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.

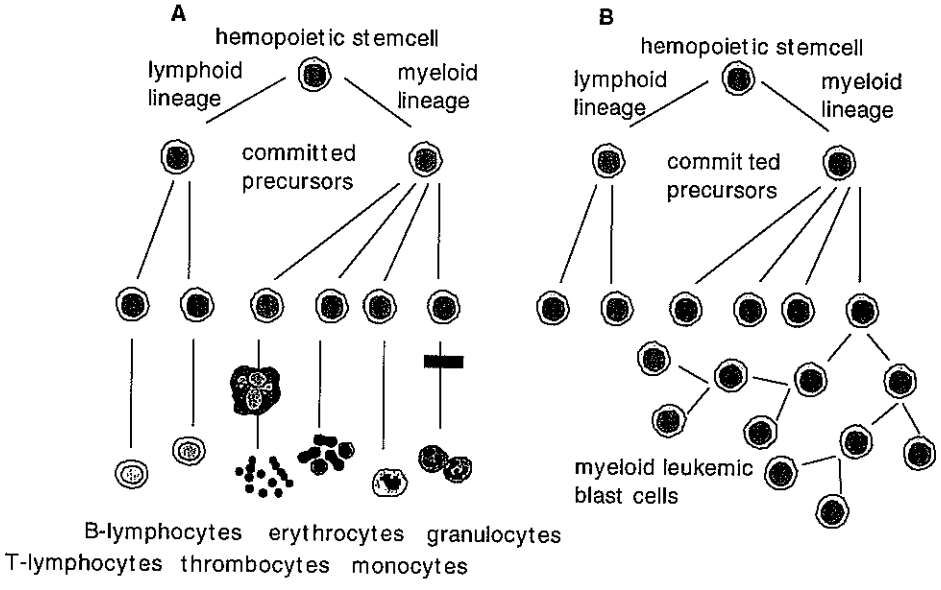

Figure 1.1
Hemopoietic lineage map with postulated leukemia development. Leukemia develops when a block occurs that prevents normal differentiation to fully functional blood cells (as indicated by a solid black bar in the differentiation pathway of granulocytes in panel A). Leukemic cells, from which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

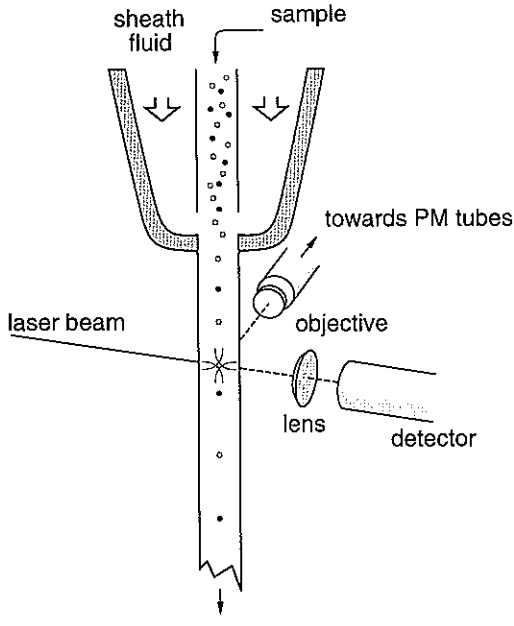

Figure 1.2
Principle of flow cytometric measurement. Particles from the sample are focused hydro-dynamically in the nozzle. One by one they pass one or two lasers at high speed. At the laser interception points light scatter and fluorescence parameters provide information about size, complexity and amount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

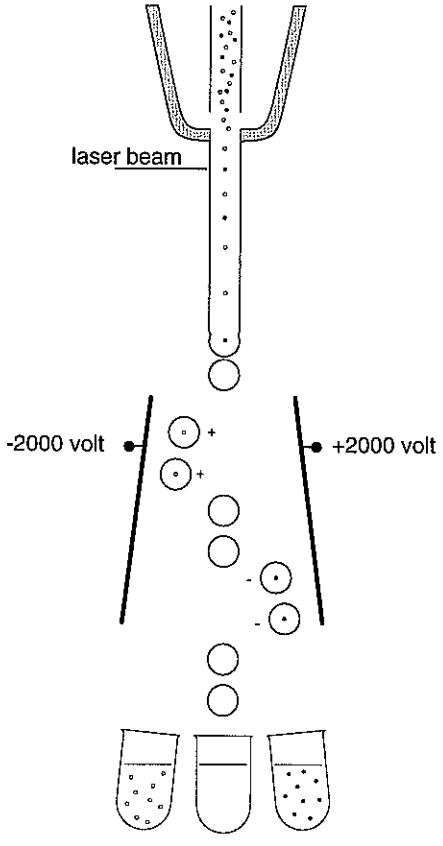

Figure 1.3
Principle of flow sorting. Particles enclosed in droplets can either be sorted to the left or the right when the droplets are charged and thereby deflected when they pass the electro-magnetic field created by the deflection plates.

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.

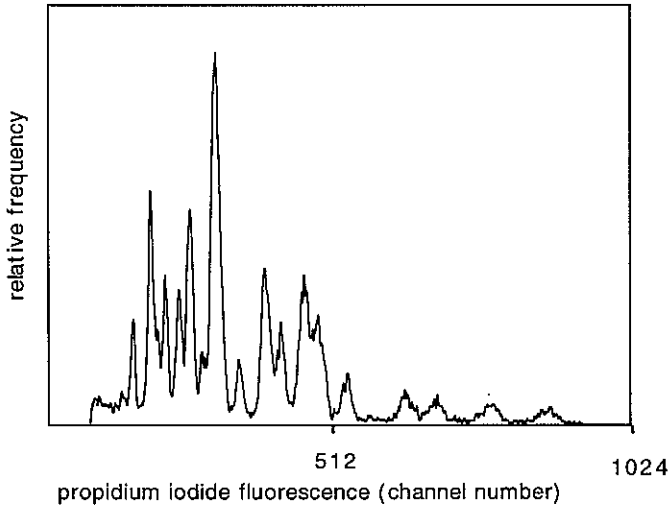

Figure 1.4
Fluorescence distribution of chromosomes from rat leukemia cells (LT-12) stained with the fluorescent dye propidium iodide (PI). The size of each individual chromosome is reflected by the amount of PI fluorescence.

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).

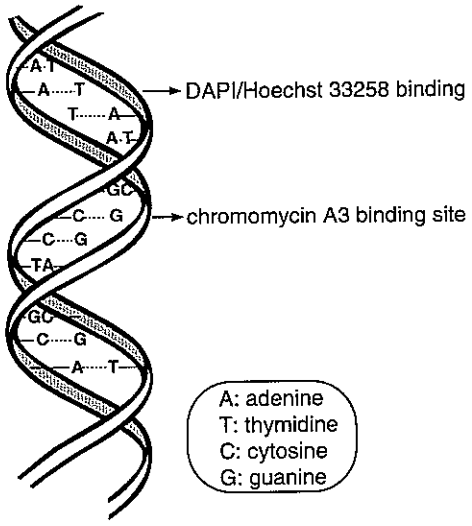

Figure 1.5
Binding of two base pair specific dyes. Hoechst 33258 or DAPI will bind to areas in the chromosome that are A-T rich; chromomycin will bind to areas with relatively high amounts of G-C base pairs

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.

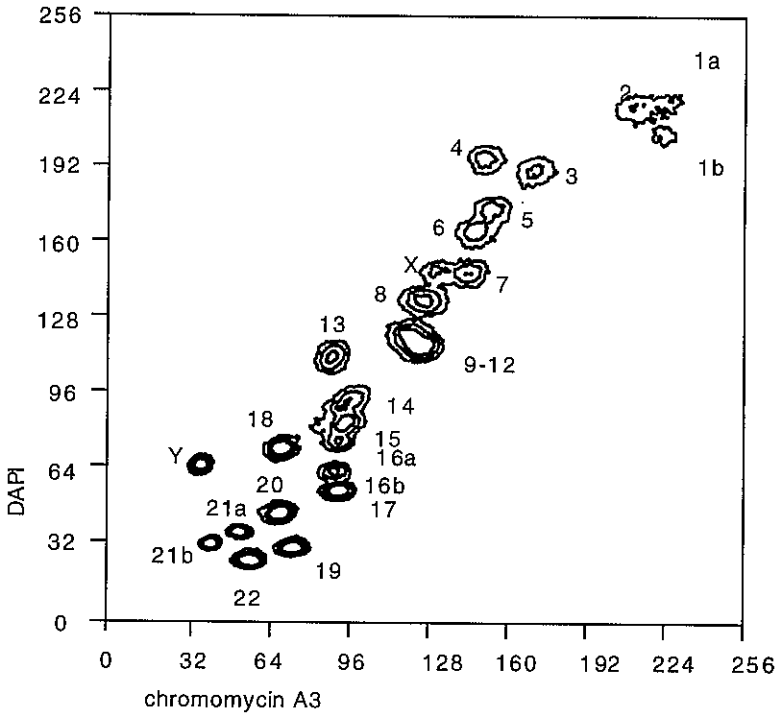

Figure 1.6
Bivariate flow karyogram from a normal male (peripheral blood lymphocytes were stimulated with PHA). Each cluster is indicated by the number of the chromosome that it represents. In several cases individual homologues of chromosome pairs can be seen (as indicated by a and b).

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)

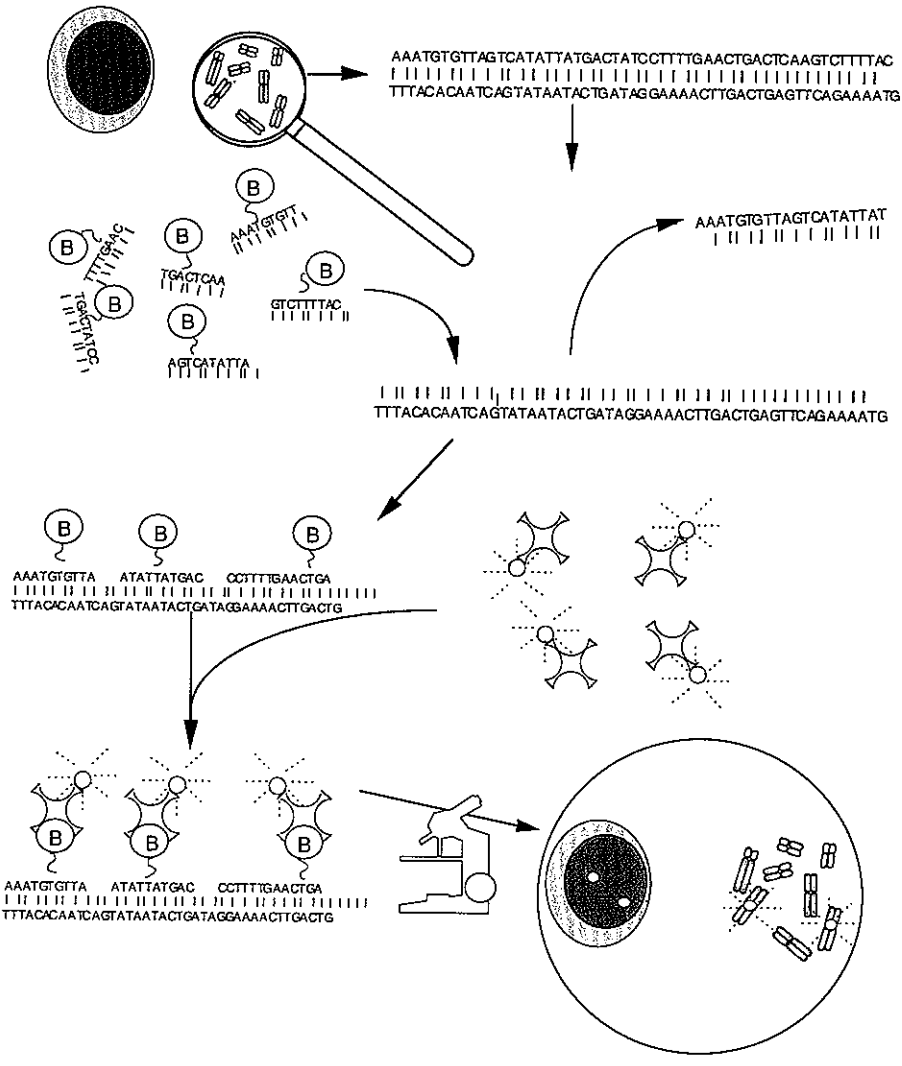

Figure 1.7
Principle of fluorescence in situ hybridization (FISH). DNA in nuclei or chromosomes is denatured without loss of nuclear or chromosome morphology. Hybridization takes place with complementary labeled DNA. The labeled DNA is visualized by binding to fluorescently labeled conjugate. Microscopic 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.

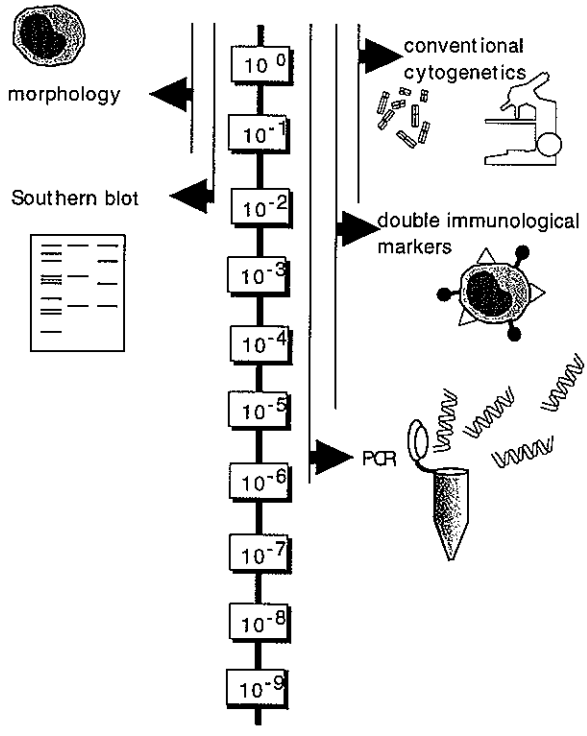

Figure 1.8
Levels of detection of leukemic cells in human leukemia.

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.