Chapter III

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

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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).

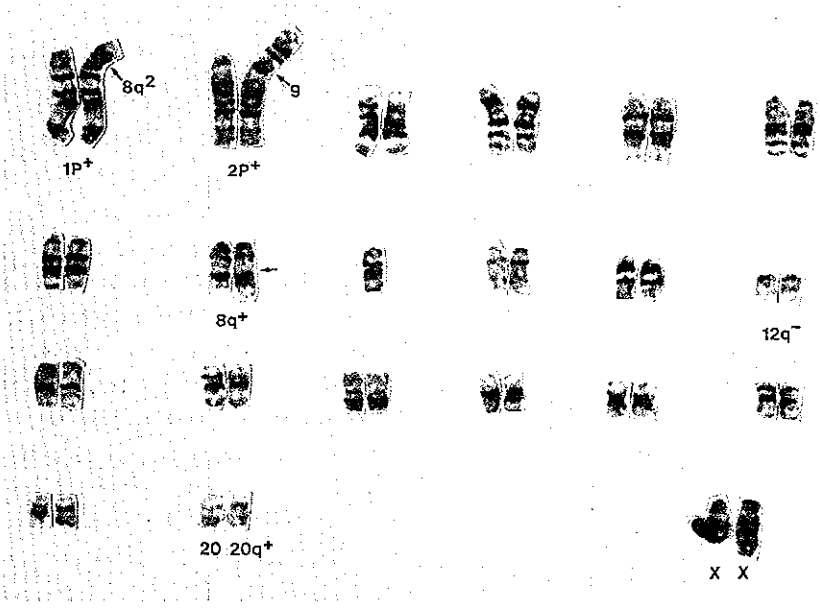

Figure 3.1
G-banded metaphase chromosomes from the BNML originating from leukemic bone marrow.

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.

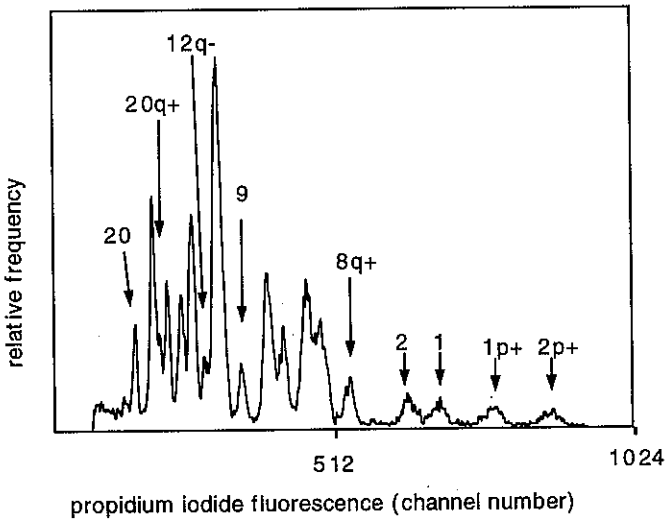

Figure 3.2
Univariate flow karyogram (PI stained) of the BNML in vitro cell line. Numbers indicate the expected chromosome positions.

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.

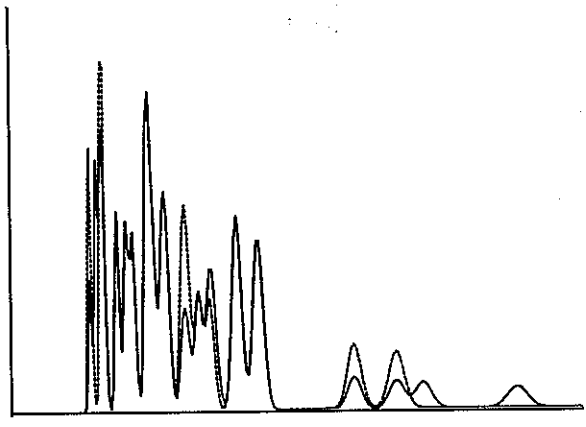

Figure 3.3
Computer simulated flow karyogram of the BNML (solid line) and the normal BN female (dotted line). Simulation was based on the mean relative length measurements of the chromosomes on standard metaphase microscope slides.

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.

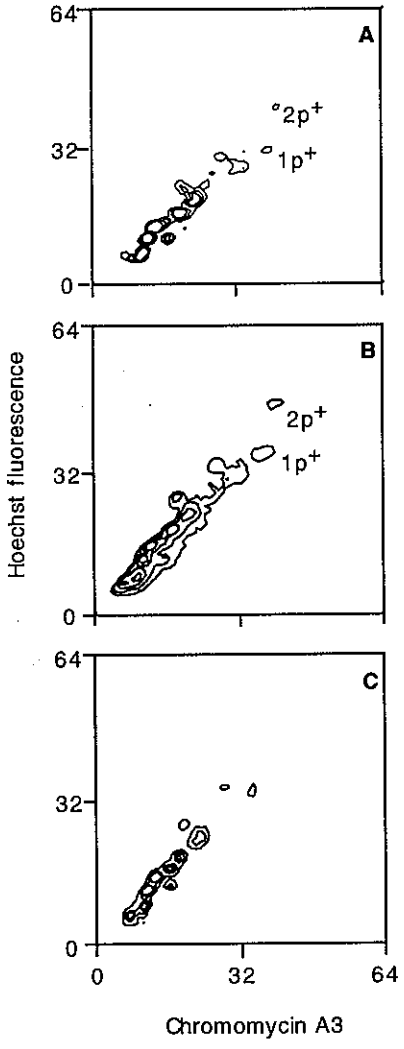

Figure 3.4
Contour plot from the bivariate flow karyogram of the in vitro BNML cell line (A), ex vivo BNML cells (B) and PHA stimulated lymphocytes of the normal BN female (C).

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.

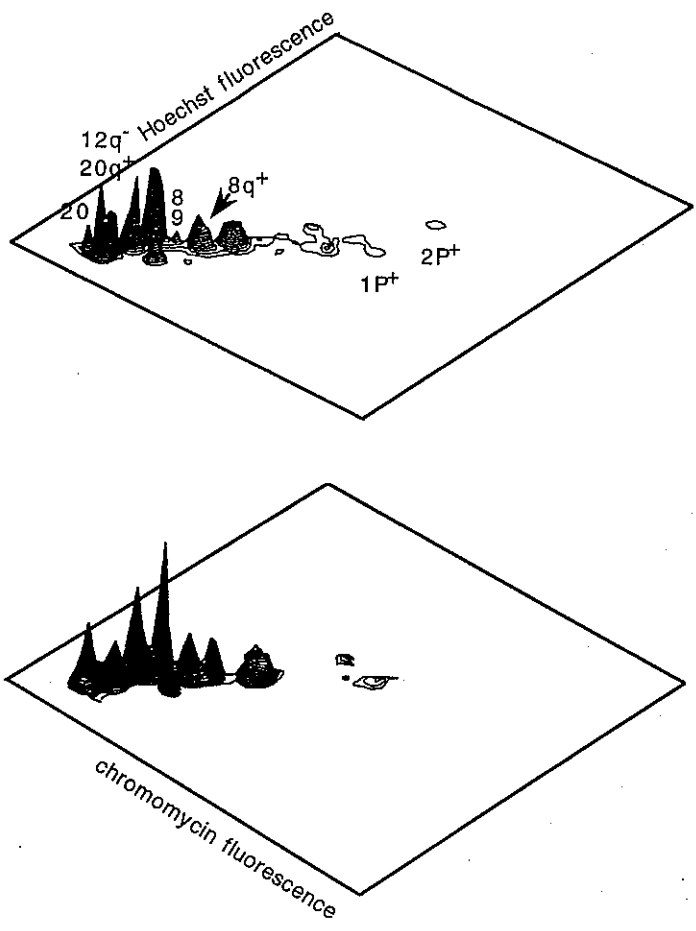

Figure 3.5
Three-dimensional representation of the bivariate flow karyogram of the in vitro BNML cell line (A) and normal PHA stimulated female BN lymphocytes (B). Arrows indicate the area where the differences due to chromosomal changes in the BNML cell line can be observed.

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).

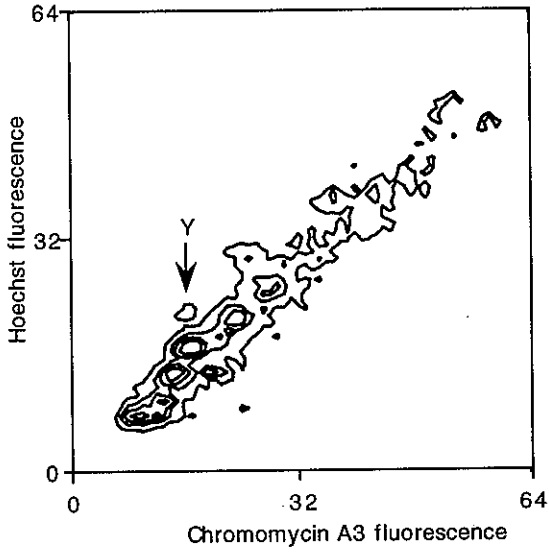

Figure 3.6
Contour plot from the bivariate flow karyogram of a density of 1.070 g/cm^3 containing predominantly recipient normal male BN cells originating from the same gradient as in Figure 3.4 B.

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.