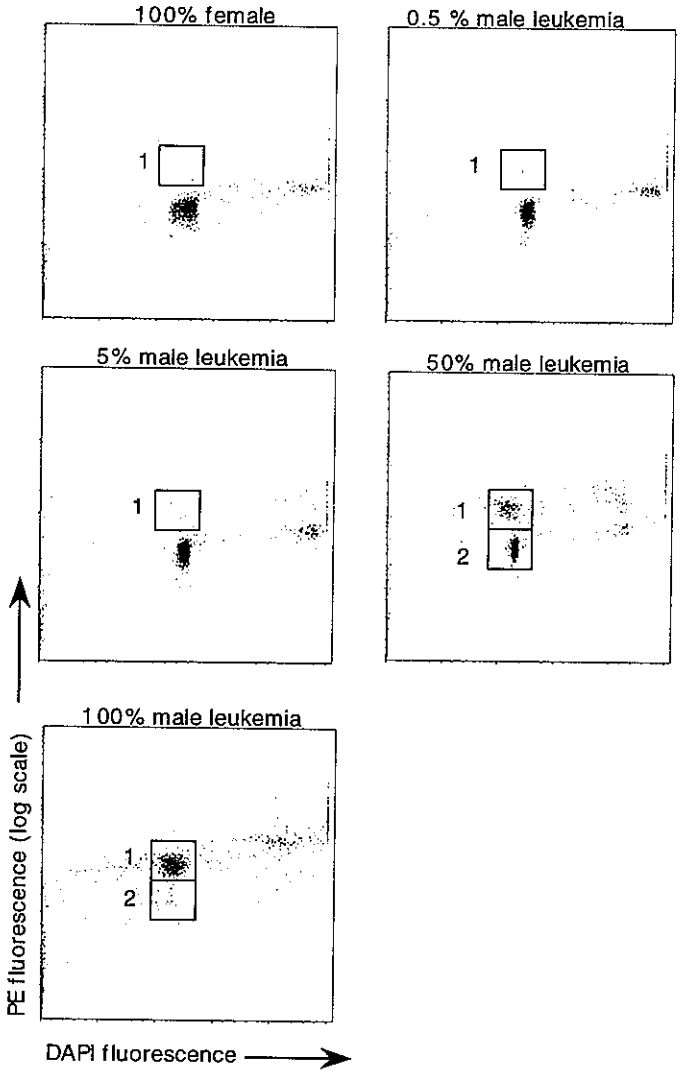

Figure 7.3
Bivariate dot plot of mixtures of male leukemia and normal female nuclei hybridized in suspension with biotin-labeled Y chromosome specific DNA probe and DIG-labeled probe specific for chromosome 8 . Fluorescence detection of the Y probe binding and axes are as in Figure 7.1. Sort windows for Y positive and Y negative clusters are indicated as 1 and 2 respectively.

Y positive and Y negative nuclei were sorted on slides (Figure 7.3; region 1 or 2). After staining of the probe 8 hybridization with Anti-DIG-FITC the presence of numerical aberrations for chromosome 8 was determined with fluorescence microscopy. In some of the sorted fractions the presence or absence of Y probe hybridization was verified by incubation of the sorted fractions on slides with avidin-FITC. The first column in Table 7.3 shows the mixtures that were made.

Table 7.3 Recovery of male leukemic cells after in situ hybridization in suspension (8 and Y probe), flow sorting on the basis of Y probe binding followed by microscopical analysis for chromosome 8 specific probe binding

			sorting of Y positive window		sorting of Y negative window	
male leukemic cells in mixture (%)	expected cells with 3 or more spots in mixture (%)[1]	cells in Y positive window (%)	cells with 3 or more spots for probe 8 (%)	cells reanalyzed for the presence of 1 Y spot (%)	cells with 3 or more spots for probe 8 (%)	cells reanalyzed for the presence of 1 Y spot (%)
0	0	0.1	0.39	0	-	-
0.5	0.4	0.5	40.9	-	-	-
5	3.5	1.9	68.4	-	-	-
50	35.2	29.9	71.3	-	1.7	-
100	70.4	96.8	68.1	99.0	68.4	78.0

1: Calculated from conventional FISH on slides (70.4% of all cells have 3 or more spots).

Column 2 shows the expected percentage of nuclei with three or more spots for chromosome 8 in the mixture based on the observation that with FISH on slides 70.4% of the cells in the leukemia sample contained 3 or more spots. In column 3 the percentage of Y positive nuclei is shown as measured by flow cytometry. Column 4 shows the percentage of nuclei with 3 or more spots for chromosome 8 after sorting of the Y positive area. In the fractions sorted from the 5%, 50% and 100% male cell populations, approximately 70% of the nuclei has three or more spots per nucleus for chromosome 8. In the fraction sorted from the 0.5% male cell sample, the percentage of nuclei with 3 or more fluorescent spots for 8 is 40.9. This indicates that a more than 100-fold enrichment is achieved for this fraction. In the sample that contained no male leukemic cells, samples sorted from the Y positive area yielded nuclei with false positive trisomies for chromosome 8 at the background level (0.39%). Evaluation by microscope, indicated that none of the recovered nuclei from this fraction contained a Y chromosome (Table 7.3 column 5). In the Y negative fraction sorted from the 50% sample, 1.7% of the nuclei had 3 or more fluorescent spots for chromosome 8 (Table 7.3; column 6) indicating that a small percentage of trisomy 8 containing leukemic cells lost the Y chromosome. The sample consisting of 100% male cells showed 68.1% and 68.4% nuclei with 3 or more fluorescent spots for chromosome 8 in the Y positive and the Y negative window, respectively. In the same sorted fractions approximately 100% of the nuclei was expected to show one spot for the Y probe when reanalyzed by microscope, albeit present in a very low percentage of the total population in region 2. Although the sort fractions of Y positive area from the 100% male sample contained, as expected, 99% nuclei with 1 spot for the Y probe, the sorted fraction of the Y negative population from this sample, showed that 22% (100% minus 78%) of this sub-population lost the Y

chromosome. The number of events in the Y negative window was 3.2% of the whole population (Table 7.3 column 3; 100% minus 96.8%). Therefore, FISH in suspension combined with flow cytometry and sorting indicates that 0.7% of the total leukemic cell population lost its Y chromosome (i.e. 22% of 3.2%).

7.5 Discussion

The presence of host derived cells in blood and bone marrow from sex-mismatched bone marrow transplanted leukemia patients might be either indicative for mixed chimerism or for the presence of (residual) leukemic cells. The detection of residual leukemic cells with numerical chromosomal aberrations using chromosome specific probes and FISH on slides becomes difficult once the target population drops below 1-3%. In normal cell populations a small percentage of cells is found that shows an aberrant chromosome number when analyzed by FISH (Table 7.1). This phenomenon remains largely unexplained. Either cells contain a real aberrant chromosome number or the hybridization procedure itself might be the cause of erroneous observations. Microscopically based analysis using a combination of immunophenotype and the genotype of a cell was develloped (Price et al. 1992) This technique could succesfully be employed to monitor the kinetics of mixed chimerism in subpopulations of leucocytes from patients, for the analysis of residual host cells and for the characterization of relapse, at different stages of BMT (Kögler et al. 1995).

Compared to microscopy, flow cytometry allows processing of large numbers of nuclei. Furthermore, nuclei of interest can be sorted and studied for the presence of additional aberrations. Therefore in suspension hybridization was performed to be able to analyze nuclei by flow cytometry. To exclude that the hybridization in suspension as applied in this study yields more nuclei with aberrant chromosome numbers, FISH in suspension was compared to FISH on slides using nuclei from a healthy individual. Hybridization of nuclei in suspension and analysis by fluorescence microscopy did not lead to a greater fraction of aberrant cells than conventional FISH on slides. Though successful in situ hybridization in suspension was achieved for all the probes tested, only hybridization with the Y probe resulted in reproducible fluorescence differences when mixtures of male and female cells were analyzed by flow cytometry. There are several explanations for this. First of all, the Y probe that was used recognizes a large part of the long arm of the Y chromosome resulting in fluorescence intensity that is higher than that observed with the other probes. Furthermore, the use of the Y probe on a mixture of male and female cells shows the difference between 0 or 1 spot. Because of the variation in fluorescence distribution measured on the nuclei with 1, 2, 3 or more spots, it becomes gradually more difficult to detect the difference in fluorescence intensity between 1 and 2, 2 and 3 or more spots, which is required for the detection of numerical aberrations of the other chromosomes. Finally, the use of PE as a fluorescent conjugate appeared to

give more reproducible results for the detection by flow cytometry than FITC conjugated biotin (data not shown).

In the flow cytometric studies of the samples consisting of 100% female, 0.3% of the nuclei appear in the Y positive window. In the 100% male sample 0.5% of the nuclei appear in the Y negative window. Basically these figures reflect the lower detection level for male or female cells in mixtures when flow cytometry is performed alone. The possibility to sort selected subpopulations enables a further reduction of the detection level. Sorting of the Y positive area from the 100% female sample and analysis for X probe hybridization by microscope, shows that 0.7% from this 0.3% (= 0.002%) has one fluorescent spot for the X probe. Sorting of the Y negative area from the 100% male sample shows that 1% of the 0.5% (= 0.005%) contained 2 spots for the X probe.

The ability to measure sex differences between cells is highly relevant for leukemia patients that received a sex-mismatched bone marrow transplantation (SMM-BMT). In Figure 7.2 the use of the Y probe and in suspension hybridization followed by FCM showed a high percentage of host cells. The indication for leukemia relapse was confirmed by morphological criteria. However, low amounts of host derived cells often occur for years in the bone marrow of SMM-BMT patients without clinical signs of relapse. To be able to further characterize these cells as being leukemic or not, the presence of additional leukemia associated markers can be analyzed after sorting. As a model, male leukemic cells with numerical aberrations for chromosome 8 were mixed with normal female cells, hybridized in suspension, analyzed by flow cytometry and sorted. In the 100% female sample 0.1% of the nuclei appeared in the Y positive area. From this fraction 0.39% contained an apparent trisomy for chromosome 8 (Table 7.3). This implies that approximately 1 nucleus per 250,000 (0.39% from 0.1%) would be falsely classified as belonging to the leukemic host cell population. This ratio indicates the lower detection level of the combined approach of flow cytometry and FISH for the experiment as performed in this paper. The following consideration has to be made. When nuclei are sorted at high purity with an efficiency of 95% and approximately 300 nuclei are required on a slide to perform a significant FISH analysis by microscope, it would require at least 316 leukemic cells in the analyzed sample. At a 1 to 250,000 ratio this means that at least 79 x 10^6 total cells should be processed in one sample. This indicates that the amount of cells that can be processed in one sample is a limiting factor.

The calculations of the ratios of male and female cells by flow cytometry were performed on G1/G0 clusters only (area 1 and 2 in all the dot plot figures). Based on the observations of the dot plots it is highly unlikely that more than 50% of the cells were not in G1/G0. The selection of the G1/G0 will at most lead to an underestimation of a factor of 2.

Several factors are of influence to the successful hybridization in suspension. First of all a proper fixation with 4% paraformaldehyde for 16 hours. Secondly a protein digestion with 0.25% trypsin to detach cell clumps, and thirdly the detection of probe hybridization in flow using PE instead of FITC. FISH in suspension as described in this paper allows clear discrimination between positive and negative nuclei when analyzed by flow cytometry. The additional value of FISH combined with flow cytometry and sorting and its value for detection of residual leukemic cells is demonstrated. Other applications, like non-invasive prenatal diagnosis, are envisaged. Further developments resulting in discrimination of signals with other chromosome specific probes will further expand the area of applications.

Chapter VIII

Summary and discussion

The three major stages in the treatment strategy of leukemia are 1) diagnosis, 2) remission-induction and 3) eradication of minimal residual disease. Leukemia diagnosis depends to some part on cytogenetic analysis. In a number of leukemias aberrant gene products or expression levels are directly correlated to biological behavior. Therefore, the cytogenetic aberrations found in leukemic cells also provide information with regard to treatment strategies and prognosis. In this thesis, detection of aberrant chromosomal patterns are discussed in the context of the detection of low numbers of leukemic cells in blood and/or bone marrow from leukemia patients

The most straightforward way to obtain cytogenetic information is to look at chromosomes through the microscope. This method has proven to be effective and suitable for the detection of aberrations of various kinds. The visual component in microscope analysis is strong; the actual observation of a translocated chromosome in a metaphase is the strongest evidence for the aberrant karyotype of a leukemia. This technique is commonly used and accepted.

In this thesis two techniques are discussed that offer a different approach to study chromosomes; i.e. flow karyotyping and fluorescence in situ hybridization employing chromosome specific alpha satellite DNA probes. Both techniques have their advantages and their shortcomings when compared to the microscope approach.

Flow karyotyping offers the possibility to analyze signals that are representative for chromosomes. These signals are indicative for the DNA content per chromosome (length of the chromosome) and base pair ratio. The sum of these signals forms a flow karyogram, which is as representative for a karyogram as the visual information obtained by microscope (Chapter I).

Model studies show that chromosomes can be obtained from cell lines as well as from ex-vivo derived leukemic cells. In this material the cluster configuration in the flow karyogram clearly shows the presence of aberrant chromosomes. Quantitative analysis, which can be performed with moderate accuracy, shows which chromosomes are involved in a translocation or which chromosomes are over- or underrepresented (Chapter III).

To be able to recognize aberrant clusters, the normal pattern must be thoroughly known. The human bivariate flow karyotype is well established and the position of each chromosome in the cluster pattern is determined. Therefore, deviations from this pattern are rapidly recognized. In Chapter IV a series of CML patients is studied in which the cytogenetic hallmark (i.e. the Philadelphia chromosome) can be clearly discriminated. Chromosomes required for flow karyotyping can only be derived from the proliferating fraction of the cell sample. Whether cells proliferate depends

on the presence of the right stimuli (growth factors). Thus, flow karyotyping only provides cytogenetic information on the proliferating fraction of a cell sample. Therefore, flow karyotyping does not provide a true measure of the absolute amount of leukemic cells in the original sample. This is clearly demonstrated in Chapter V, where bone marrow or blood samples from leukemia patients were grown in two fractions with different types of stimulation.

Like all cytogenetic approaches that require chromosomes, the success of flow karyotyping depends on the possibility to obtain cells in mitosis. One requirement for this is that cells proliferate for at least a short period of time in vitro. Theoretically, for flow karyotyping there are no lower limits to the mitotic index. The lower the MI the more cells have to be analyzed to obtain an average bivariate flow karyotype of 20.000 events. A total of 20.000 human chromosomes stands for approximately 430 mitotic cells. To be able to perform such a measurement, a margin has to be taken into account for setting up and adjusting of the cytometer and the fact that not all mitotic cells release their content as single chromosomes in suspension. Therefore, to measure and store the appropriate number of 20,000 chromosomes, a factor of 10 higher mitotic cells than the above calculated 430 would at least be require; i.e. 4300 mitotic cells. Assuming a cell culture with mitotic index of 0.1% this would require at least 4,300,000 cells in the culture. This is not a dramatic high amount of cells and in reality higher amounts of cells were often cultured .

Theoretically, the ability to specifically stimulate leukemic cells would imply that small numbers of cells can grow out in vitro to establish a population that is large enough to be analyzed by flow karyotyping. In such case MRD detection studies yield qualitative rather than quantitative data. However, since there are no growth factors to which leukemic cells are the sole responders or to which each and every leukemia cell will respond, overgrowth of normal bone marrow cells is likely to occur.

On the other hand flow karyotyping has a number of advantages over microscope analysis. The amount of chromosomes that can be analyzed is much higher. Furthermore, it allows an objective interpretation of the cluster positions in bivariate analysis. A third aspect that makes flow karyotyping an attractive technique is the ability to sort chromosomes to high purity. Although not implemented in this thesis, sorting is being applied successfully for several purposes. It is envisaged that the ability to sort chromosomes will be the major future application for flow karyotyping. Especially the introduction of the PCR technique has contributed to this perspective. Numerous possibilities are applied or envisaged; to name a few:

- Sorting of unidentified chromosomes, DNA amplification and "reverse painting' on normal metaphases for the purpose of detailed marker analysis.
- Sorting of translocated chromosomes and the normal chromosomal counterparts for the purpose of differential cloning and breakpoint analysis and cloning.
- In high resolution flow karyotypes some homolog chromosomes can be sorted separately. This might open ways to study in more detail the aspects that concern inheritance of paternal or maternal origin and may add new perspectives to, e.g., imprinting studies.

To overcome the quantitative limitations of flow karyotyping, additional information was sought by using interphase cytogenetic techniques. The direct use of interphase cells from blood or bone marrow implies that no selective outgrowth of subpopulations of cells takes place and that each individual cell in the sample is examined on a per cell basis. For this purpose FISH was applied with success. The flow karyotypes are indicative for the presence of leukemia as well as normal cell populations and FISH provides information about the real number of leukemic cells in the bone marrow (Chapter V). The interphase cytogenetic approach is a powerful tool to screen large number of cells in a relatively short period of time with the above mentioned advantages. FISH is easily applicable for the detection of numerical aberrations. Using chromosome specific DNA probes the number of hybridization signals per nucleus represents the number of chromosomes present in each cell (Chapter VI). Conventional cytogenetic knowledge is a starting point. Once a leukemia is characterized by a numerical aberration, the follow up of a patient is relatively easy and can be performed on at least a ten fold higher amount of cells than with conventional cytogenetics. Though FISH is very useful for this purpose there are shortcomings as well. One is the fact that conventional cytogenetics is obligatory in the first place. Because of the small amount of metaphases that are studied, certain aberrations might remain unnoticed. Secondly, FISH on interphase cells remains mainly applicable on numerical aberrations. The analysis of structural aberrations in interphase by FISH is restricted to the limited number of well defined translocations from which the breakpoints can be visualized by the appropriate probe combinations. This might be resolved when interphase cytogenetics could be extended in such a way that chromosomes can be observed in cells that are in G1/G0 state. This can be performed using the premature chromosome condensation (PCC) technique. In addition to that, FISH might be used on the prematurely condensed chromosomes. Potentially, this would provide insight into numerical as well as structural aberrations in interphase cells; cells that would hardly or never go in cell cycle when cultured for metaphases. Although both FISH and PCC have been described and applied numerous times, it appears to be difficult to realize a combination of both techniques (Y. L. Lu and G.J.A. Arkesteijn, unpublished). Apparently, too many difficulties are encountered with the combination of the techniques. Little has been published about its development (Brown et al. 1992, Brown et al. 1993, Pandita et al. 1994)

and until now it has not been reported to be used on a routine basis for experimental or clinical research.

In this thesis FISH was applied for the analysis of cells in leukemia samples carrying numerical aberrations. The lower detection level of FISH is set by the occurrence of cells with an aberrant FISH pattern in healthy individuals. Considering the most frequently applied repetitive DNA probes, the lower detection level ranges between 0.1 to 5%. For the purpose of minimal residual disease detection this brings the detection level at best one log down as compared to morphological analysis of the bone marrow. The FISH method is, however, very well suited for repeated follow up of patients in various stages of the disease. Using the numerical aberrations as markers, FISH provides information about treatment efficacy, regression or regrowth of leukemia.

To improve the detection level, a second independent leukemia-associated parameter should be added. A second hybridization with another chromosome specific probe is only applicable when multiple numerical chromosomal aberrations are present. In the group of leukemia patients who have received a Sex-mismatched bone marrow transplantation, there is a unambiguous correlation between the presence of either sex chromosomes. This makes the group an ideal target for double simultaneous hybridizations, utilizing both X and Y chromosome specific probes. The resulting detection level is the product of both separate levels (provided that both hybridizations occur independent in one cell). This could bring the theoretical detection level down to one in 100,000. Yet, two obstacles remain. One is the need to further classify the residual host cells as being leukemic or not. The other is, that the detection of 1 in 100,000 requires a multitude of 100,000 cells to be analyzed.

At this point the flow cytometry experience, obtained earlier in the course of the research program, came of help. Flow cytometry allows the analysis of large number of cells in a short period of time. Furthermore, cell populations can selectively be enriched. One obstacle had yet to be taken. To be able to analyze FISH by flow cytometry cells or nuclei had to be adequately hybridized in suspension. This technique was developed with success (Chapter VII). In mixtures of male and female cells that were hybridized with a Y chromosome specific probe the cells with a Y positive hybridization signal could be discriminated from the Y negative population. For chimerism studies these cells were hybridized simultaneously with an X chromosome specific probe. Now, the lower detection level is determined by the number of cells with a male hybridization pattern in a 100% female cell sample or vice versa after two subsequent selection steps. One is the selection of the cells appear as false positive or negative in the sort window and the second is the analysis by FISH with the second probe in the sorted fraction for the presence of false hybridization signals. For example from the 100% female sample, the area is sorted where Y positive cells would normally appear. The cells that scantily appear in this

area are sorted and analyzed by fluorescence microscopy for the presence of one X probe hybridization signal (indicative for male cells). This procedure implies that a selection of a selection is made and the detection level is reduced likewise. The ability to measure sex differences between cells is highly relevant for leukemia patients that received a SMM-BMT. However, small numbers of host derived cells are often observed for years in the bone marrow of SMM-BMT patients without clinical signs of relapse. To be able to further characterize these cells as being leukemic or not, the presence of additional leukemia associated markers can be analyzed after sorting. It is possible to perform a hybridization with a Y chromosome specific probe combined with a probe specific for chromosomes that have a numerical aberration in the leukemic cells. Sorting can be performed on the basis of the presence or absence of the Y probe hybridization, thus making a selection of recipient type of cells. Analysis of the sorted cells for the presence of numerical aberrations will enable to classify them as belonging to the leukemic population or not. Studies using this technique revealed that a theoretical detection level could be achieved in the order of 1 leukemic cell per 250,000 normal cells.

The lower detection level of both flow karyotyping and interphase cytogenetics by FISH are restricted to approximately 1% because of the occurrence of background events. In this respect, both techniques do not differ greatly from conventional cytogenetics and Southern blot analysis. The combination of flow cytometry, sorting and in situ hybridization yields a detection level in the order of 0.001% (Fig. 8.1). Each of the methods in given in figure 8.1 can be applied to a restricted number of leukemia types and depends on the type of markers (either immunological, genetical or cytogenetical) present.

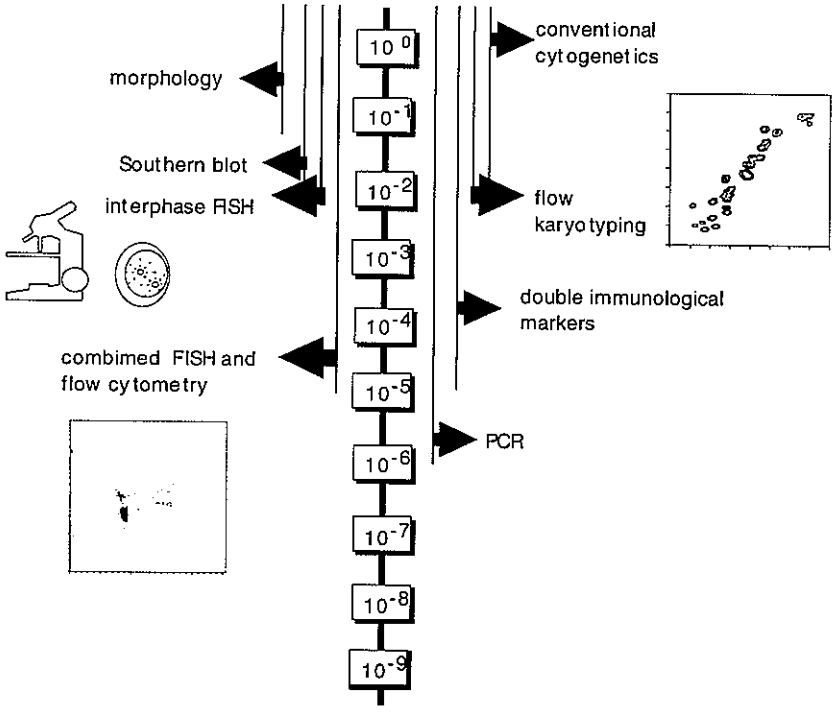

Figure 8.1 Levels of detection of leukemic cells in human leukemia

Cytogenetic aberrations are often present in leukemic cells and can be used as markers for their presence. The experimental studies as performed in this thesis contributes to the understanding of theoretical and practical detection levels using these markers. The knowledge gained, may hopefully contribute to the application of detection of residual leukemic cells. It certainly forms the basis for further experimental research that is already showing its contours, i.e. flow karyotyping and sorting of chromosomal markers from leukemia, amplification of the sorted DNA and analysis of the markers by 'reverse painting FISH' on normal metaphases.

The combined knowledge gained in the fields of conventional cytogenetics, interphase cytogenetics, flow karyotyping and in-situ hybridization will contribute to a more refined analysis of tumor cytogenetics, resulting in a better understanding of biological behavior of leukemic cells.

Samenvatting en discussie

De drie belangrijkste stadia bij de behandeling van leukemie zijn 1) diagnose, 2) remissie-inductie en 3) eliminatie van residuele leukemiecellen. De diagnose van leukemie wordt mede gesteld aan de hand van cytogenetische analyse. In een aantal leukemieën zijn afwijkende genproducten of veranderde genprocuctienivo's direct gecorreleerd aan het biologisch gedrag. Derhalve kunnen de cytogenetische afwijkingen die gevonden worden in leukemie eveneens een bijdrage leveren wanneer het gaat om behandelstrategieen en prognose. In dit proefschrift worden aberrante chromosoom patronen gebruikt in de context van detectie van lage aantallen leukemiecellen in bloed en beenmerg van leukemiepatiënten.

De meest voor de hand liggende manier om cytogenetische informatie te verkrijgen is door middel van kijken naar chromosomen door het microscoop. Deze methode heeft zijn effectiviteit bewezen en is toepasbaar voor het herkennen van afwijkingen van verschillende aard. De visuele component is de kracht van de analyse met het microscoop; het letterlijk waarnemen van een translocatie in een metafase is het sterkste bewijs voor een afwijkend karyotype bij leukemie. Deze techniek is derhalve veel gebruikt en algemeen geaccepteerd.

In dit proefschrift worden twee technieken besproken die een andere benadering bieden om chromosomen te bestuderen. Deze technieken zijn flow-karyotypering en fluorescente in situ hybridisatie met chromosoomspecifieke alpha satelliet DNA probes. Ten opzichte van de 'microscoop benadering' hebben beide technieken duidelijke voordelen, maar er zijn ook tekortkomingen.

Flow-karyotypering biedt de mogelijkheid tot het analyseren van signalen die representatief zijn voor de chromosomen. Deze signalen zijn indicatief voor DNA inhoud per chromosoom (gerelateerd aan de lengte van het chromosoom) en de basepaarsamenstelling. De som van deze signale vormt het 'flow-karyogram', welke net zo representatief is voor het karyotype van een individu of celtype, als de metafase informatie verkregen door middel van microscoop analyse (Hoofdstuk 1).

Model studies laten zien dat chromosomen, geschikt voor flow-karyotypering, verkregen kunnen worden van cellijnen zowel als van ex-vivo verkregen leukemiecellen. In zulk materiaal laat de clusterconfiguratie van het flow-karyogram duidelijk zien dat er afwijkende chromosomen aanwezig zijn. Kwantitatieve analyse van de clusters, wat met redelijke nauwkeurigheid kan worden uitgevoerd, laat zien welke chromosomen bij (reciproke) translocaties betrokken zijn of welke chromosomen over- of ondervertegenwoordigd zijn (Hoofdstuk 3). Om in staat te

zijn afwijkende clusterpatronen in het flow-karyogram aan te wijzen moet het normale patroon goed bekend zijn. Het normale menselijke bivariate flow-karyogram is uitvoerig vastgelegd en de positie van ieder chromosoom in het clusterpatroon is bepaald. Derhalve worden afwijkingen in dit patroon snel herkend. In Hoofdstuk 4 is een serie CML patiënten bestudeerd waarvan het cytogenetische kenmerk (het Philadelphia chromosoom) duidelijk onderscheiden kan worden.

De chromosomen die nodig zijn voor flow-karyotypering kunnen alleen verkregen worden van de delende fractie van de cellen. Of cellen groeien hangt af van de juiste stimulus (o. a. groeifactoren). Flow-karyotypering geeft daarom geen volledig inzicht in het absolute aantal leukemiecellen in het oorspronkelijke beenmerg of bloed monster. In Hoofdstuk 5 wordt dit duidelijk aangetoond. Beenmerg- of bloedmonsters werden gekweekt in twee fracties onder verschillende stimulerende condities. In theorie houdt specifieke groeistimulatie van leukemiecellen in dat uit kleine aantallen cellen voldoende nakomelingen gekweekt kunnen worden voor chromosoomanalyse door middel van flow-karyotypering. In dat geval zou de detectie van MRD kwalitatieve in plaats van kwantitatieve resultaten opleveren. Het probleem is echter dat er a) geen groeifactoren bestaan waarop uitsluiten de leukemiecellen reageren en b) er geen groeifactoren zijn waarop elke leukemiecel reageert. Daarom is het in zeer waarschijnlijk dat van de beenmergmonsters uit MRD situaties in de in-vitro cultures de normale beenmergcellen de leukemie zullen overgroeien.

Aan de andere kant biedt flow-karyotypering een aantal voordelen ten opzichte van analyse per microscoop. Het aantal chromosomen dat geanalyseerd kan worden is vele malen hoger. Verder is het mogelijk om tot een objectieve interpretatie te komen van de clusterpositie in het bivariate flow-karyogram. Een derde aspect dat flow-karyotyping een aantrekkelijke techniek maakt is de mogelijkheid om chromosomen met een hoge graad van zuiverheid uit te sorteren. Alhoewel niet geimplementeerd in dit proefschrift, wordt het sorteren van chromosomen met succes toegepast voor verschillende doeleinden. De mogelijkheid om chromosomen te sorteren zal in de toekomst één van de belangrijkste toepassingen van flow-karyotyperings zijn. Met name de introductie van de polymerase-ketting-reactie (PCR) heeft bijdragen aan dit toekomstperspectief. Een veelvoud aan mogelijkheden ligt in het verschiet of wordt reeds toegepast. Bijvoorbeeld:

- Sorteren van niet of moeilijk te definieëren chromosomen, DNA amplificatie en omgekeerde 'painting' op normale metafase chromosomen voor gedetailleerde markeranalyse.
- Sorteren van chromosomen welke bij een translocatie betrokken zijn en de respectievelijke normale chromosomen met als doel differentieële clonering van de verschillen tussen normaal en afwijkend DNA met breukpunt analyse als doel.

- In flow-karyogrammen met hoge resolutie zijn soms de homologe chromosomen apart te sorteren. Deze mogelijkheid opent de weg om in meer detail aspecten te bestuderen die te maken hebben met paternale of maternale overerving (imprinting).

Door de kwantitatieve beperking van flow-karyotypering werd aanvullende informatie gezocht door het gebruik van z. g. interfase cytogenetica technieken. Het directe gebruik van interfase cellen uit bloed of beenmerg houdt in dat er geen selectieve groei in vitro heeft plaatsgevonden van subpopulaties van cellen en dat elke individuele cel bestudeerd wordt. Voor dit doel werd met succes fluorescente in situ hybridisatie (FISH) toegepast. De flow-karyotypes geven een aanwijzing voor de aan- of afwezigheid van leukemie terwijl FISH de informatie verstrekt over het werkelijke aantal leukemiecellen in het beenmerg (Hoofdstuk 5). De benadering met interfase cytogenetica levert een krachtige mogelijkheid om grote aantallen cellen in een relatief korte periode te bekijken met behoud van het eerder genoemde voordeel om een kwantitatief oordeel te kunnen geven over het oorstpronkelijke aantal leukemiecellen. FISH is eenvoudig toepasbaar voor de detectie van cellen met numerieke afwijkingen. Wanneer chromosoomspecifieke DNA probes worden gebruikt, is het aantal signalen dat per kern zichtbaar wordt representatief voor het aantal kopieën van het betreffende chromosoom per cel (Hoofdstuk 6). Het uitgangspunt is de informatie afkomstig van conventionele cytogenetisch onderzoek. Nadat een bepaalde leukemie eenmaal gekarakteriseerd is met numerieke afwijkingen kan een patiënt op relatief eenvoudige wijze gevolgd worden door bestudering van ten minste een tienvoud hoger aantal cellen dan bij conventionele cytogenetica. Hoewel FISH uitermate geschikt is voor dit doel zijn er ook tekortkomingen. Eén ervan is dat conventionele cytogenetica noodzakelijk is als uitgangspunt. Door het kleine aantal metafases dat daarmee bestudeerd wordt kunnen sommige afwijkingen onopgemerkt blijven. Verder blijft FISH op interfase cellen voor het hier beschreven doel hoofdzakelijk beperkt tot numerieke afwijkingen. De analyse van strukturele afwijkingen in interfase met behulp van FISH is beperkt tot een beperkt aantal duidelijk gedefinieerde translocaties waarvan de breukpunten gevisualiseerd kunnen worden door middel van geschikte probe combinaties. Dit probleem zou opgelost kunnen worden als interfase cytogenetica zodaning uitgebreid zou kunnen worden dat afzonderlijke chromosomen zichtbaar gemaakt kunnen worden in G1/G0 cellen. Een techniek die dit toestaat is 'premature chromosoom condensatie' (PCC). In aanvulling hierop zou FISH dan gebruikt kunnen worden op de prematuur gecondenseerde chromosomen. Dit zou inzicht kunnen verschaffen in de aanwezigheid van numerieke zowel als strukturele afwijkingen in interfase cellen, inclusief de cellen die zelden of nooit in celcyclus zouden komen als ze voor metafase studies gekweekt zouden worden. Zowel FISH als PCC zijn als afzonderlijke technieken veelvuldig beschreven. De combinatie van beide technieken bleek echter moeilijk te realiseren (Y. L. Lu and G. J. A. Arkesteijn, ongepubliceerd). Te veel (onbekende) factoren verhinderen een routinematig

gebruik. Slechts weinig is over de ontwikkeling van deze combinatie gepubliceerd (Brown et al. 1992, Brown et al. 1993, Pandita et al. 1994) en tot nu toe zijn er weinig publicaties die routinematig gebruik beschrijven voor experimentele of klinische toepassing.

In dit proefschrift is FISH gebruikt voor de analyse van cellen in leukemie monsters met numerieke afwijkingen. De laagste detectiegrens die met FISH te bereiken is wordt bepaald door het voorkomen van numeriek aberrante FISH patronen in normale cellen van gezonde individuen. Wanneer de meest gebruikte DNA probes in ogenschouw worden genomen, ligt die grens tussen 0. 1 en 5%. De meest optimistische schatting zou FISH dus een log onder het detectienivo brengen van morphologische beenmerganalyse. De FISH methode is daarentege uitermate geschikt voor vervolgstudies van patiënten in verschillende stadia van leukemie. Op deze manier kan FISH informatie verschaffen over efficiëntie van behandeling, regressie of teruggroei van leukemie.

Om tot een verbetering van het detectienivo to komen is een tweede, onafhankelijke parameter nodig. Een tweede hybridisatie met een andere chromosoomspecifieke probe is slechts mogelijk als meerdere numerieke afwijkingen aanwezig zijn. In de groep patiënten die een zg. sex-mismatched beenmergtransplantaat hebben ontvangen bestaat een eenduidige relatie tussen de aan- of afwezigheid van één van beide geslachtchromosomen. Dat maakt deze doelgroep bij uitstek geschikt voor dubbel-hybridisaties met X en Y chromosoomspecifieke probes. Het resulterende detectienivo is dan het product van detectienivo's van de afzonderlijke probes (vooropgesteld dat beide hybridisaties in één cel onafhankelijk van elkaar plaats vinden). Theoretisch kan dit de onderste detectiegrens verlagen tot 1 cel per 100,000. Er blijven echter nog twee obstakels over. De ene is dat er dan nog steeds vastgesteld moet worden of een gastheercel wel of geen leukemiecel is. De andere is dat voor het waarnemen van 1 cel per 100. 000 ten minste een veelvoud van 100. 000 cellen geanalyseerd dient te worden.

Op dit punt komt de flow-cytometrie-ervaring, eerder opgedaan in dit onderzoeksprogramma, van pas. Met flow-cytometrie kunnen grote aantallen cellen in een korte periode geanalyseerd worden. Verder kunnen celpopulaties selectief verrijkt worden door ze uit te sorteren. Om FISH met behulp van flow-cytometrie te kunnen meten moeten cellen in suspensie gehybridiseerd kunnen worden. Deze techniek werd met succes ontwikkeld (Hoofdstuk 7). Mengsels van mannelijke en vrouwelijke cellen werden gehybridiseerd in suspensie met een Y chromosoomspecifieke probe. Met behulp van flow-cytometrie konden positieve en negatieve signalen van respectievelijk de mannelijke en vrouwelijk kernen onderscheiden worden. Voor chimerisme studies werden dubbelhybridisaties uitgevoerd met Y- en X chromosoomspecifieke probes. Het laagst haalbare detectienivo werd nu bepaald door het aantal cellen met een mannelijk hybridisatiepatroon in een monster bestaande uit 100% vrouwelijk cellen of vice

versa na twee opeenvolgende selectiestappen. Eén selectiestap wordt gevormd door het aantal cellen dat als vals positief of negatief verschijnt in het uit te sorteren gebied en de tweede is de FISH analyse van de gesorteerde fractie op de aanwezigheid van valse hybridisatie signalen met behulp van de tweede probe. In een monster dat bijvoorbeeld voor 100% bestaat uit vrouwelijke cellen, wordt het gebied uitgesorteerd waar de Y positieve cellen zouden verschijnen. De spaarzaam aanwezige cellen die in dit gebied voorkomen worden vervolgens met behulp van FISH en microscopie geanalyseerd op de aanwezigheid van 1 X chromosoom (indicatief voor mannelijke cellen). Dit houdt in dat een selectie van een selectie is gemaakt wat een corresponderende reductie van het detectienivo tot gevolg heeft. De mogelijkheid om sex-verschillen te kunnen meten is hoogst relevant bij patiënten die een zg. 'sex-mismatched' beenmergtransplantatie hebben ondergaan. Echter, kleine aantallen ontvanger cellen komen vaak voor in beenmerg van dergelijke patiënten zonder dat er tekenen zijn van een recidief. Om nu in staat te zijn deze cellen verder te klassificeren als zijnde leukemisch of niet kunnen andere leukemie-geassocieerde merkers na sortering geanalyseerd worden. Het is bijvoorbeeld mogelijk om hybridisatie met de Y chromosoomspecifieke probe te combineren met een probe die specifiek is voor een bepaalde numerieke chromosomale afwijking in de leukemiecellen. Op basis van aan- of afwezigheid van het Y hybridisatiesignaal kunnen cellen gesorteerd worden waarna analyse op de aanwezigheid van numerieke afwijkingen in de gesorteerde fractie de mogelijkheid biedt tot classificatie als leukemiecel of niet. In dit proefschrift wordt aangetoond dat op deze wijze een theoretisch detectienivo rond de 1 op 250. 000 kan worden bereikt.

Het laagste detectienivo dat met zowel flow-karyotypering als interfase cytogenetica met FISH bereikt kan worden beperkt zich tot ca. 1%. Vanuit dit oogpunt verschillen deze technieken niet veel van conventionele cytogenetica en Southern blot analyse. De combinatie van flow-cytometrie, sorteren en in situ hybridisatie resulteert in een detectienivo rond de 0,001% (Fig. 8. 1). Elk van de in figuur 8. 1 genoemde methodes kan toegepast worden op een beperkte groep leukemiepatiënten die voldoet aan specifieke kenmerken (ofwel immunologisch, genetisch of cytogenetisch)

Leukemiecellen worden vaak gekarakteriseerd door cytogenetische afwijkingen welke als merkers voor hun aanwezigheid kunnen fungeren.

De experimentele studies die beschreven zijn in dit proefschrift dragen bij aan de kennis omtrent theoretische en practische detectienivo's met deze merkers. Hopelijk zal de opgedane kennis een bijdrage leveren aan de toepassing van detectie van leukemie. Het vormt zeker de basis voor verder experimenteel onderzoek waarvan de contouren zich reeds aftekenen; nl. flow-karyotypering en sorteren van merker chromosomen, vermeerdering met behulp van PCR van het gesorteerde DNA en analyse van de merkers door middel van omgekeerde 'painting' op normale metafasen.

De combinatie van conventionele cytogenetica, interfase cytogenetica, flow-karyotypering en in-situ hybridisatie zal bijdragen aan een verfijning van de analyse van de tumor cytogenetica, wat uiteindelijk zal resulteren in een beter kennis van het biologische gedrag van leukemiecellen.

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List of frequently used abbreviations

A-T	adenine-thymidine
ALL	acute lymphocytic leukemia
alpha-MEM	alpha-minimal essential medium
AML	acute myelocytic leukemia
BM	bone marrow
BMT	bone marrow transplantation
BN	Brown Norway rat
BNML	Brown Norway acute myelocytic leukemia
BSA	bovine serum albumine
CA3	chromomycin A3
CM	conditioned medium
CML	chronic myelocytic leukemia
CV	coefficient of variation
DAPI	4'-6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FLS	forward light scatter
G-C	guanine-cytosine
GCT-CM	giant cell tumor conditioned medium
GM-CSF	granulocyte macrophage-colony stimulating factor
HHBBSS	Hanks hepes buffered balanced salt solution
Ho	Hoechst 33258
IFN	interferon
IL	interleukin
ISH	in situ hybridization
LT12	in vitro growing rat cell line derived from the BNML
MI	mitotic index
PB	peripheral blood
PBS	phosphate buffered saline
PCC	Premature chromosome condensation
PCR	polymerase chain reaction
Ph^1	Philadelphia chromosome; t(9;22)
PHA	phytohemagglutinin
PI	propidium iodide
SMM	sex-mismatched
SRBC	sheep red blood cells
SSC	standard saline citrate: 0.15 M Na/Cl + 0.015 M Na-citrate
TPA	12-O-tetradecanoylphorbol-13-acetate
UV	ultra violet

Dankwoord

In de tijd dat ik aan het onderzoek heb gewerkt heb ik veel steun ondervonden van een ongelofelijke hoeveelheid mensen. Toch begin ik met enige aarzeling mijn dankwoord. Niet dat jullie het niet verdienen, in tegendeel. Het is echter moeilijk om te bepalen wie het eerst te noemen. Laat ik dus bij deze met jullie afspreken dat de volgorde niet bepalend is voor de mate van waardering voor de betreffende persoon. Laten we het pad chronologisch doorlopen.

Mijn eerste stappen op het pad van de flow cytometrie en mijn eerste pogingen om chromosomen te meten op een flow-cytometer, uitgerust met een hogedruk-kwiklamp, heb ik ondernomen op de afdeling Pathologie van de RU te Leiden. Cees Cornelisse, onder jouw bezielende leiding kwamen we er achter dat je dit soort dingen beter op een "laser-based" machine kunt uitvoeren, wat ons uiteindelijk toch nog het resultaat opleverde wat we wilden. Door bij jou de richting "flow-karyotypering" te nemen heb ik dit pad verder kunnen bewandelen. Mijn dank hiervoor.

Via deze afslag kwam ik terecht op de flow-cytometrie route. Op het RBI-TNO te Rijswijk hadden Ger van den Engh, Jan Visser en Barbara Trask een ware meerbaans snelweg aangelegd waarop ik alleen maar hoefde in te voegen. Dit kon doordat er een door het KWF gesubsidieerd project beschikbaar was waarop ik aan de slag kon. Ton Hagenbeek, mijn dank gaat dus zeker naar jou uit omdat jij mij het vertrouwen hebt geschonken dit project uit te voeren. Goed en wel voortrazend op deze weg ontdekte ik dat er veel medereizigers waren, in de vorm van collega's, die allemaal dezelfde kant op raasden. Sommigen kwamen pas later op de weg, sommigen namen eerdere afslagen, sommigen haalden mij in en sommigen werden door mij ingehaald. Verder vond ik altijd wel een praatpaal als ik pech onderweg had. De voltallige TNO-bemanningen die destijds reden met de nummerborden: "Hagenbeek", "Visser" of "Bauman" worden zeer bedankt voor hun collegialiteit en hulpvaardigheid. Niet zonder de anderen te kort te doen wil ik uit deze periode met name Carla van Marrewijk bedanken voor de extra benzine die ik van haar gekregen heb.

Na de in-situ wisselstrook te hebben genomen reed ik een tijdje parallel aan de flow cytometrie, maar, ook aan die strook kwam een einde op het punt waar in situ en flow weer één baan worden. Dit punt viel samen met een bocht in de weg die mij voerde naar de afdeling Hematologie van de Erasmus Universiteit te Rotterdam. Hoewel het asfalt anders aanvoelde bleef de koers en snelheid gelijk. Niet in de minste plaats omdat een aantal collega's op dit stuk de route tot een aangenaam vertoeven wisten te maken. Steven Erpelinck, je nimmer aflatende enthousiasme en inventiviteit zullen me nog lang bijblijven. Anton Martens, door je regelmatige

controle van de bougies is de motor altijd goed blijven draaien. Esther Jumelet, pas kort bijrijder, maar ik weet nu al dat je een prima kaartlezer bent. Met jou erbij zal ik de weg niet snel kwijtraken. Ton Hagenbeek, in je helikopter hou je boven de weg in de gaten waar zich de files voor doen en wat de beste route is. Ted Kloosterman en Jan Bayer, jullie hebben alle twee een afslag eerder genomen, maar we zien elkaar vast nog wel eens in een wegrestaurant en dan gaan we natuurlijk voor een 'patatje oorlog met Bosniers'. Pieter Moelker en Paul Weststrate; bedankt voor het binnenloodsen gedurenden het laatste stukje en Mirjam Bosman bedank ik voor haar hulp bij het mooimaken van het eindprodukt.

Het zou oneerlijk zijn nu te stoppen want dan zou ik mijn ouders ernstig te kort doen. Op afstand hebben zij altijd met interesse gevolgd welke afslagen en opritten ik nam. In goede zowel als in slechte tijden heb ik altijd op ze kunnen rekenen. Door bij hen regelmatig een onderhoudsbeurt te laten uitvoeren ben ik op de weg gebleven.

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Ik mag het dan wel geschreven hebben, maar jij hebt het gemaakt.

Curriculum Vitae

De schrijver van dit proefschrift werd geboren te Rijswijk (ZH) op 6 januari 1956. Hij behaalde in 1975 het VWO-b diploma aan het St. Stanislas College te Delft. In hetzelfde jaar werd een aanvang gemaakt met de studie biologie aan de Rijksuniversiteit te Leiden. In 1978 werd het kandidaatsexamen B4 (biochemie) afgelegd. Het doctoraalexamen werd in 1982 behaald met als hoofdvakken Genetica en Immunologie en de met succes gevolgde onderwijscursus. Na de studie werd de militaire dienstplicht vervuld. Vanaf 1984 werd in kort lopend dienstverband aan de Rijksuniversiteit te Leiden onderzoek verricht naar de mogelijkheden van flow-karyotypering voor tumorcellen (Dr. C.J. Cornelisse). Vanaf medio 1985 werd aangevangen met onderzoek wat betrekking had op de mogelijkheden om met behulp van flow-karyotypering residuele leukemiecellen op te sporen in bloed en beenmerg (Prof. Dr. A. Hagenbeek). Dit onderzoek werd uitgevoerd op het Radiobiologisch Instituut-TNO te Rijswijk. De resultaten zijn in het eerste deel van dit proefschrift beschreven. In de lijn van dit onderzoek werd vanaf 1990 een onderzoek gestart waarin de detectie op basis van cytogenetische afwijkingen werd onderzocht door middel van fluorescerende in-situ hybridizatie (Prof. Dr. A. Hagenbeek). Dit onderzoek werd grotendeels uitgevoerd op het ITRI-TNO (voorheen RBI-TNO) en gedeeltelijk op het instituut Hematologie van de Erasmus Universiteit te Rotterdam. Vanaf 1 december 1993 is de schrijver werkzaam bij het instituut Hematologie op een project waarbij eveneens cytogenetische aspecten van leukemiecellen een belangrijk onderdeel vormen en waarbij technieken als flow-karyotypering, chromosoom sorteren, en in-situ hybridizatie gebruikt worden.

STELLINGEN
behorende bij het proefschrift

FLOW KARYOTYPING AND FLUORESCENCE IN SITU HYBRIDIZATION
New cytogenetic approaches for the detection of leukemia cells

van G.J.A. Arkesteijn

1 Met flow karyotypering kunnen structurele en numerieke chromosomale afwijkingen gedetecteerd worden. Voor het bepalen van het aantal leukemiecellen in het bloed of beenmerg biedt flow karyotypering geen meerwaarde ten opzichte van reeds bestaande technieken. *(Dit proefschrift)*

2 Dankzij de mogelijkheid om DNA random te amplificeren heeft 'flow sorting' van chromosomen een centrale rol gekregen als het gaat om het verkrijgen van zuiver chromosomaal DNA. *(Telenius et al. 1992, Genes Chromosom Cancer 4(3): 257-263)*

3 Amplificatie van zuiver chromosomaal DNA dat door sorting van marker chromosomen is verkregen kan leiden tot painting probes die compleet nieuwe chromosoom-translocatiepatronen aan het licht brengen welke met conventionele cytogenetisch onderzoek vooralsnog verborgen blijven.

4 Een afwijkend aantal FISH signalen in perifere bloedcellen van gezonde individuen kan veroorzaakt worden ofwel doordat er bij gezonde individuen inderdaad cellen met afwijkende chromosoomaantallen te vinden ofwel doordat de FISH techniek in een laag percentage van de gezonde cellen afwijkende aantallen zien terwijl ze er niet zijn. Onafhankelijk van welke verklaring de juiste is, wordt door dit fenomeen de laagste limiet van FISH bepaald voor de detectie van numerieke aberraties*(Dit proefschrift)*.

5 Binnen de grenzen van de detectielimiet van FISH voor de verschillende repetitieve DNA probes geldt dat, als FISH gebruikt wordt voor het opsporen van numerieke afwijkingen in leukemie, een afwijkend aantal spots per kern altijd de aanwezigheid van leukemiecellen betekent. Het omgekeerde (geen afwijkend hybridizatiepatroon) betekent echter niet dat er geen leukemie aanwezig is *(Dit proefschrift)*.

6 De detectiegrens van FISH in suspensie in combinatie met flow cytometrie is gelijk aan het produkt van de twee technieken apart *(Dit proefschrift)*.

7 De chromosoomspecificiteit van alpha satelliet DNA probes veroorzaakt door de 'higher-order repeats' speelt een ondergeschikte rol bij een DNA probe fragmentlengte die kleiner is dan de lengte van de higher-order.

8 'Comparative genomic hybridization' (CGH) laat niet toe verschillende subclones in een populatie van tumorcellen te identificeren *(Kallioniemi et al. 1993, Semin Cancer Biol 4(1): 41-46)*. Met 'single-cell sorting', amplificatie van het DNA van indivuduele tumorcellen en het toepassen van CGH op het verkregen materiaal zou het mogelijk zijn om deze informatie wel te krijgen.

9 Het verfraaien of uitbreiden van data zonder dat dit wezenlijk bijdraagt aan een betere of andere conclusie, is als het oppoetsen van geld: het ziet er wel aardig uit maar het is niet meer waard geworden.

10 Niet de wetenschappelijke ontwikkelingen op het gebied van prenatale geslachtsbepaling zijn de oorzaak van de vele abortussen in sommige landen, maar de opvatting dat het ene geslacht waardevoller zou zijn dan het andere.

11 De kosten per verslaafde die de maatschappij zou moeten dragen als zg. 'hard-drugs' legaal verkrijgbaar zijn is vele malen kleiner dan de kosten die nu opgebracht worden voor medische zorg en ter compensatie van criminele activiteiten die als gevolg van de illegaliteit ontstaan.

12 Mensen die pretenderen werk te scheppen door er alleen maar over praten, moesten maar eens een schop krijgen. (naar Koos van Zomeren; NRC Handelsblad).

Ger Arkesteijn

Rotterdam, 12 mei 1995