Chapter IV

Bivariate flow karyotyping in human Philadelphia-positive chronic myelocytic leukemia

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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).

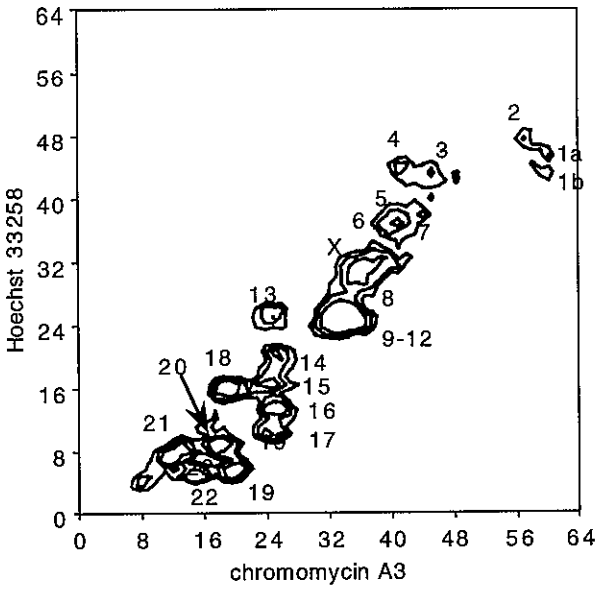

Figure 4.1
Contour plot from the chromosomal distribution of a bivariate flow karyogram from a female CML patient in the chronic phase (patient 1). The karyogram was obtained from peripheral blood cells that were stimulated with PHA. It shows the normal distribution of chromosomes without the specific 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.

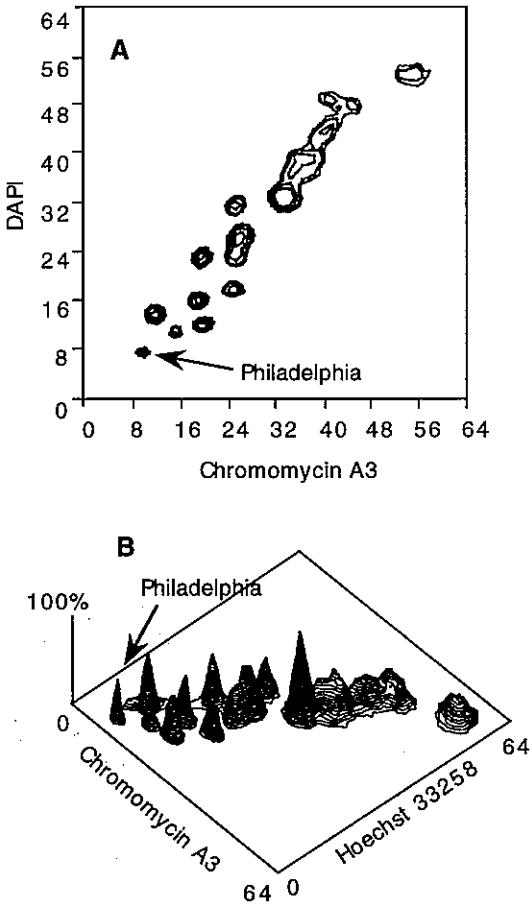

Figure 4.2
Chromosomal distribution of a bivariate flow karyogram from a female CML patient in chronic phase (patient 4). White blood cells were stimulated with GCT-CM. Contour plot (A) and three-dimensional representation (B) display a Ph^1 chromosome as the only aberration in this karyogram.

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.

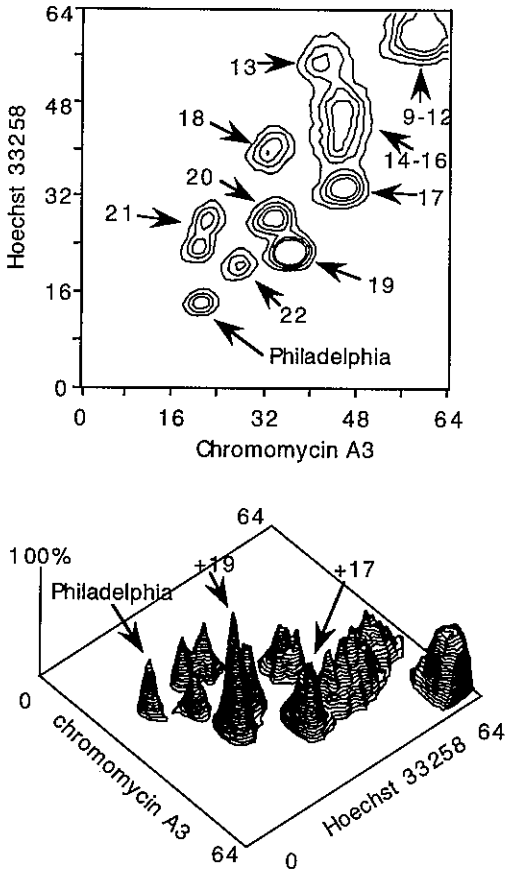

Figure 4.3
Chromosomal distribution of a bivariate flow karyogram from a female patient in accelerated phase (patient 3). Amplifier gains were adjusted so that only the small chromosomes are displayed. The flow karyogram was obtained from bone marrow cells that were stimulated with PHA. Contour plot (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.

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