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.

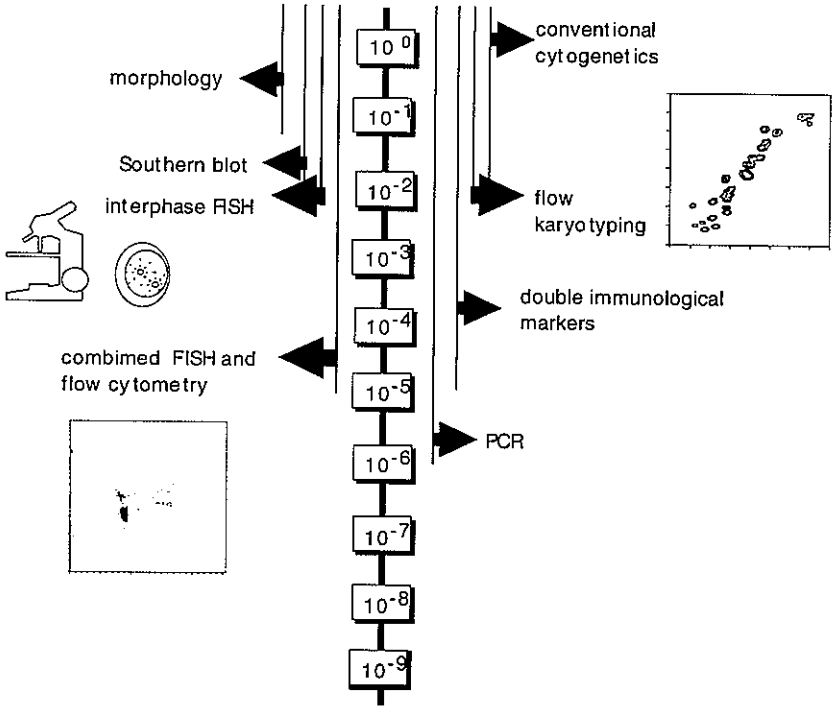

Figure 8.1 Levels of detection of leukemic cells in human leukemia

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.