NORMAL AND LEUKEMIC STEM CELLS DURING MINIMAL RESIDUAL DISEASE

Studies in an experimental rat leukemia model (BNML)

A.C.M. Martens

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STELLINGEN

1. De veelgehoorde stelling, dat een experimenteel dierleukemie-model slechts representatief zou zijn voor één individuele leukemie-patient, getuigt van een onderschatting van de flexibele inzetbaarheid van het diermodel voor de beantwoording van concreet geformuleerde deel-problemen betreffende het leukemisch proces.

2. De heterogeniteit in de hemopoietische stamcelpopulatie van de muis zou reeds veel eerder zijn opgemerkt indien de miltkolonieaantallen op de verschillende dagen zouden varieren, zoals dat is geconstateerd bij gebruik van de muis voor het meten van de hemopoietische stamcel van de rat.
(dit proefschrift)

3. De veronderstelling dat leukemie per definitie tot de zich snel verspreidende tumoren behoort en dat tijdens de remissie een enkel beenmergpunktieonderzoek volstaat voor het vaststellen van de zich in dit compartiment bevindende tumor-massa, kan op basis van proefdiermodel studies als onaannemelijk worden beschouwd.
(dit proefschrift)

4. De milt van de rat biedt niet de juiste omgeving om het pluripotente karakter van hemopoietische stamcellen te bepalen.
(dit proefschrift)

5. Ten onrechte wordt door Peters en medewerkers gesuggereerd dat fractionering van totale lichaamsbestraling voor de conditionering van leukemiepatienten voorafgaande aan beenmergtransplantatie, het anti-leukemisch effect onveranderd zou laten.
Peters e.a.. (1979), Radiology 131, 269-276
Hagenbeek & Martens (1981), Int.J. Rad. Oncol. Biol. Phys. 7, 1075-1079

6. In tegenstelling tot verbetering van "purging-methoden" ter verwijdering van resterende leukemiecellen uit het autologe beenmergtransplantaat van leukemiepatienten, zal verbetering van de conditioneringsbehandeling wel leiden tot een verlaagd percentage leukemie recidief.

7. Tijdens de discussies over detectiegrenzen van "residual disease" wordt lichtzinnig over "exponenten" gesproken als ware het slechts lineaire grootheden.

8. Het is aannemelijk, dat het opheffen van zogenaamde "multi-drug-resistance" bij kankerpatienten m.b.v. stoffen die de cellulaire efflux van cytostatica verhinderen, niet zal leiden tot een hoger percentage genezingen.

9. De "drie-dimensionele reconstructie" methode in combinatie met in situ hybridisatie met chromosoom-specifieke probes, biedt in principe een grotere potentie dan in situ hybridisatie van chromosomen in suspensie voor de detectie van chromosoomtranslocaties die onder het detectie-niveau liggen van de standaard cytogenetische methode van onderzoek.

10. De door Spangrude en medewerkers onlangs voorgestelde methode voor zuivering van pluripotente hemopoietische stamcellen uit muizebeenmerg, is omslachtig, niet vernieuwend en leidt, in tegenstelling tot hun conclusies, niet tot zuivere stamcelpreparaten.
Spangrude e.a., (1988) Science 241:58-62

11. Amplificatie van getransfecteerde beta-globine genen, zoals door Rund en medewerkers nagestreeft voor gentherapie bij beta-thalassemie en sikkelcel-anaemie, leidt met grote waarschijnlijkheid tot alpha-thalassemie.
Rund, D., e.a. (1987) Blood 70: 733-739
Grosveld, F., (1987) Cell 51: 975-985

12 De houding t.a.v. "roken" is pas wezenlijk veranderd, wanneer alom in plaats van "roken verboden", "roken toegestaan" wordt aangegeven.

A.C.M. Martens, 27 september 1988

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PROEFSCHRIFT

ter verkrijging van de graad van Doctor
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hoogleraar in de Faculteit der
Wiskunde en Natuurwetenschappen,
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College van Dekanen
te verdedigen op
dinsdag 27 september 1988
te klokke 14.15 uur

door

ANTONIUS CORNELUS MARIA MARTENS

geboren te Gilze-Rijen in 1952

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CHAPTER 1

STUDIES IN THE BN ACUTE MYELOCYTIC LEUKEMIA WITH RELEVANCE FOR CLINICAL APPLICATIONS

1.1 INTRODUCTION

The progress in the understanding of normal and pathological processes in the biology of man has benefited to a great extent from the study of animal models. In cancer research a large variety of animal species as well as many different types of animal tumors have extensively been characterized and studied. Each of them served a different, specific purpose.
The main objectives of studies with animal models are:
1. to gain basic knowledge of the underlying biological phenomenon
2. to exploit the results for application in solving clinical problems.

Comparative studies between the human and the animal are required to translate clinical problems to laboratory models. For the development of animal models for cancer research one should realize that many different types of tumors are known to occur in man and, even between tumors of the same type, differences are found due to variation between individuals.

Nevertheless, similarities in characteristics should be looked for and these should serve as selection criteria for a particular animal model. It is a general concept in animal model research that, depending on the specific question that has to be answered, a particular animal model has to be selected.

The studies which are discussed in this thesis are focused on acute leukemia, which is a disease of the hemopoietic system. Therefore, this particular malignancy can best be studied in relation to normal hemopoiesis. As studies on human hemopoiesis are mainly limited to in vitro experiments, animal models are indispensable.

The animal model which has extensively been investigated in our institute in recent years is the transplantable acute myelocytic leukemia in the Brown Norway rat (BNML).
The objectives of the studies which will be discussed are twofold:
1. The translation of clinical problems in human acute leukemia to a laboratory animal model and
2. Exploration of the animal model for developing new diagnostic and therapeutic tools to be applied clinically.

Obviously this requires extensive knowlegde of the human disease in relation to its counterpart in the experimental animal [Hagenbeek, 1987b]. The BNML leukemia model was introduced in 1977 in the Radiobiological Institute TNO in Rijswijk [Hagenbeek & Van Bekkum, 1977a, and Van Bekkum & Hagenbeek, 1977a]. In the years thereafter the model disseminated to a number of Dutch, European and American leukemia research centers (Table 1-1). In the rat model various aspects which have clinical relevance were studied. It is the intention to give, in this chapter, a summary of what has been achieved by the various research groups that have employed the BNML model. This review comprises the joined efforts to increase the knowledge of:

-the biology of leukemia growth;
-the interaction of leukemia with normal hemopoiesis;
-development of new approaches for experimental treatment; and
-the characteristics of Minimal Residual Disease (MRD).

TABLE 1-1

RESEARCH CENTERS WHICH EMPLOY THE BNML LEUKEMIA MODEL

-Radiobiological Institute TNO, Rijswijk, The Netherlands,
-Erasmus University, Rotterdam, The Netherlands,
-Leiden State University, Leiden, The Netherlands,
-Groningen State University, Groningen, The Netherlands,
-State Institute for Public Health, Bilthoven, The Netherlands,

-University of Zürich, Zürich, Switserland,
-Institute de Pathologie Cellulaire, Hôpital de Bicêtre, Paris, France

-ASTA-Werke, Bielefeld, W.Germany,
-Finsen Institute, Copenhagen, Denmark,
-University of Münster, W.Germany,
-University of Leuven, Belgium,

-M.D. Anderson Hospital and Tumor Institute, Houston, Tx, USA,
-Johns Hopkins University, Baltimore, Md, USA,
-Rush Presbyterian St. Luke Medical Center, Chicago, Ill, USA,
-Northwestern University, Chicago, Ill, USA,
-McGill University, Montreal, Canada,
-Lawrence Livermore National Laboratories, Livermore, CA, USA,
-Ohio State University, Columbus, Ohio, USA,
-Case Western Reserve University, Cleveland, Ohio, USA.

1.2 BIOLOGY OF LEUKEMIA GROWTH IN THE RAT

1.2.1 In vivo characteristics.

The transplantable myelocytic leukemia in the Brown Norway rat was introduced as a model for studying human acute myelocytic leukemia by Hagenbeek and Van Bekkum [1977a]. The most important characteristics of the BNML model are listed in Table 1-2 and schematically illustrated in Figure 1-1.

TABLE 1-2

MAJOR CHARACTERISTICS OF THE BROWN NORWAY ACUTE MYELOCYTIC LEUKEMIA

- induced by dimethyl-benzanthracene (DMBA),
- analogy with human (pro-) myelocytic leukemia:
  - cytology and cytochemistry,
  - slow growth rate (10^7 cells i.v. survival time 21-25 days),
  - growth fraction initially 100% decreasing to 40-50%,
  - severe suppression of normal hemopoiesis (CFU-S decrease)
  - diffuse intravascular coagulation (DIC),
  - prolonged blood transit time of leukemic cells (34-36 hours),
  - response to chemotherapy as in human AML,
  - presence of leukemic clonogenic cells,
  - no specific leukemia-associated antigens,
  - no virus involved.

The leukemia was classified as an acute pro-myelocytic leukemia (Figure 1-1a), with signs of diffuse intravascular coagulation as the leukemia progresses [Hilgard, 1977; Donati, 1977; Colucci 1983]. The leukemia can be transferred by intraperitoneal (i.p.), subcutaneous (s.c.), intrathecal (i.t.) or intravenous (i.v.), injection of single cell suspensions. The i.p. injection of leukemic cells results in the formation of greenish coloured colonies of leukemic cells in the mesenterium (chloromas), followed eventually by disseminated leukemia growth. No ascites fluid is formed. The s.c. transfer of leukemic cells results in the slow development of a subcutaneous tumor which eventually results in disseminated leukemia growth. The i.p. and s.c. transfer of leukemia cells were not investigated in depth, in contrast to the i.t. transfer of leukemic cells [Hoogerbrugge & Hagenbeek, 1985] which was studied extensively with the purpose of the development of a model for central nervous system (CNS) leukemia.

Most studies, however, were done after intravenous transfer of leukemia. The survival time after i.v. injection of leukemic cells is linearly correlated with the injected cell number (Figure 1-1b). After injection leukemic cells spread to all

organs, but they predominantly home to the bone marrow, liver and spleen where they start to proliferate and increase in number [Hagenbeek & Martens, 1980a]. The ED50, which represents the number of cells that is required to induce leukemia in 50 % of the animals, was found to be 25 cells. The leukemic cells are transferred in a syngeneic system, i.e. the in-bred BN rat strain and were found not to be immunogenic.

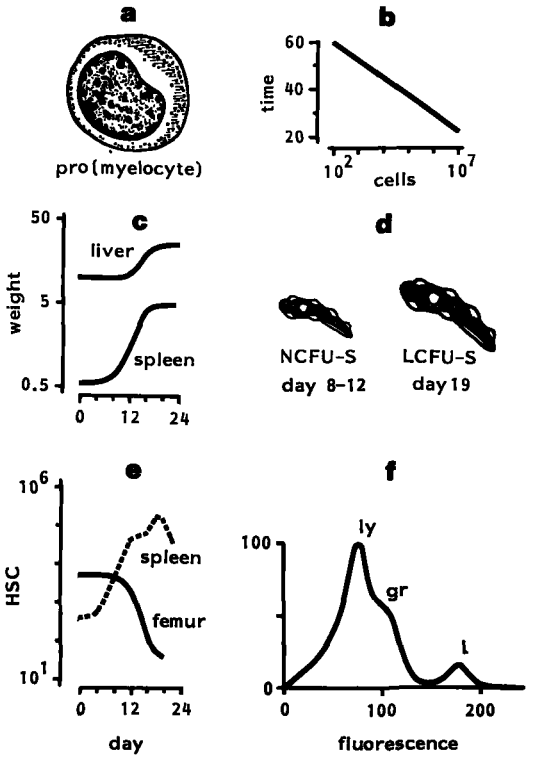


Figure 1-1:
Main characteristics of the BNML model.
Schematically are represented:
a (pro)myelocyte;
b dose-survival relationship;
c increase in liver and spleen weight during leukemia progression;
d possibility to detect the normal hemopoietic stem cell (CFU-S) and, the clonogenic leukemic cell (LCFU-S);
e the decrease of the normal stem cells in the bone marrow and the increase in the spleen during the progression of leukemia; and
f the possibility to detect leukemic cells with a monoclonal antibody and flow cytometry (ly: lymphocytes; gr: granulocytes; l: leukemic cells).

The growth pattern of the leukemia appeared to be highly reproducible throughout a long period of exploitation.

The BNML could be successfully transferred to certain F1 hybrid combinations with varying degrees of hybrid resistance [Vaughn et al., 1978; Williams et al., 1980; Singer et al., 1980; Burke et al., 1986a; Tutschka et al., 1987].

The liver and spleen increase in size and weight during the progression of the disease (Figure 1-1c). During the development of leukemia the bone marrow is rapidly replaced by leukemic blast cells. At this stage leukemic cells are appearing in the peripheral blood leading to increased numbers of peripheral white blood cells, predominantly leukemic blast cells.

The fate of the pluripotent hemopoietic stem cells during this process has been studied. Hemopoietic stem cells are detected by a spleen-colony-assay (SCA) (Figure 1-1d; see also Chapter 2). Concurrently with the replacement of the normal bone marrow, the number of hemopoietic stem cells decreases and reaches very low levels during the terminal stage of the disease. Simultaneously, the number of stem cells in the spleen and in the peripheral blood increases (Figure 1-1e).

Another important characteristic of the BNML is the clonogenic property of the leukemic cells. The injection of low numbers of cells (less than 10^5) results in the development of leukemic colonies in the spleen, defined as LCFU-S (leukemic-colony-forming-unit-spleen) clearly visible on the surface 19-20 days after injection (Figure 1-1d). Every viable leukemic cell has clonogenic potential [Van Bekkum et al., 1978]. However, after the i.v. injection, a certain proportion of the cells home to places which are unfavorable for leukemic cell growth. This explains the discrepancy between an ED 50 value of 25 cells and a clonogenic potential of close to 1.

Finally, the BNML has the advantage that the leukemic cells can be discriminated from the normal cells by the use of a mouse anti-BNML monoclonal antibody (MCA). The MCA reacts with an antigen, present in low quantities on normal granulocytes and in high quantities on leukemic blast cells, but which is absent on normal lymphocytes and normal blast cells. By using flow cytometry this enables the study of the growth of the leukemic cell population (Figure 1-1f).

1.2.2 In vitro characteristics.

One of the characteristics of human acute myeloid leukemia (AML) is the limited capability of the leukemic blast cells to form colonies in <u>in vitro</u> culture systems which facilitate colony forming cells of normal bone marrow origin to grow. A similar observation was made in the BNML model [Van Bekkum et al.,;Van Bekkum & Oosterom,1976b] Be it after a many attempts, it was possible to generate <u>in vitro</u> growing cell lines from the BNML.Two different approaches

were used. One was based on stimulation of cells in long term cultures containing 4% rat serum and 6% fetal calf serum (v/v). The cells were derived from the spleen of leukemic animals [Glynn & Sullivan, 1983]. Autonomously growing cells were derived, which were blastic variants, morphologically distinct from the parent line; i.e. they were less mature myeloblasts. When transferred into rats these cells behaved differently from the original leukemia as indicated by the formation of tumor nodules in the epidural space accompanied by hind limb paralysis. This growth behaviour offered an interesting system for studying CNS involvement in leukemia [Brox et al., 1984].

A second successful approach for the establishment of an in vitro growing cell line was based on culturing BNML cells in the presence of a stromal layer. The presence of both fetal calf serum and horse serum was important for the formation of a stromal layer composed of fibroblasts, macrophages and adipocytes, which could support the BNML cells until they were capable of autonomous growth [Lacaze et al., 1983]. In this case the promyelocytic nature of the BNML cells was retained. By using a collagen in vitro culture system, the plating efficiency appeared to be 60-90% for the in vitro established cell line, when cells were taken during log phase growth. If, however, the cell line was first transferred to rats and then cultured in the collagen layer assay, directly out of the animal, the plating efficiency was in the order of 6 %. This suggests that in vivo subpopulations of leukemic cells are formed, that are characterized by a different capability to form colonies in vitro [Lanotte et al., 1984] .

1.2.3 Methods for detection and enumeration of leukemic cells

Based on the previously discussed growth characteristics, five independent methods are available by which the leukemic cell content in animals, organs or cell suspensions can be determined. This enables to evaluate the efficacy of a given treatment in a quantitative way.

a. Cytology/cytochemistry.

Simple total nucleated cell counting in combination with May-Grünwald-Giemsa staining for differential cell counting is sufficient to determine leukemic cells numbers when they are present in frequencies between 1% and 100 % (Figure 1-2). Cytochemical characterization revealed that the leukemic cells were promyelocytes (peroxidase reaction ++; esterase +; Sudan Black +++; acid phosphatase ++; alkaline phosphatase -).

Obviously it makes a difference which type of organ is studied. In spleen cell suspensions the detection level will be low (1%) because promyelocytes are rarely observed in the spleen under normal conditions. In the bone marrow however, detection of leukemic promyelocytes will be difficult when they are

present in a frequency below 5 %, because this is the background value of normal promyelocytes.

b. The organ weight parameter

The growth of leukemic cells in liver and spleen results, during the later stages of leukemia development, in an increase in the weight of both organs. It was found that 1 gram increase in weight corresponds to an increase of 10^9 cells (schematically shown in Figure 1-3).

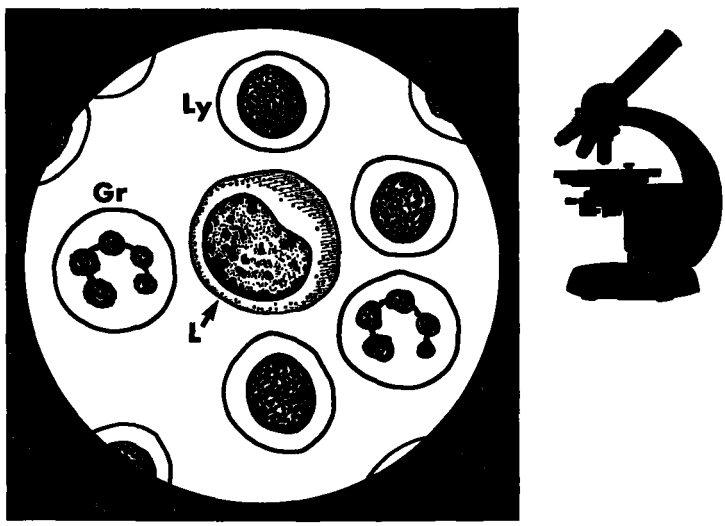


Figure 1-2:
Schematical representation of the microscopical method for the detection and enumeration of BNML cells.
Ly: lymphocytes; Gr: granulocytes; L: leukemic cells.

Once the organ weight is significantly increased, the leukemic cell number can be deduced from it. The spleen increases in weight from about 500 mg to 3-4 grams towards the terminal stages of leukemia. The liver increases in weight from 10 to 20-25 grams. For the spleen the increase has to be at least 200 mg (corresponding to 2×10^8 cells) to become significantly different from the normal background organ weight. For the liver the increase has to be at least 1 gram to become significant, which corresponds with 1×10^9 cells. By weighing the spleen and the liver the effectiveness of a treatment can be quantified. The method is valuable for studying advanced disease. Changes in the tumor load in the range of 5×10^8 to $1\text{-}2 \times 10^{10}$ can be studied corresponding to one and a half decade (expressed as 1.5 log).

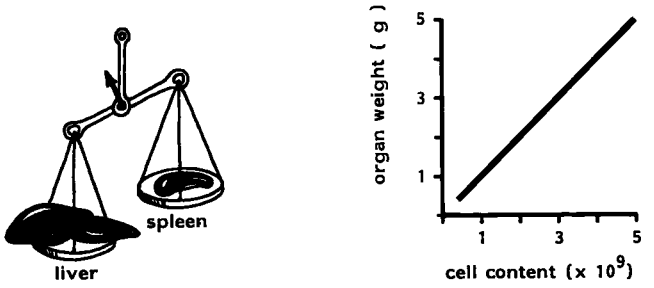


Figure 1-3:
Schematical representation of the method for measuring the leukemic cell content of spleen and liver based on organ weights.
One gram increase in tissue weight corresponds with 10^9 leukemic cells.

c. The survival time assay

A more sensitive method is based on the relation between the survival time and the number of injected cells. It is schematically illustrated in Figure 1-4. A single cell suspension can be prepared from the total content of a femoral bone by flushing the marrow cavity with a physiological fluid (e.g. phosphate buffered saline). The total volume of this suspension is measured as well as its cell content. A fraction of the total volume e.g. 1/10 is injected i.v. into normal recipient rats. If leukemic cells are present in the cell suspension, the animals will eventually die from leukemia. The survival time is recorded. Based on the known relation between the number of leukemic cells injected and the survival time, the originally injected leukemic cell content can be deduced. To determine the leukemic cell content of any organ, the survival time assay can be used which implies that the total leukemic cell content for each organ of choice can be determined.

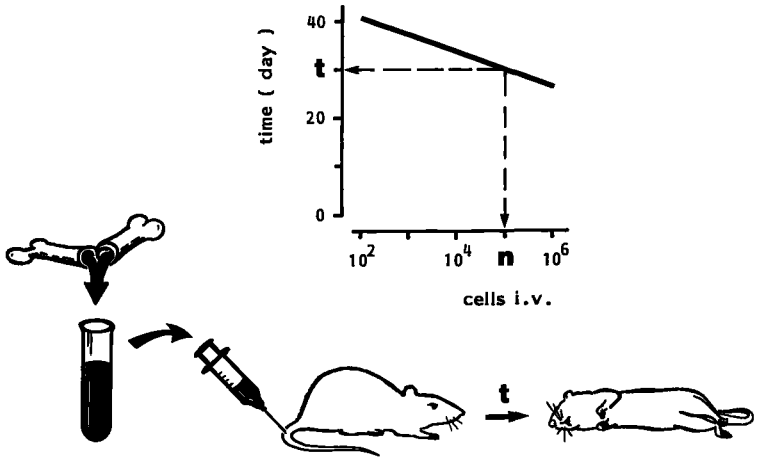


Figure 1-4:
Schematical representation of the method for measuring leukemic cell numbers based on the correlation between the number of leukemic cells injected and the corresponding survival time.
A decrease in the survival time with 4 days corresponds with a factor of 10 (1 log) increase in cell number.

For very low numbers of leukemic cells (i.e. below 100 cells) the survival times deviate from the straight line: the survival times increase disproportionally [Van Bekkum et al., 1977a]. However, by using the results from the cell dose-survival curve, the leukemic cell number can be deduced from the percentage of animals that develop leukemia.
The limits of detection of this dose-survival bioassay spans 8-9 logs.

d. Leukemic Colony Forming Unit-Spleen assay (LCFU-S).

After the i.v. injection of low numbers of leukemic cells into normal recipient rats they distribute to all tissues of the body. Cells seeding into the spleen form colonies. When monitored at the appropriate time, i.e. 19-20 days after the injection, these colonies (composed of leukemic cells) are clearly visible on the surface of the rat spleen. In analogy to spleen colony formation of normal stem cells it is assumed that each colony is derived from one clonogenic leukemic cell, termed leukemic colony forming unit spleen (LCFU-S). The fraction of leukemic cells which enters the spleen could be determined. This so-called f-factor was found to be 0.002 [Van Bekkum et al., 1977b]. By counting the number of spleen colonies and multiplying this value with the f-factor, the number of clonogenic cells in a cell suspension can be calculated. The limits of detection of this method spans 5-6 logs.

The LCFU-S assay shows a strong resemblance with the spleen colony assay by which normal hemopoietic stem cells can be measured. Normal stem cells also form colonies on the surface of the spleen and are therefore named colony forming unit spleen (CFU-S). However, there are two major differences between normal- and leukemic spleen colony formation: i.e. 1. to determine the normal CFU-S the recipients have to be conditioned with lethal whole body irradiation while the LCFU-S assay is performed in non-irradiated recipients and 2. the normal CFU-S colonies can be counted between days 7 and 14 after injection of the cells [see also Chapter 3], while in the LCFU-S assay the colonies are counted at day 19-20. This indicates that normal hemopoietic stem cells proliferate approximately twice as fast as clonogenic leukemic cells. These discriminative assays are schematically shown in Figure 1-5.

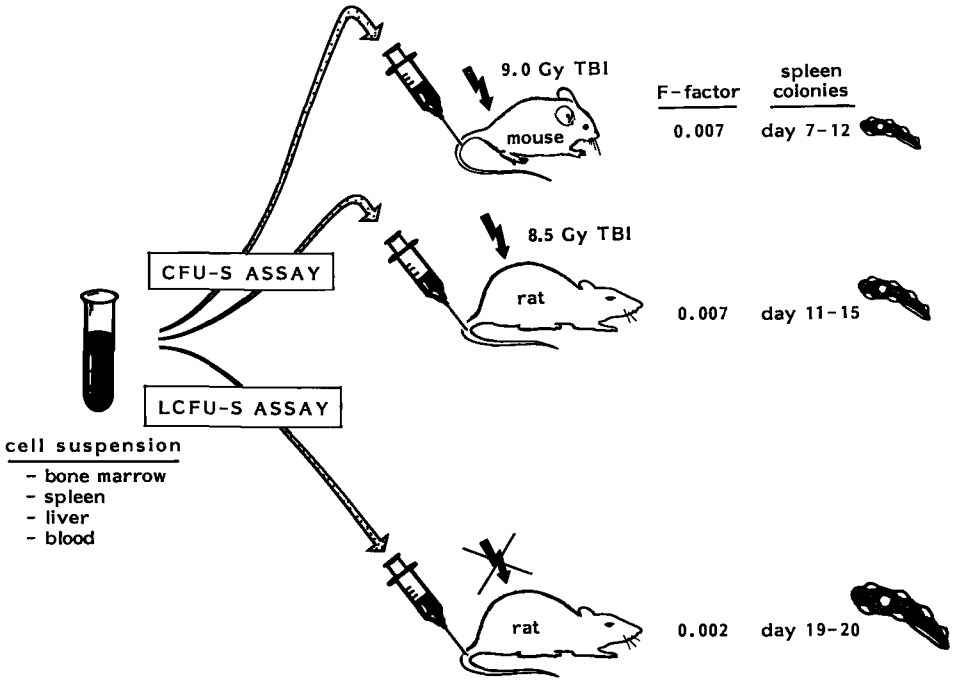


Figure 1-5:
Schematical representation of the assays for measuring the numbers of normal hemopoietic stem cells (CFU-S) and the leukemic clonogenic cells (LCFU-S), based on their ability to form colonies on the surface of the spleen.
Normal stem cells are detected in animals (rats or mice) that received lethal total body irradiation (TBI) before they were injected with cell suspensions, derived from various organs. Leukemic cells are forming spleen colonies in non-irradiated recipient rats.

In summary, one of the great advantages of the BNML is the fact that it is possible to enumerate the normal CFU-S as well as the LCFU-S in the same cell suspension by choosing the appropriate spleen colony assay. These two methods allow the

determination of the therapeutic index of all treatments which depend on the differences in response between leukemic cells and normal hemopoietic cells. Chemotherapy and total body irradiation have extensively been studied with these methods while, current research with recombinant hemopoietic growth factors will indicate whether stimulation of hemopoietic cells also results in stimulation of the growth of leukemic cells.

e. Monoclonal antibody labeling and flow cytometry.

A monoclonal antibody (MCA) was raised against BNML cells i.e. MCA-Rm124 (Dr. R.J. Johnson, Johns Hopkins University, Baltimore, Md, USA), which detects an antigen that is present in a high density on leukemic cells, in a low density on normal granulocytes and absent on lymphocytes and normal blast cells [Martens et al., 1984 and Chapter 5]. After labeling of cells with the FITC conjugated MCA under standard conditions it is possible to discriminate between normal and leukemic cells on the basis of fluorescence intensity using a flow cytometer. The principle of the method is schematically shown in Figure 1-6.

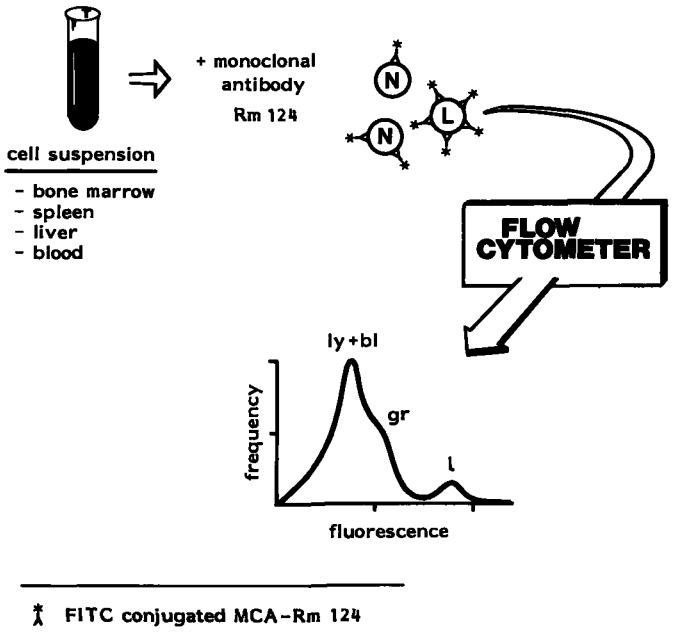


Figure 1-6:
Schematical representation of the method for measuring the number of leukemic cells after labeling with the monoclonal antibody Rm124 and subsequent flow cytometrical determination of the fluorescence intensity.
The leukemic cells are found in the fraction with the highest fluorescence intensity.
L: leukemic cells; N: normal cells; ly+bl: lymphocytes and blast cells; gr: granulocytes.

A typical fluorescence distribution histogram of a bone marrow cell suspension containing low numbers of leukemic cells is shown in Figure 1-7. The fraction of leukemic cells can easily be determined.

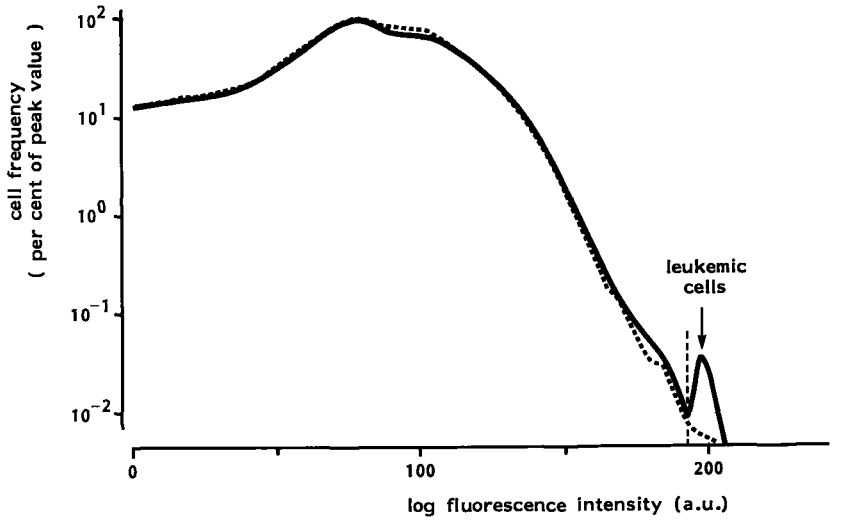


Figure 1-7:
Typical histograms of normal bone marrow and bone marrow containing a low fraction (0.01%, i.e., 1 per 10,000) of leukemic cells:
Dashed line: normal bone marrow
Solid line: bone marrow containing 0.01% leukemic cells
The dashed vertical line indicates the boundary between normal and leukemic cells

This type of analysis allows the exact quantitation of the leukemic cell content in cell suspensions prepared from any organ. If, however, the frequency of leukemic cells is low, e.g. in minimal residual disease (MRD), accurate analysis is hampered due to the presence of dead cells which aspecifically bind the MCA. The addition of Propidium Iodide (PI) to counter-stain the dead cells with the use of a special setting on the FACS II (using a 570 nm di-chroic mirror and the FITC filter combination, Figure 1-8), enabled selective measurement of the fluorescence intensity of the viable cells while retaining the possibility to measure two other parameters i.e. foreward light scatter (FLS) and perpendicular light scatter (PLS).

The detection limit of leukemic cells using flow cytometry is in the order of 1 leukemic cell per 10,000 to 100,000 normal cells and thus it spans 4 to 5 decades.

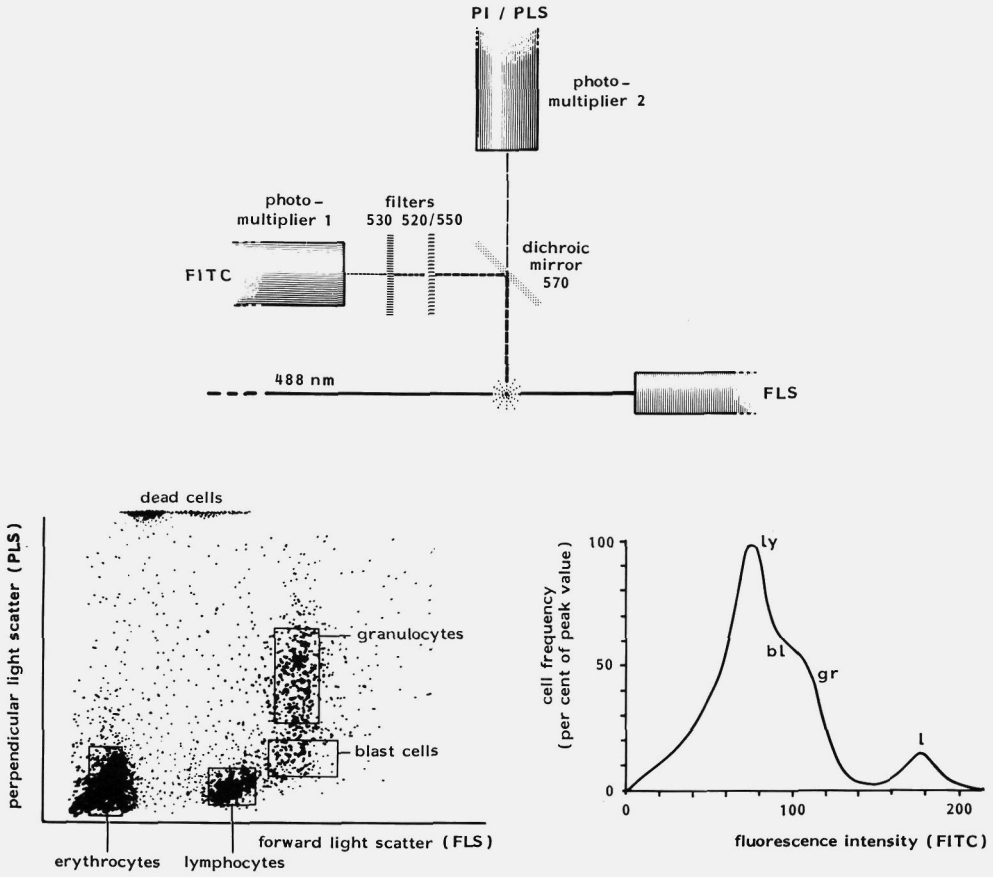


Figure 1-8:
Configuration of the FACS II flow cytometer for measuring the fluorescence intensity of the viable cell fraction (see paragraph 1.2.3 for explanation)

FLS: forward light scatter;
PLS: perpendicular light scatter;
PI: propidium iodide;
FITC: fluorescein-isothiocynate (fluorescent dye);
PM: photomultiplier tube; 488 nm: laser-beam wavelength;
BP: band-pass-filter 520-550 nm; 530 LP: 530 nm long-pass filter; DM 570 dichroic mirror 570 nm: >570 nm passes, < 570 nm is deflected 90 degrees;
ly: lymphocytes;
bl: blast cells;
gr: granulocytes;
l: leukemic cells.

All five methods, independently of each other, allow the enumeration of leukemic cell numbers in various organs [Martens & Hagenbeek, 1985]. The sensitivity is

however different for each of the methods described. The lower limits and the range of detection of the various methods are shown in Figure 1-9.

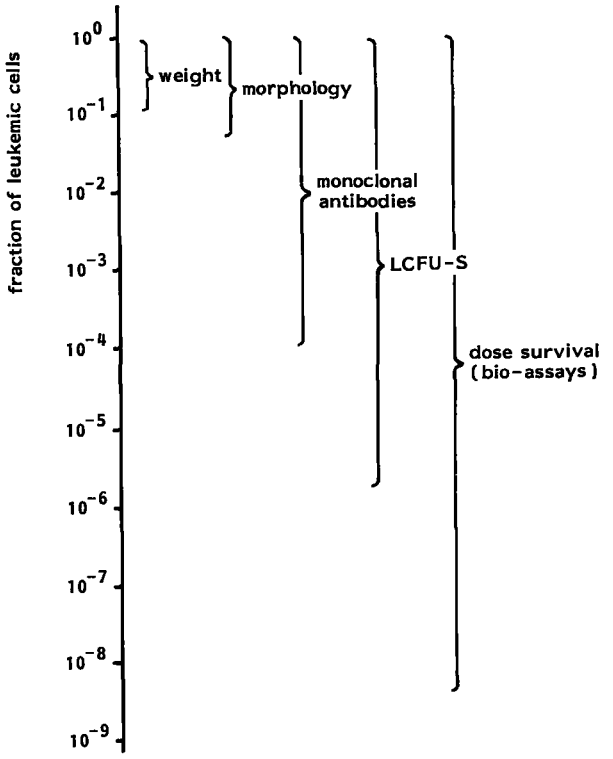


Figure 1-9:
Limitations of the available methods for the detection and quantification of leukemic cells in the BNML model.
LCFU-S: leukemic colony forming unit spleen.

1.2.4 Cytogenetics of the BNML.

BNML cells appeared to be characterized by a typical pattern of chromosomal aberrations i.e., 1p+, 2p+, 8q+, -9, 12q-, 20q+, XX. A complete karyogram for the BNML is shown in Figure 1-10 [Dr. A. Hagemeijer, Erasmus University Dept. of Cell Biology and Genetics, Rotterdam, The Netherlands]. The in vitro growing cell line ICP-81 [Lacaze, 1983], later LT-12, was also characterized cytogenetically and compared to the parent BNML line [Arkesteijn et al., 1987] the typical chromosomal aberrations of the in vivo growing parent line were preserved in the LT-12 cell line. The cell line appeared to be most valuable for

studying various aspects of flow karyotyping of leukemia cells, e.g., for the development of chromosome isolation and staining procedures [Arkesteijn et al., 1987; 1988]. Examples of the univariate and bivariate flow karyograms as well as a three-dimensional distribution of chromosomes derived from the LT-12 cells are shown in Figures 1-11, 1-12 and 1-13.

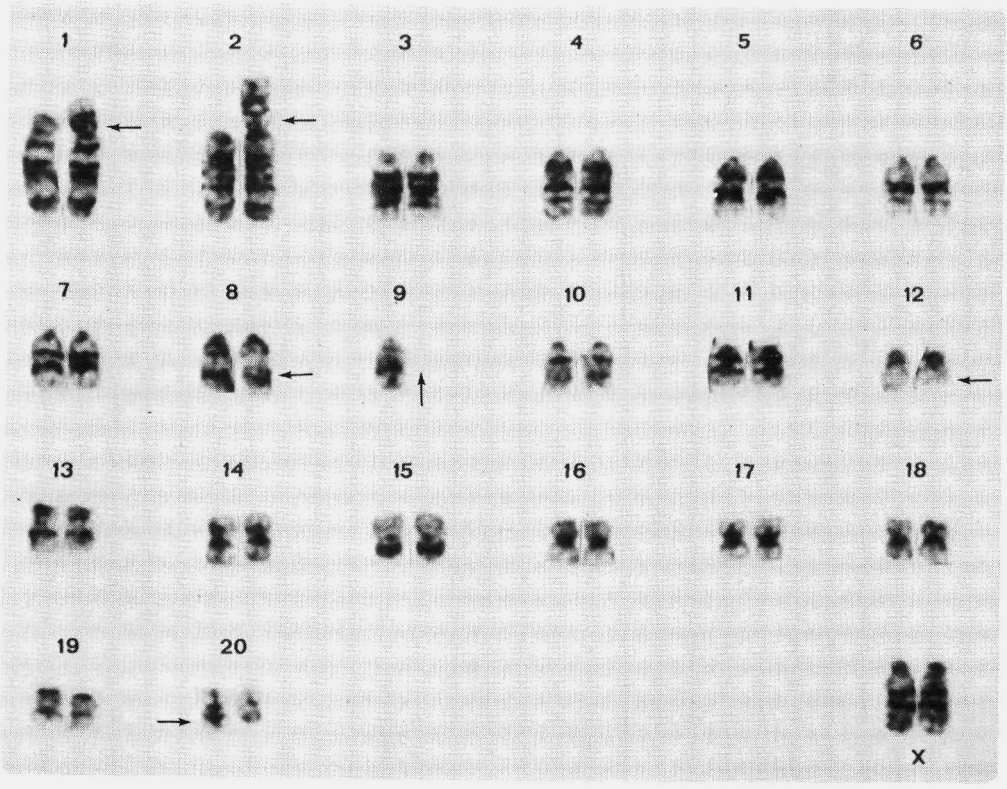


Figure 1-10:
Conventional cytogenetic analysis of the BNML.
The arrows indicate the chromosomes with aberrations: i.e.,
1p+, 2p+, 8q-, -9, 12q+ and 20 q-.
(Dr. A. Hagemeijer, Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands)

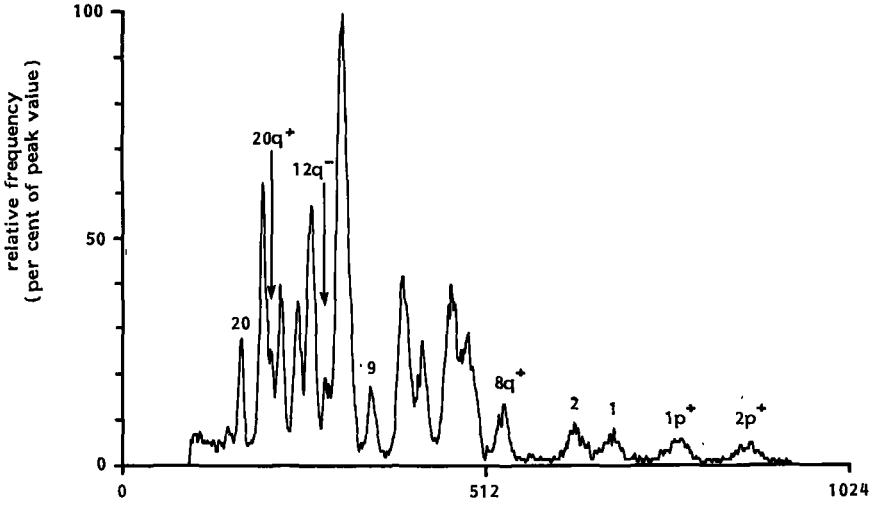


Figure 1-11:
Flow cytometrical cytogenetic analysis of the in vitro growing subline from the BNML (LT12) after chromosome isolation and staining with propidium iodide.
An univariate flow karyogram is shown. The positions of some of the normal chromosomes as well as all aberrant chromosomes are indicated.

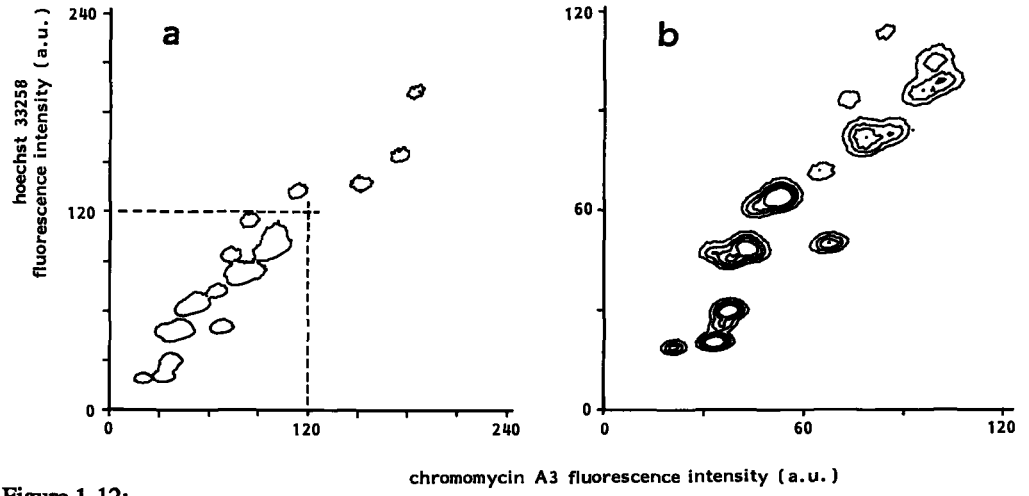


Figure 1-12:
Bivariate flow karyogram of the in vitro growing subline from the BNML (LT12).
Chromosomes are isolated and stained with the fluorescent dyes Chromomycin A3 (binds specifically to CG-rich regions in the DNA) and Hoechst 33258 (binds specifically to AT-rich regions in the DNA). The Chromomycin A3 fluorescence is plotted on the x-axis and Hoechst 33258 fluorescence is plotted on the y-axis. Contour lines are plotted at selected intervals to show the chromosome cluster pattern. The left panel (a) shows all chromosomes; the right panel (b) shows the "smaller" chromosomes (surrounded by the dashed line in the left panel) at full scale resolution.

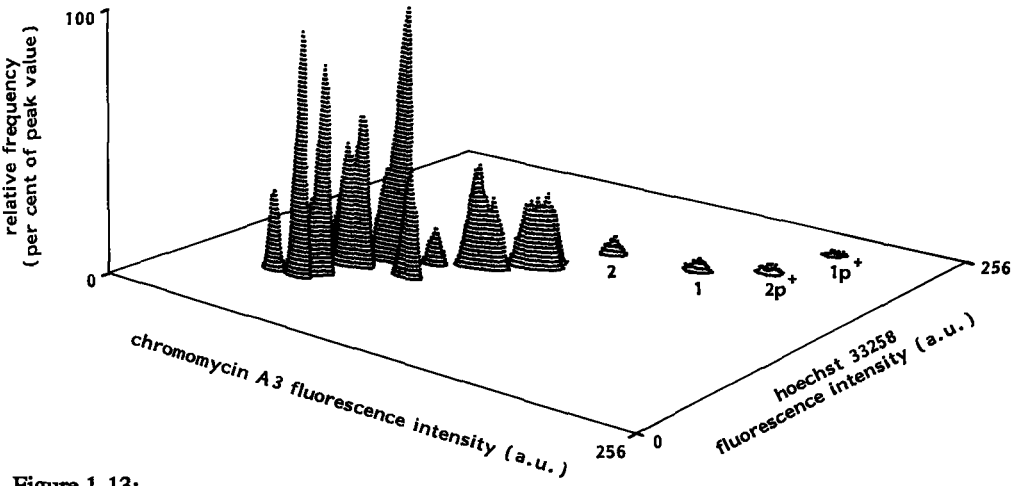


Figure 1-13:
Three-dimensional representation of the flow karyogram of the chromosomes of the in vitro growing BNML subline (LT12).
Resolution of the matrix is 256 x 256 channels.
The marker chromosomes 1p+ and 2p+ and the normal chromosomes 1 and 2 are indicated.

1.2.5 Cell cycle kinetics of the BNML

The BNML leukemia belongs to the group of "slowly" growing experimental tumors, predominantly because the growth fraction decreases from 1 to 0.4 during the development of the leukemia. At the same time the cell loss factor increases from 0.26 to 0.88. The cell kinetic parameters for the BNML leukemia have been determined by means of autoradiographic methods (tritiated thymidine percent labeled mitosis curve) [Hagenbeek et al., 1977b]. The distribution of cells over the cell cycle was determined by means of flow cytometry (FCM). Typical DNA histograms of Propidium Iodide (PI) stained leukemic cell suspensions are shown in Figure 1-14. The cell suspensions as well as the isolated nuclei were treated with RNAse to digest RNA. It is important that double stranded RNA (dsRNA) is removed because PI does not discriminate between DNA and dsRNA. At the same time, the amount of dsRNA in the nuclei as well as in the cytoplasm, can be estimated. An increased amount of RNA is a common finding in acute promyelocytic leukemia.

The cell cycle time of the BNML cells in vivo is 14.0 hours comprising a G1 phase of 0.8 hours, an S-phase of 10.0 hours, a G2+1/2M phase of 3.2 hours, respectively, leading to a doubling time during the exponential growth phase of 19 hours, given a growth fraction of 1 and a cell loss fraction of 0.26 [Hagenbeek et al., 1977b].

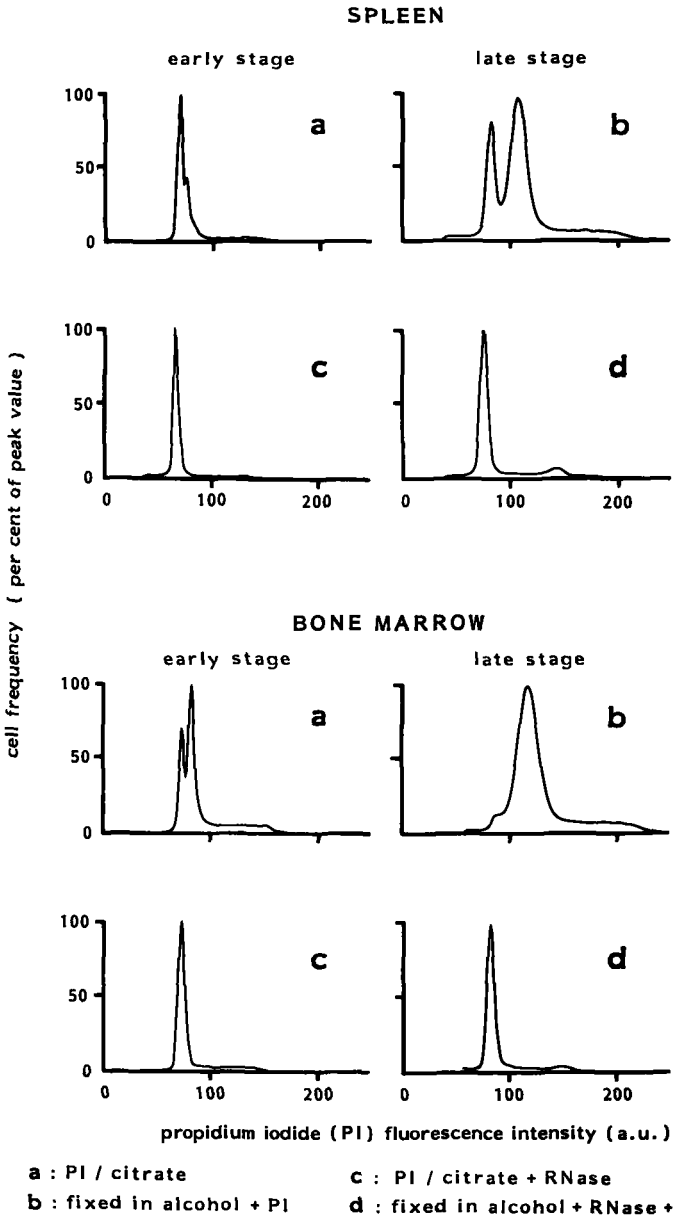


Figure 1-14:
The contribution of dsRNA to DNA fluorescence histograms of BNML cells derived from the spleen or from the bone marrow during early and late stage leukemia development when stained with the intercalating dye propidium iodide.

The population doubling time of 19 hours was confirmed in later studies which were focused on mathematical modelling of leukemic cell growth by using the survival and L-CFUS bio-assays [Schultz et al., 1985c, 1986a, 1986b, 1987a] . The growth curves for the BNML cell population could best be described using a non-linear, least squares computer algorithm. The growth curve consists of an initial exponential part (constant doubling time of t= 19 hours) which changes into a Gompertz curve, leading to a steady state plateau phase as shown in Figure 1-15.

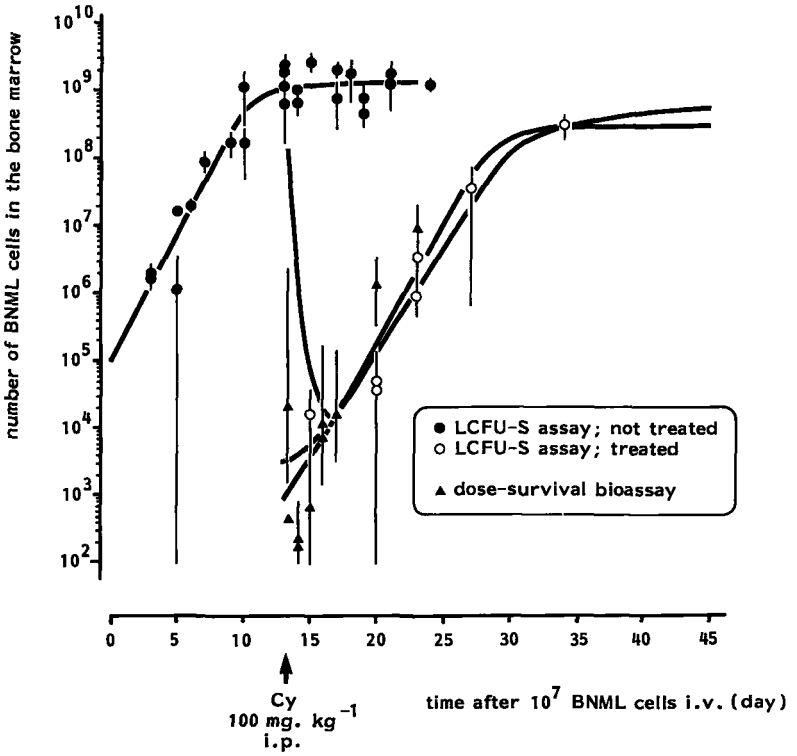


Figure 1-15:
Computer derived growth curves, fit to datapoints of BNML cell content determinations in the bone marrow compartment before as well as after the induction of a complete remission with cytostatic drug treatment.

This figure also shows the regrowth curve after a single dose treatment with cyclophosphamide. It was concluded that the drug treatment resulted in an instantaneous reduction in tumorload. This was followed by a regrowth pattern resembling leukemic growth before treatment although the tumor population doubling time increased from 19 hours to 24 hours. This suggests that either there is a more efficient elimination by cyclophosphamide of the faster cycling cell population or that during the leukemic regrowth there is increased cell death

resulting in an increased cell-loss factor and thus a slower increase of the leukemic cell population.

1.2.6 Interaction with normal hemopoiesis.

The suppression of normal hemopoiesis is a prominent feature of human acute leukemia as well as during the development of the BNML [Van Bekkum et al., 1976a; 1978; Hagenbeek et al., 1977d; 1977e; Martens & Hagenbeek, 1987 and Chapter 4]. Therefore the interaction between leukemic cells and normal hemopoietic cells has been extensively studied in the BNML model. One of the main questions was: are normal cells replaced by leukemic cells or do leukemic cells inhibit normal hemopoietic cell growth by means of humoral factors? The absolute numerical decrease in the number of in vitro clonogenic hemopoietic precursor cells (CFU-C) as well as of pluripotent stem cells in the marrow (CFU-S, detected in a spleen colony assay) during leukemia growth, support the replacement theory [Van Bekkum et al., 1976a]. In vitro mixing experiments and in vivo diffusion chamber studies excluded that either (short-range) humoral factors or direct cell-to-cell interaction played a significant role in the inhibition of normal hemopoiesis [Van Bekkum & Oosterom, 1976b].The mechanism responsible for this replacement was studied by determining the homing characteristics of BNML cells after labeling with radio-isotopes. In this way the exact location of leukemic cells could be determined in femoral marrow cross-sections [Prins & Van Bekkum 1981; Van Bekkum et al., 1981]. After the i.v. injection of BNML cells they lodged predominantly near the endosteal sites in the bone marrow (Figure 1-16).

This behaviour of the myelocytic BNML cells was completely distinct from the behaviour of cells from an acute lymphatic leukemia (the L4415 rat leukemia model) which was studied in comparison. L4415 cells were found randomly distributed in the bone marrow cavity. These findings correlate with previously reported observations using time lapse cinematophotography [Haemmerli et al., 1983 and Sträuli et al., 1981]. These investigators found that cells from myelocytic leukemias and lymphocytic leukemias behaved completely different with respect to their in vitro motility and mobility, as well as in the mode of infiltration of feederlayers of mesenteric cells.

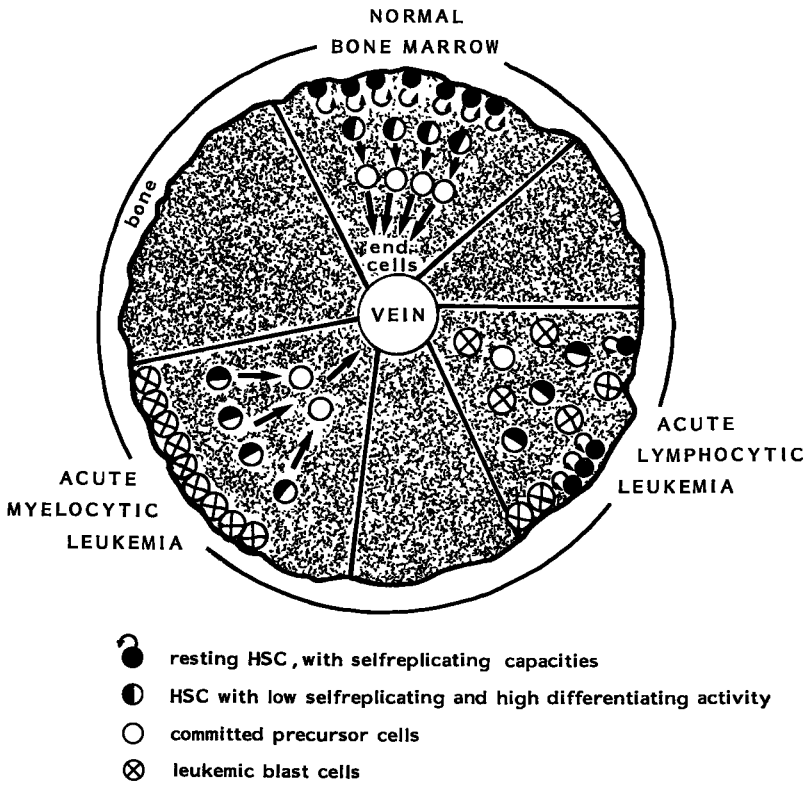


Figure 1-16:
Schematic representation of a transverse section of a rat femur showing specific homing of acute myelocytic leukemia cells (AML) leading to replacement of normal hemopoietic stem cells and random homing of acute lymphocytic leukemia cells (ALL) cells.

With respect to blood cell production the pluripotent hemopoietic stem cell is of crucial importance. Stem cells are located in the bone marrow near the endosteal sites exactly at the location where the BNML cells were found to lodge. This specific location may be associated with the presence of "niches" in the marrow cavity where the microenvironment controls the proliferation of pluripotent hemopoietic stem cells [Schofield et al., 1980; Lord, 1986; Gordon et al., 1987]. The more differentiated descendants from normal stem cell proliferation migrate out of these niches towards the centre of the marrow cavity. The leukemic cells have lost the ability to differentiate and may as a consequence fail to migrate, thereby competing for the favourable microenvironment with the normal hemopoietic stem cells. This could very well be the reason why stem cells i.e. the

colony forming unit spleen (CFU-S) and the in vitro detectable colony forming cells (CFU-C) decrease in numbers so early in the development of leukemia. Although the decrease of normal stem cells in the marrow is accompanied by an in increase in CFU-S numbers in the spleen (Figure 1-17), the liver and the peripheral blood, the absolute number of CFU-S in the animal decreases during the terminal stage of leukemia [Van Bekkum et al., 1978; Hagenbeek, 1977e; Colly, 1980; Chapter 4].

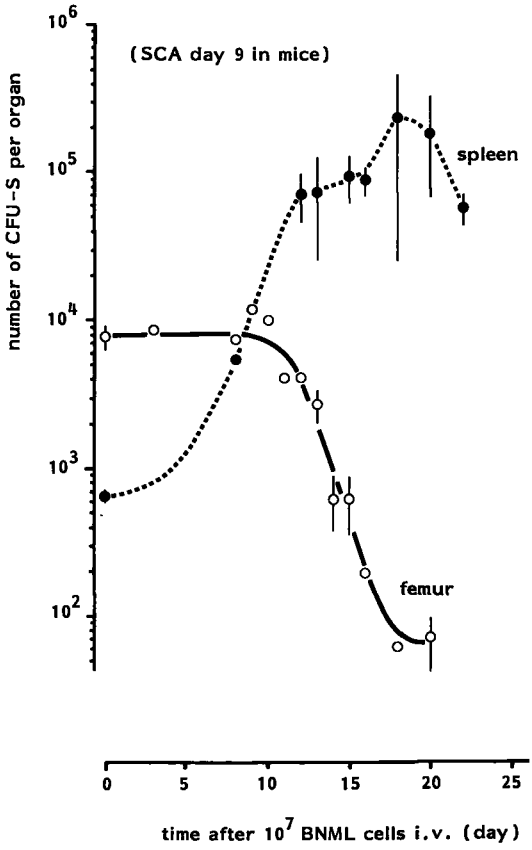


Figure 1-17:
The fate of the hemopoietic stem cells in the bone marrow and in the spleen during the development of leukemia.

The determination of the kinetic status of the CFU-S in various organs indicated [Hagenbeek & Martens, 1981e] that, in the marrow of leukemic animals the fraction of CFU-S in cycle as well as the absolute number decreased. In the spleen however, the number of CFU-S increased rapidly while they retain a high cycling activity. The rise of CFU-S in the spleen can be explained in two ways:

1. by the local production, in response to insufficient production in the marrow, 2. by transportation of dislocated marrow stem cells to the spleen.

During the terminal stage of leukemia, diffuse intravascular coagulation occurs resulting in the formation of leukemic cell thrombi which obstruct the vascular capillaries of the lungs. This eventually leads to the death of the animal. This phenomenon is also typical for human promyelocytic leukemia.

1.3 EXPERIMENTAL TREATMENT OF THE BNML LEUKEMIA

The BNML leukemia model has been used extensively in attempts to develop new treatment approaches. The effect of treatment was studied at various phases of leukemia development. A number of different treatment modalities e.g. high-dose chemotherapy, total body irradiation followed by bone marrow transplantation, immunotherapy, as well as combinations, were investigated at various stages of leukemia development. In Figure 1-18 the leukemic cell load at these various stages is shown.

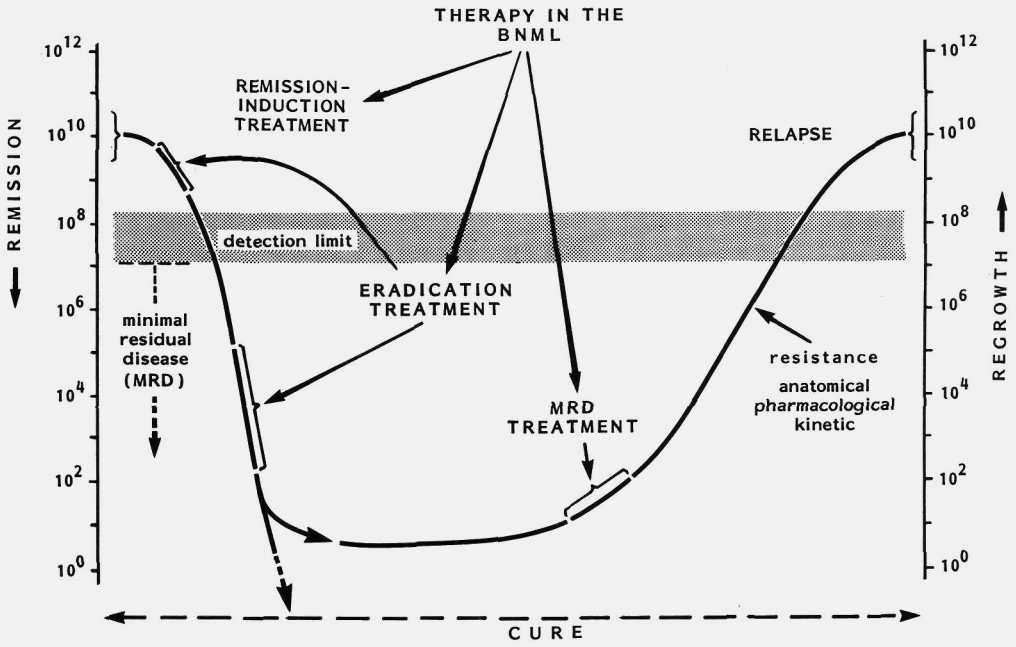


Figure 1-18:
Schematic representation of the various stages of leukemia development in the BNML during which certain treatment modalities were explored.

The model was particularly used for studying problems that cannot adequately be investigated in patients. An overview of the various treatments studied, is given in Tables 1-3 and 1-4. The parameters for evaluation of the therapeutic ratio for the various treatment modalities have been discussed in section 1.2.3.

TABLE 1-3

STRATEGIES FOR THE DEVELOPMENT OF NEW REMISSION INDUCTION CHEMOTHERAPY

treatment	purpose/question
ECIB	efficacy of extracorporeal irradiation of the blood as alternative for remission-induction chemotherapy
Adriamycin/ Daunomycin	anti-leukemic effect/therapeutic index pharmacokinetic studies drug uptake kinetics using flow cytometry development of a multi-compartment computer analysis program for pharmacokinetics of anthracycline combination chemotherapy with Ara-C
AMSA	anti-leukemic effect/therapeutic index
Cytosine-Arabinoside	anti-leukemic effect/therapeutic index cell kinetic response/timed-sequential chemotherapy combination chemotherapy with - methotrexate - adriamycin
Dinaline	anti-leukemic effect/therapeutic index
5-Fluorouracil	anti-leukemic effect combination chemotherapy with nitrous oxide
Glutaminase/ Asparaginase	anti-leukemic effect/therapeutic index
Methotrexate	anti-leukemic effect combination chemotherapy with Ara-C
Nitrous Oxide	anti-leukemic effect via inactivation of cobalamine (vitamin B12)
Thymidine	anti-leukemic effect of high dose treatment

TABLE 1-4

BONE MARROW TRANSPLANTATION

A. CONDITIONING REGIMENS

treatment	purpose/question
Total body irradiation (TBI)	determination of *in vivo* irradiation sensitivity anti-leukemic effect of TBI comparison of flash- and fractionated TBI anti-leukemic effect of the combination of TBI and high-dose chemotherapeutic agents
Eradication chemotherapy "conditioning" with a variety of drugs i.e. -Cyclophosphamide -Busulphan -Piperazinedione -Peptichemio -Chlorambucil -Melphalan	determination of the maximally tolerated dose in normal and leukemic animals after single or split-dose treatment; the bone marrow function is fully restored by "autologous" bone marrow transplantation

B. ELIMINATION OF RESIDUAL LEUKEMIC CELLS FROM THE MARROW GRAFT

treatment	purpose/question
Biophysical elimination of leukemic cells using - discontinuous density gradient centrifugation - *velocity sedimentation separation* - free-flow-cell electrophoresis - combinations	
In vitro chemotherapy	selective elimination of leukemic cells with *in vitro* active metabolites of cyclophosphamide

C. TREATMENT AFTER BONE MARROW TRANSPLANTATION DURING MINIMAL RESIDUAL DISEASE (MRD)

treatment	purpose/question
Ara-C	cell kinetic status of residual cells
Cyclophosphamide	eradication chemotherapy of MRD
Dinaline	eradication chemotherapy of MRD
Peptichemio	eradication chemotherapy of MRD
BCG	a-specific immunotherapy of MRD
Interferons alpha and gamma	a-specific immunotherapy of MRD

1.3.1. Remission-induction therapy

The first attempt to improve the treatment of human leukemia was the study of the effect of extracorporeal irradiation of the blood (ECIB). In view of the necessity to apply an extracorporeal arterial shunt, the smallest experimental animal that can be used for such purpose is the rat [Hagenbeek, 1977f; 1979b]. ECIB was studied as an alternative for chemotherapy because at that time remission-induction treatments were not extremely successful. The method is non-invasive and normal tissues are not exposed to the treatment. In case that an adequate exchange of leukemic cells between the organs and the circulating blood could be achieved, ECIB might be expected to eventually eliminate all leukemic cells in the organism. It was soon found, that this was not the case under normal conditions. Therefore ECIB treatment was combined with leukocyte mobilizing agents i.e. polymethacrylic acid and dextransulphate, substances which cause a 3-4 fold increase in circulating white blood cell numbers. Simultaneously functional cell compartment analysis was performed in relation to changes under the influence of ECIB with or without the leukocyte mobilizing agents [Hagenbeek et al.,1976;Hagenbeek & Martens,1977c;1979a,Hagenbeek et al., 1976].

In the BNML leukemia model it was found that although a substantial reduction in tumor load (between 1 and 2 logs) could be achieved in combination with the mobilization of leukemic cells, it could not compensate for the leukemic cell production [Hagenbeek& Martens 1981a; 1981c].

The studies of ECIB have provided considerable insight in the traffic of leukemic cells between the various cell compartments upon depletion of only one compartment, i.e. the blood. In the course of these studies the BNML model became rather well defined and was recognized as a suitable model for studying a variety of remission-induction treatments. The model was used for studying the effectiveness of newly developed drugs in order to determine whether or not they might lead to improvement in the treatment of leukemia.

Furthermore, many frequently used drugs were studied as well, mainly to compare the various dose schedules and combinations, since in many cases the clinical results did not allow an accurate evaluation of their comparative effectiveness.

The results of these studies are discussed in alphabetical order.

AMSA

The anti-tumor drug acridinyl-anisidide (AMSA) appeared to be effective in the treatment of patients with refractory leukemia yielding remission-induction in 20 % of the patients. This led to the introduction of the drug in first line remission-induction chemotherapy. In the BNML leukemia model the anti-leukemic effect of single high dose and multiple split-dose treatment was evaluated and compared

with the toxicity for the normal hemopoietic stem cell [Hagenbeek & Martens, 1986a]. A high therapeutic ratio was found as illustrated in Figure 1-19. Split-dose treatment was found to be superior to single-dose treatment: it resulted in a higher leukemic cell kill while there was an improved survival of the normal hemopoietic stem cells, hence an improved therapeutic ratio.

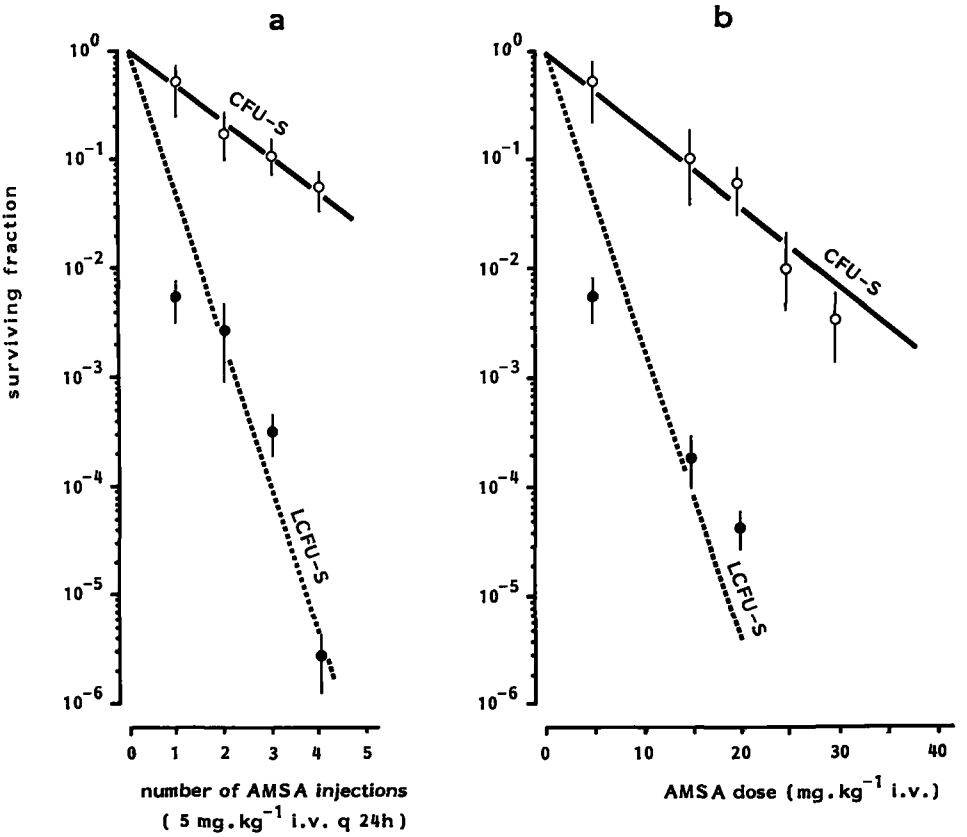


Figure 1-19:
Comparison of split-dose and single dose treatment of normal and leukemic rats with AMSA.
Panel a: Response of the hemopoietic stem cells (CFU-S) and the clonogenic leukemic cells (LCFU-S) to repeated injections with AMSA on 4 subsequent days (day 0, 1, 2, 3). The treatment of leukemic rats with AMSA was started on day 13 after injection of 10^7 BNML cells i.v.
Panel b: Response of the hemopoietic stem cells (CFU-S) and the clonogenic leukemic cells (LCFU-S) to single dose treatment with AMSA on day 13 after the injection of 10^7 BNML cells i.v.

The results obtained in the BNML model supported the inclusion of AMSA in the primary treatment of AML in man.

Anthracyclines and Cytosine Arabinoside (Ara-C).

The cytostatic drugs Adriamycin, Daunomycin and Cytosine-arabinoside (Ara-C) were extensively studied in the BNML model to compare their anti-leukemic effectiveness, to determine their interaction with the tumor cells, to study their pharmacokinetics and to analyze the kinetic response of the leukemic cells in an attempt to optimize treatment protocols.
The study of these drugs in the rat model were relevant because in particular these two groups of drugs have in recent years contributed to the improved success rate in the treatment of human acute leukemia.

a. Anthracyclines.

The distribution kinetics of both Adriamycin [Sonneveld, 1980] and Daunomycin [Nooter et al., 1984] were studied in the BNML in comparison with normal rats. Organ specific differences were found, using HPLC analysis methods [Sonneveld et al., 1981a; 1981c, Nooter et al., 1983; 1986], as well as a reduced uptake of the anthracyclines in organs of leukemic animals [Sonneveld et al., 1981d]. Anthracycline uptake levels appeared to be inversely related to the tumor load (Nooter et al., 1985], which may be one of the underlying reasons for the large variation in response in human leukemia.

Anthracyclines are rather unique because of the fluorescent property of the drugs upon exposure to light of the appropriate wavelength. Based on this property, anthracycline uptake of bone marrow cells can easily be monitored with flow cytometry (FCM). In vitro daunomycin uptake studies showed differences between various cell types, i.e. low levels in lymphocytes, higher levels in granulocytes while the highest levels were found in the blast cells, equally high in both normal and leukemic blasts [Sonneveld et al., 1981a; Nooter et al., 1985]. Resistance to the action of anthracycline is frequently associated with reduced levels of intracellular anthracycline uptake in clinical leukemia.
The combined data were used to develop a mathematical model for the distribution of anthracyclines in vivo [Sonneveld 1980; Schultz et al., 1985b; 1987b; 1987c]

b. Cytosine-arabinoside (Ara-C)

In the BNML leukemia model many studies were conducted with the cytostatic agent Cytosine-arabinoside (Ara-C). The main interest in the study of Ara-C was on the cell kinetic level because its anti-tumor activity depends on the inhibition of DNA polymerase during DNA-synthesis.
Animals were studied at a stage of fully developed leukemia. The standard bio-assays i.e. organ weights, LCFU-S and survival prolongation [Colly et al., 1977a; 1977b; 1978b] were used for the determination of the anti-leukemic effect of Ara-C. Besides these, various other methods were also employed for studying the mode of action of Ara-C on BNML cells (i.e. perturbation of the cell cycle) e.g. radio-

labeled thymidine [Aglietta et al., 1978; Burke et al., 1980], DNA analysis with flow cytometry [Martens & Hagenbeek, 1977; Colly et al., 1978a; Schultz et al., 1985] and the measurement of stimulating or inhibiting humoral factors [Burke et al., 1980, 1986a].

Treatment of the rat leukemia with Ara-C resulted in an initial block in cell proliferation, a reduction in the percent of S-phase cells, followed by a recruitment of resting G_0 cells, which subsequently moved as a cohort of cells through the cell cycle ending in mitosis and than re-entering G_1/G_0 phase [Aglietta et al., 1979a; 1979b; Burke et al., 1982a; 1982b; Colly 1980]. Figures 1-20 and 1-21 show the most crucial results of how autoradiographic and flow cytometric studies led to these conclusions.

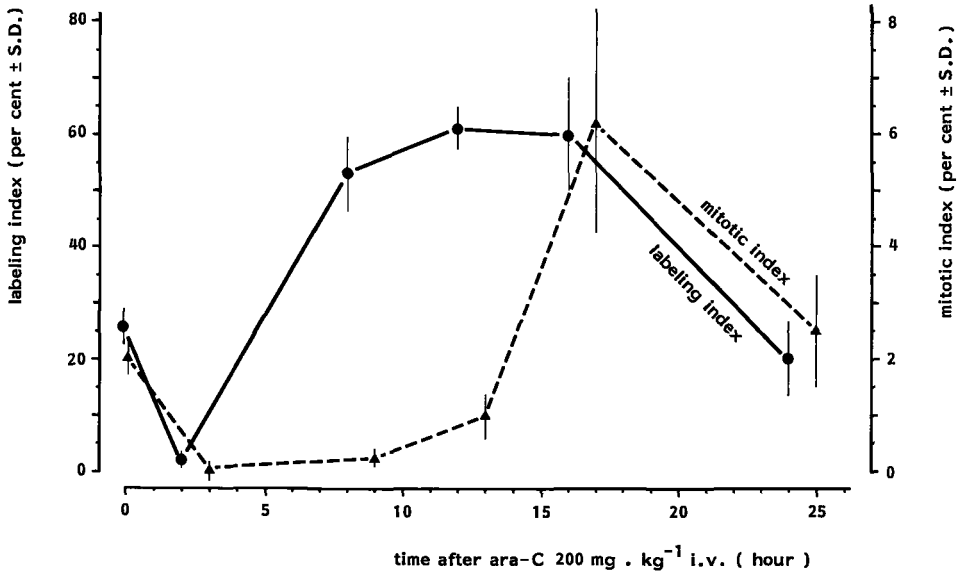


Figure 1-20:
Effect of a single dose of Ara-C (200 mg.kg^{-1}) i.v. at day 13 after the injection of 10^7 BNML cells i.v. on the labeling index and the mitotic index of the leukemic cells.
The cells are "pulse-labeled" by injecting the leukemic animals with radioactively labeled thymidine. The labeling index is a measure for the percentage of cells which were actually synthesizing DNA (S-phase) at the time of investigation; the mitotic index reflects the percentage of cells which is in the phase of cell division during mitosis (M-phase).

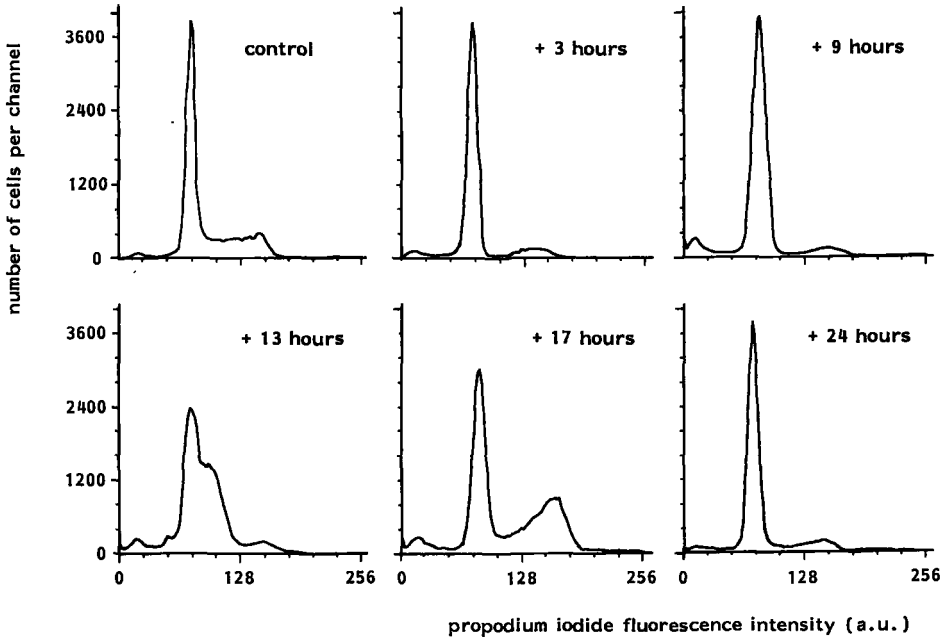


Figure 1-21:
DNA histograms of femoral bone marrow at various time intervals after the i.v. injection of Ara-C (200 mg.kg^{-1}) at day 13 after the i.v. injection of 10^7 BNML cells.
Cells were fixed in alcohol (70%); treated with RNAse (1 mg.ml^{-1})and subsequently stained with propidium iodide (50 μg.ml^{-1}). The histograms are normalized to the area under the curve.

Based on the time course of these phenomena of recruitment and synchronization of leukemic cells, maximal therapeutic responses could be achieved by timed-sequential application of multiple injections of Ara-C. By using a 12 hours time interval a considerable larger tumor load reduction was achieved compared to a treatment with 24 hours time interval [Aglietta et al., 1979b; Colly et al., 1984b].

In view of the therapeutic index it was important to study the effects of various dose schedules on normal tissues as well. Indeed timed-sequential therapy with short intervals, i.e. 6-8 hours, proved to be highly toxic for the intestinal mucosa [Colly et al., 1986; Burke et al., 1982a]. However, the reduction in the number of pluripotent hemopoietic stem cells appeared to be independent of the interval between Ara-C administration [Colly et al., 1984a; 1986]. The relevance of cell kinetic analysis for optimal timed sequential scheduling of chemotherapy was clearly illustrated in these studies.

Ara-C treatment was combined with methotrexate to investigate a possible synergistic effect as well as the most optimal treatment schedule for the combination of the two drugs [Aglietta & Sonneveld, 1978]. Separated administration proved to be most effective, however the time interval between the

two drugs was of major importance. The highest tumor load reduction was achieved by sequential administration with an 8-12 hours time interval.
This correlated with the presence of high numbers of leukemic cells in S-phase, as a result of the treatment with the first drug. Because both drugs have an S-phase specific anti-tumor effect, the second drug effectively eliminated the recruited leukemic cells [Aglietta & Colly, 1979a].

The studies of Burke et al., indicated that the increase in the labeling index coincided with a so-called "Humoral Stimulation Activity (HSA)" which in turn was an indication that the "Tumor-Associated-Inhibitory-Activity (TAIA)" was overcome [Burke et al., 1980; 1981; 1982a].

The conclusions of the many cell kinetic studies which were performed in the BNML leukemia model contributed to the improvement of combination chemotherapy protocols for the treatment of human acute leukemia both in adults and in children [Burke et al., 1981; 1986a; Colly, 1980; 1982; Vaughn et al., 1983; 1984; Smets et al., 1983].

Dinaline

A possibly new development in the treatment of leukemia is the introduction of a new oral drug against acute leukemia, i.e., 4-amino-N(2'-aminophenyl) benzamide, Dinaline (NSC-328786, Goe-1734). At present, its mode of action is unknown. Repeated daily administration resulted in an 8 log reduction in the leukemic cell load, while less than a 1 log reduction of hemopoietic stem cells was observed (Figure 1-22). Although slightly more toxic, split dose treatment proved to be even more effective [Hagenbeek et al., 1988]. The therapeutic index is exceptionally high so that clinical phase I/II studies are certainly to be recommended.

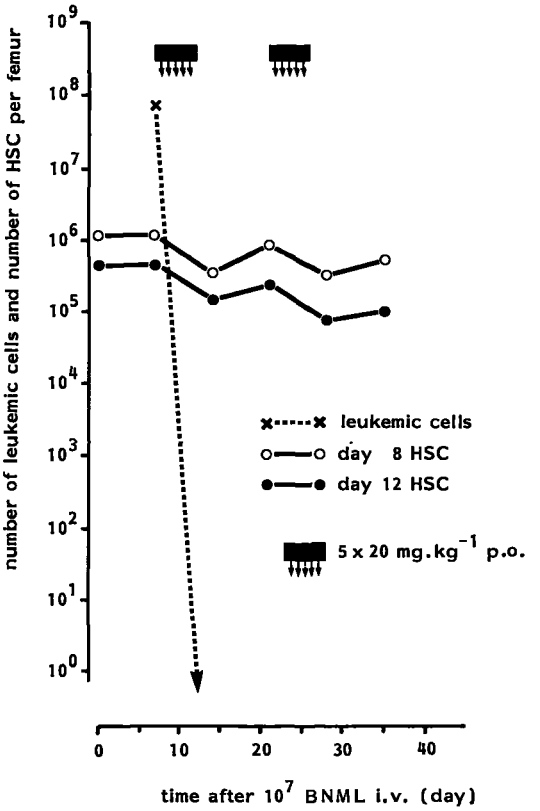


Figure 1-22:
Survival of pluripotent hemopoietic stem cells (CFU-S day 8 and CFU-S day 12) compared to the survival of clonogenic leukemic cells, after 1 or 2 cycles of daily treatment with Dinaline (GOE-1734).
HSC: hemopoietic stem cells.

5-Fluorouracil

The limited studies which have been performed with 5-fluorouracil (5-FU) in the BNML model indicate that a significant reduction in leukemic cell load can be achieved [Ermens et al., 1986]. Various treatment schemes were tested, of which split-dose treatment (3 x 15 mg.kg^{-1} or 3 x 25 mg.kg^{-1} at days 7, 12 and 17 after leukemic cell transfer) appeared to be the most effective: a 4 log cell kill was obtained with an acceptable degree of toxicity. No bone marrow transplantation (BMT) was required with the dosages used which indicates that the treatment may

even be intensified. So far, the use of 5-FU for the treatment of clinical leukemia is limited.

A characteristic of 5-FU is the selective elimination of the more mature type hemopoietic progenitor cells while it spares the most primitive pluripotent stem cells [Hodgson & Bradley, 1979]. It was concluded that based on this discriminative action of 5-FU it should be considered to include this drug in clinical treatment protocols for acute leukemia.

Succinylated Glutaminase-Asparaginase.

Anti-tumor activity of L-Asparaginase has been reported in leukemia and lymphomas. Treatment with a preparation having a glutaminase-asparaginase ratio of 1.2 showed a higher biological activity in in vitro tumor cell tests. The in vivo activity of the succinylated form of Acinetobacter-derived glutaminase-asparaginase was tested in the BNML model. A dose-related anti-leukemic effect was observed, based on the reduction in spleen and liver weights. A seven day course of treatment resulted in a 2-3 fold reduction in tumor load in spleen and liver [Sonneveld et al., 1979]. This implies that this drug has a strong anti-leukemic effect. The maximally tolerated dose and the corresponding anti-leukemic effect, as well as the toxicity for the hemopoietic stem cells, have not yet been determined.

Nitrous oxide

Nitrous oxide (N_2O), a frequently used gas for anaesthesia, influences the level of cobalamine (vitamin B12). Through that mechanism it might effect the production of hemopoietic cells and it also may have effects on the production of leukemic cells. Such an effect was investigated in detail in the BNML rat leukemia model [Kroes, 1987]. The leukemic rats were continuously exposed to a mixture of 67% nitrous oxide and 33% oxygen from day 7 after leukemia transfer until the death of the animals. This may cause some practical problems for clinical application of this method.It was observed that indeed the cobalamine levels decreased upon continuous exposure to nitrous oxide and that there was a weak tumor load reducing effect indicated by a reduction in spleen and liver weights [Kroes et al., 1984a]. However, no signs of a remission were observed. Nitrous oxide treatment was combined with various other cytostatic drugs i.e. cycloleucine [Kroes et al., 1984b], methotrexate [Kroes et al., 1986a], and 5-fluorouracil [Kroes et al., 1986b]. In all three cases a synergistic action was observed.

Based on the results obtained in the rat leukemia model it has been proposed to include nitrous oxide in clinical studies [Kroes, 1987].

Thymidine

In clinical phase I/II studies high-dose thymidine (HD-TdR) treatment revealed a serious treatment-related toxicity and a low anti-tumor effect in spite of the observation that a variety of tumors responded in vitro. Hence, there was a need for the exact determination of the surviving fractions of normal compared to leukemic stem cells after HD-TdR treatment for which the BNML model was used.

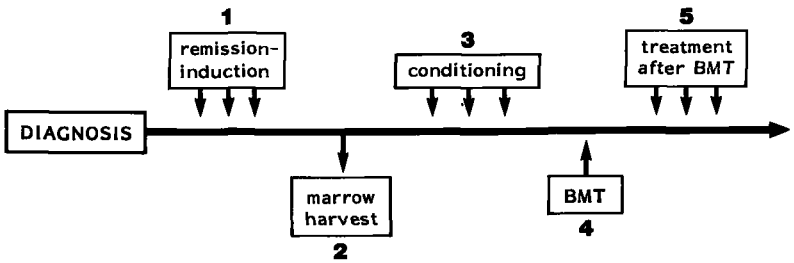
In the BNML model these observations were confirmed: i.e. HD-TdR treatment given either intravenously, intraperitoneally or as a continuous infusion did not affect the leukemic cell load. The relative increase in the number of CFU-S which was observed after the first dose of HD-TdR suggests that the resting stem cells are recruited. However, after continued treatment for five days with a relatively small dose of thymidine, CFU-S numbers were considerably reduced.

The results in the BNML are in agreement with the clinically observed hematological toxicity [Sonneveld et al., 1981b].

1.3.2 Autologous bone marrow transplantation

A substantial contribution to the increased success rate in the treatment of leukemia can be attributed to the application of marrow ablative chemo/radiotherapy. Treatment of leukemia with high-dose chemotherapy alone or in combination with total body irradiation requires the restoration of the normal bone marrow function by means of bone marrow transplantation. There are two possibilities i.e. allogeneic or autologous marrow transplantation. So far, allogeneic bone marrow transplantation is however an option for only a minority i.e. 30% of the patients, due to the relative scarcity of HLA-identical siblings.

It has convincingly been demonstrated in mice and in rhesus monkeys that T-cell depletion of the graft efficiently prevents GVHD in HLA-identical combinations as well as in MHC mismatched combinations [Wagemaker et al., 1982; Dicke & Van Bekkum, 1970]. However, clinicians are still reluctant to use other than HLA-identical siblings as BM donors. This implies that for the remaining group of patients the alternative treatment may be autologous bone marrow transplantation (ABMT). In the case of (AMBT), high dose chemoradiotherapy is given to eliminate all residual leukemia cells. An additional objective of this pretreatment for allogeneic transplantation, is the suppression of the residual host resistance to allow the grafted marrow to take. In both cases space has to be created for the infused marrow to allow repopulation of the marrow cavity with normal bone marrow cells [Vriesendorp, 1985].



ad 1 and 3 : further intensification?
ad 2 : purging?
ad 4 : T-cell depletion?
ad 5 : low dose chemotherapy - growth factors - BRM?

Figure 1-23:
Strategy for preventing a relapse after autologous bone marrow transplantation.
Each of the indicated 5 treatment components are subject to improvement.
BMT: bone marrow transplantation;
BRM: biological response modifiers.

At present, a major problem in the treatment of leukemia with ABMT is a relapse of the leukemia in about half of the patients. This might be due to residual leukemia cells which have survived the conditioning treatment, or to leukemic cells reinfused with the graft (or a combination of the two). Obviously, a significant reduction in the relapse rate might be achieved by developing more effective tumor-load reducing regimens prior to ABMT and/or by eliminating leukemic cells from the marrow graft and/or by giving maintenance treatment after ABMT provided that this has relatively low myelotoxicity.

All three approaches were studied in the BNML which is schematically illustrated in Figure 1-23 and in Table 1-4.

1.3.3 Conditioning regimens prior to bone marrow transplantation in the BNML

In man, the number of different pre-transplantation conditioning regimen is large and the design of controlled, randomized clinical trials to determine the best regimen is a difficult process. In an attempt to answer the question illustrated in Figure 1-24, with respect to the "best" ablative treatment, a preclinical animal model such as the BNML may help in determining the relative effectiveness of various treatment modalitiesThemodelhasbeenstudied extensively

in this respect . Most studies were done at a stage of leukemia development comparable to the phase of relapse in man.
In another series of experiments similar treatment modalities were evaluated during the phase of Minimal Residual Disease.

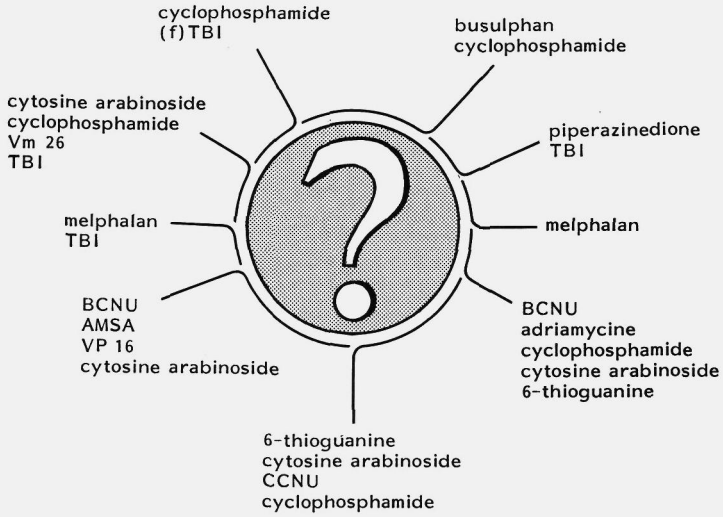


Figure 1-24:
A selection of the various ablative conditioning regimens prior to bone marrow transplantation in acute leukemia.
The questionmark symbolizes the difficulty in selecting the "best" regimen.

Total body irradiation (TBI):

Total body irradiation (TBI) is included in most pre-transplantation conditioning regimens for the treatment of leukemia. Nevertheless, a randomized clinical trial for the determination of the best schedule for the elimination of the leukemic cells was never initiated. A large variety of TBI regimens is employed, varying from large single dose irradiation upto highly fractionated irradiation with either high or low dose rates. The fractionation of the TBI dose was based on the observation that the survival curve of mouse CFU-S did not contain a so-called "shoulder" [Peters et al., 1979]. The assumption was made that the leukemic cells would respond similarly and that the observed reduction in toxic side-effects after fractionated irradiation would increase the therapeutic ratio. The validity of this assumption was tested in the BNML model. The radio-sensitivity of the BNML was determined in vivo for X-rays (the D_0= 0.99 Gy; N=1.7 [Hagenbeek & Martens, 1981d]) and is shown in Figure 1-25. Indeed the D_0 for the leukemic cells was in the same range as has been reported for normal hemopoietic cells. However, in contrast to the speculation of Peters et al., the survival curve did contain a shoulder

(indicating a capacity to repair sublethal DNA damage), which would predict that fractionated irradiation to be less effective for the elimination of the leukemic cells.

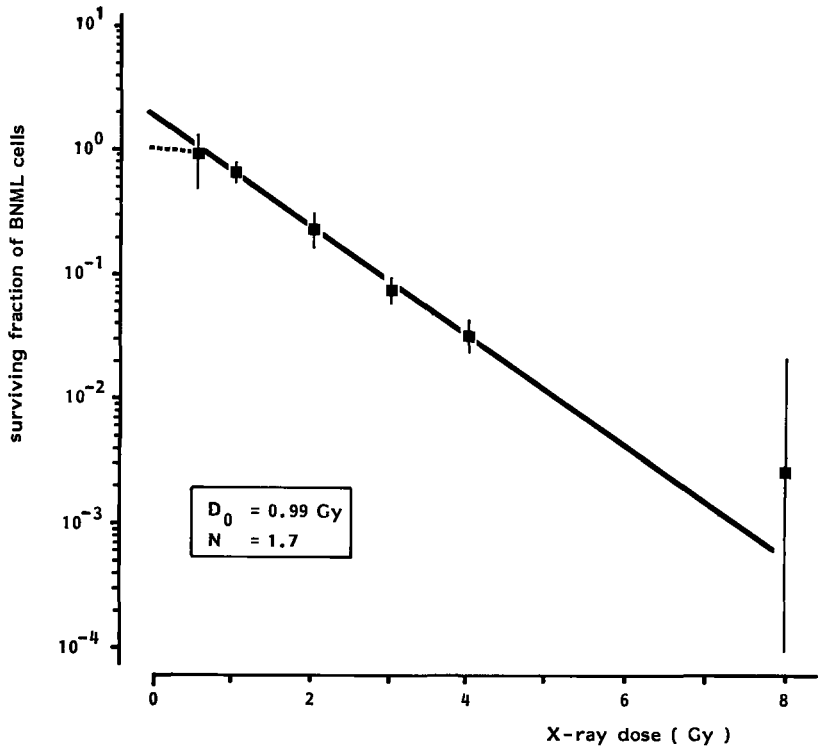


Figure 1-25:
In vivo radiosensitivity for X-rays of the BN acute myelocytic leukemia.
Animals received total body irradiation (TBI) at day 13 after injection of 10^7 BNML cells. Immediately thereafter a fraction of the content of the femur (1/10) was injected into healthy recipients and the survival time was recorded.A reference dose-survival relationship was determined for femoral bone marrow from non-irradiated control animals. The prolongation in life span of the "test" animals was used to calculate the surviving fraction of leukemic cells for the applied TBI doses (dose rate 0.12 $Gy.min^{-1}$; 300KV X-rays)

Fractionated TBI was compared in the BNML with single dose "flash" TBI, both at high dose rate (0.12 $Gy.min^{-1}$). The best results were obtained with flash irradiation (a 4 log cell kill). Due to the dose fractionation the totally applied dose could be increased, which is a standard rule in radiobiology. Only the regimen which consisted of fractionated irradiation of two large (6.0 Gy) doses gave a result comparable to the flash irradiation. Split doses of 2 fractions per day

offered no additional advantage. The more fractions were applied, the less effective the treatment became. Protracted low dose rate irradiation (0.0026 $Gy.min^{-1}$, total dose 20 Gy) was also studied and proved to be quite effective i.e. also a maximal 4 log reduction in tumor load was observed, however, at the expense of 50 % treatment related deaths (gastro-intestinal tract damage).

Usually, TBI is given in combination with chemotherapy. Apart from its immunosuppressive action, TBI is mainly of importance in sterilizing leukemic cells which are anatomically or otherwise resistant to the cytostatic drug treatment.

Chemotherapeutic agents

For a number of cytostatic drugs, the maximal tolerated dose (MTD) was determined in normal as well as in leukemic rats. As a rule syngeneic marrow transplantation followed the ablative treatment. The maximal tolerated dose was then tested for its tumor load reducing capacity in terms of log cell kill (LCK) when applied at a stage of leukemia development comparable to relapse in man. The investigated drugs include cyclophosphamide, busulphan, chlorambucil, peptichemio, piperazinedione and melphalan.

Chlorambucil

For chlorambucil not more than a 1.5 LCK was observed at the maximally tolerated single (MTD) dose of 15 $mg.kg^{-1}$ compared to a 3 LCK for a MTD for split-dose treatment of 3 daily doses of 7 $mg.kg^{-1}$. Therefore it was concluded that the anti-leukemic effect of this drug in the BNML is minimal.

Melphalan

More effective treatment was observed with the drug melphalan. Again split-dose treatment proved to be superior. The MTD of single dose treatment of normal control rats was 10 $mg.kg^{-1}$. Treatment of leukemic animals with this dose at the stage of full blown leukemia i.e. at day 13 after 10^7 cells i.v., resulted in a 9 LCK although 50 % treatment-related deaths were observed. Split-dose treatment of 2 doses of 5 $mg.kg^{-1}$ each at 24 hours interval (total dose 10 $mg.kg^{-1}$) resulted in an 8 LCK. With this scheme no treatment related deaths were observed. Split-dose treatment of 3 daily doses of 4 $mg.kg^{-1}$ (total dose: 12 $mg.kg^{-1}$) resulted in 10 LCK but at the expense of 6 animals out of 8 dying of

toxicity (gastro-intestinal tract). Treatment with melphalan has never resulted in cures in the BNML.

Peptichemio

Peptichemio, a synthetic peptide, showed a strong anti-leukemic effect, most pronounced in case of split-dose treatment. The results of the various treatment regimens which were tested are shown in Table 1-5.

TABLE 1-5

TREATMENT OF THE BNML ACUTE MYELOCYTIC LEUKEMIA WITH PEPTICHEMIO ALONE OR IN COMBINATION WITH CYCLOPHOSPHAMIDE

a. remission induction treatment

dose (x number of) injections	on days	leukemia relapse (%)	cures (%)
5 $mg.kg^{-1}$	13-17	100	0
7 $mg.kg^{-1}$ x 7	13-19	33	67

b. treatment during minimal residual disease (MRD)

dose (x number of) injections	on days	leukemia relapse (%)	cures (%)
5 $mg.kg^{-1}$ x 5 (2 cycles)	14-18 & 35-39	60	40
5 $mg.kg^{-1}$ x 5 (2 cycles)	13-17 & 27-31	63	37
5 $mg.kg^{-1}$ x 5 + cyclo 100 $mg.kg^{-1}$	13-17 27	17	83

peptichemio i.v., interval 24 hours
cyclo: cyclophosphamide i.p.
N.B.: bone marrow transplantation after the end of each cycle.

Highly effective anti-leukemic regimens always required bone marrow transplantation. Treatment was started at full blown leukemia i.e., at day 13 after 10^7 cells i.v. Administration of 5 daily doses of 5 $mg.kg^{-1}$ resulted in recurrent leukemia in 100% of the animals. Increasing the frequency to 7 daily doses of 5mg.kg-1 each resulted in cure in 67% of the animals.

Peptichemio was also tested in a situation when the animals were in a stage of MRD which was induced by a first course of peptichemio treatment of 5 times 5 $mg.kg^{-1}$. When a second course with this scheme was given from day 27-31 the cure rate was 40%. Replacement of the second peptichemio treatment in MRD by cyclophosphamide treatment, resulted in a cure rate of 83%. These results indicate that peptichemio is a very efficient drug for ablative treatment of leukemia.

Piperazinedione

The anti-leukemic activity of the drug piperazinedione was not tested as a single dose treatment but always after split-dose treatment in accordance with clinical practice. Various treatment schemes were investigated using the survival parameter as a measure for anti-leukemic effect The highest therapeutic effect with split-dose treatment was obtained with a scheme of 3 twice-daily doses of 2 $mg.kg^{-1}$ each (total dose: 12 $mg.kg^{-1}$). This treatment resulted in a 7 to more than 9 LCK which implies that the drug has a strong anti-leukemic effect. The tumor load at the start of the treatment was in the order of 10^{10}, which means that nearly all leukemic cells were eradicated However, no animals were cured with this treatment schedule [Hagenbeek & Martens, 1981b].

Cyclophosphamide and Busulphan

The remaining two drugs i.e. cyclophosphamide (Cy) and busulphan (Bu) were studied much more in depth, in view of the general use of these drugs in conditioning regimens for BMT, with special emphasis on combined treatment as well as on the sequence [Colly, 1978b; Hagenbeek & Martens, 1982b; 1983a; 1987b; Martens & Hagenbeek, 1982; Sharkis & Santos, 1977; Santos & Sharkis, 1978].

In the clinical situation the various combinations have not been tested in a randomized trial. Furthermore the sequence of the various combinations may be of great importance as well. It was observed in patients that when cyclophosphamide was given prior to busulphan this resulted in gastro-intestinal tract damage which induced vomiting thereby prohibiting adequate treatment

with the second drug i.e. busulphan [Santos, Johns Hopkins University, Baltimore, MD, personal communication]. Treatment with TBI followed by cyclophosphamide caused in patients mucositis, which was not observed in rats.

The BNML model was used to compare the anti-leukemic activity as well as the toxicity for both drugs separately, in combination or each of them combined with TBI. The effect of changing the sequence was studied as well. Both drugs show a strong anti-leukemic effect When high dosages of the drugs are used it was absolutely necessary to apply bone marrow transplantation in order to prevent death due to bone marrow aplasia. Bone marrow transplantation was given 6-8 hours after the drug treatment. The dose-response curves for cyclophosphamide and busulphan, are shown in Figure 1-26. To derive the efficacy of the treatment, the increase in the survival time was measured. An increase in the survival time of 4 days corresponds with 1 log cell kill (the method has been explained in detail in section 1.2.3) For both drugs it is clear that for the investigated dose-range there is a constant relationship between the dose and the effectiveness of the treatment is observed.

Combined treatment was studied, either cyclophosphamide with busulphan or each of them combined with TBI. The sequence of the various treatments was varied, while the dosages were kept constant. In this way the optimal schedule could be determined with regard to the anti-leukemic effect as well as to the relative toxicty for the various combinations. A summary of the results is shown in Table 1-6.

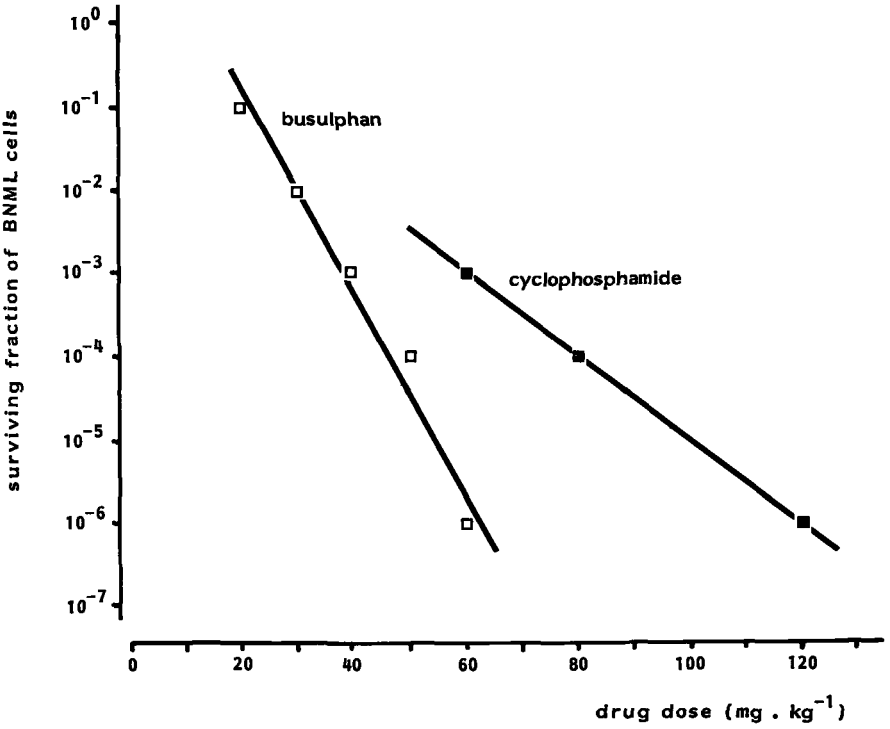


Figure 1-26:
Dose-effect relationships for cyclophosphamide and busulphan.
Leukemic animals were treated at day 13 after the injection of 10^7 BNML cells i.v., with various doses of both drugs. The surviving fraction of leukemic cells was calculated from the increase in survival time.

Cure of BNML rats carrying florid leukemia can be achieved under certain conditions with the treatment modalities indicated by ">10 LCK". A number of conclusions could be drawn with relevance to the clinical situation. The probably most effective treatment in man so far i.e. Bu followed by Cy was also the most effective, without lethal toxicity, in the BNML. In the rat it was found that TBI preceding Cy resulted in a higher LCK but also in an increased toxicity.

The toxicity of cyclophosphamide towards intestinal epithelial and lung tissue was reported to be reduced when a small, so-called "priming dose" preceded the high dose treatment. In the BNML this "priming effect" was studied for the normal as well as the leukemic cell population. A protective effect was observed against bone marrow as well as the intestinal tract.toxicity When leukemic animals were treated that were bearing a large tumor load, a priming dose of cyclophosphamide reduced the incidence of, otherwise lethal, tumor cell emboli.

Furthermore, enhanced leukemic cell kill of the second, high dose, was observed [Hagenbeek et al., 1982a; 1984a].

TABLE 1-6

ABLATIVE TREATMENT OF THE BNML WITH VARIOUS COMBINATIONS OF CYCLOPHOSPHAMIDE, BUSULPHAN AND TOTAL BODY IRRADIATION FOLLOWED BY BONE MARROW TRANSPLANTATION

	BNML		AML
treatment/sequence	LCK	lethal toxicity	relapse after BMT in man*
Bu - TBI	5	70 %	?
TBI - Bu	6	0	?
Cy - TBI	8-9	0	25%
TBI - Cy	9-10	20%	10%
Cy - Bu	10	0	?
Bu - Cy	>10	0	8%
HDAC - Cy - TBI	>10	25%	5-10%

LCK: log leukemic cell kill
*: allogeneic BMT in 1st remission AML
Cy: cyclophosphamide
Bu: busulphan
TBI: Total body irradiation.
HDAC: high dose Ara-C
?: combination not tested in AML

Another important conclusion that could be drawn from extended studies with cyclophosphamide was the fact that the tumor load at the time of treatment is a factor of major importance. When animals with a relatively small tumor load (e.g. early during leukemia development) were treated with a certain dose of cyclophosphamide, a larger fraction of cell kill was observed than was expected based on the known dose-response relation [Hagenbeek & Martens, 1982b; Chapter 7). The latter was determined at a stage of full blown leukemia during which the tumor load was much higher. If this can be extrapolated to the clinical

situation this implies that a certain treatment will be more efficient if applied during the stage of minimal residual disease (MRD). Furthermore it might provide an explanation for the observation that a similar treatment results in a different response in individual patients. Apart from the fact that their leukemia type may be different, their tumor load will most likely be different as well. For a long time the working hypothesis has been that a fixed dose of a drug with alkylating activity, will always result in a constant fraction of cell kill [Skipper, 1964]. The BNML data indicate that this hypothesis should be revised.

1.3.4 Toxicity of conditioning regimen: Early and late side effects

Ongoing clinical studies indicate that 40-50 % of the patients who are treated with high dose chemotherapy and bone marrow transplantation are being cured. These results are encouraging. If, however, the total population of leukemia patients is considered, one has to conclude that the majority of the patients are dying of factors related to their disease. This might be due to treatment-related complicating factors e.g. bleedings, infections or a natural or acquired resistance of leukemic cells to the cytostatic treatment that is being employed. Those patients which are selected to be treated with bone marrow transplantation might die of a failure to engraft, the development of Graft-versus-Host Disease (GvHD), Interstitial Pneumonitis (IP) or a combination, or an eventual relapse of leukemia. In case of syngeneic BMT (identical twins) GvHD is not observed (obviously) but also interstitial pneumonitis (IP) is not observed. In allogeneic BMT IP is a serious complication and is often fatal. When BMT is performed after T-cell depletion the reduction in the incidence of GvHD is correlated with a reduction in the incidence of IP. In the clinic the relation between the development of GvH and the development of IP is not clear. Therefore, conditions were created in the BN/BNML model to study the contribution of suspected causative factors e.g. the total dose of irradiation as well as the dose rate and the presence or absence of GvHD [Lopes-Cardozo et al., 1985a; 1985b; Varekamp et al., 1986]. The rat data indicated that the non-infectious IP is a radiation pneumonitis that is only slightly enhanced in severity by the cyclophosphamide treatment [Varekamp et al., 1987; Hagenbeek, 1985d]. Furthermore it was found that the development of GvHD contributed in the rat to the development of IP [Varekamp et al., 1987; Hightower et al., 1987a; 1987b].

For the long-term surviving patients it is of importance that a side effect of many of the cytostatics currently in use is their carcinogenic potential. Since the long term survival rate in humans is steadily increasing, the development of secondary tumors may become a serious problem. The possible contribution of cyclophosphamide and total body irradiation alone or in combination has been studied in this respect in BN rats. Compared to untreated, age-matched control

rats the treatment resulted in an increased tumor frequency. The number of observed tumors per rat were 1.42 for control rats; 1.42 for cyclophosphamide only; 2.54 for TBI only and 1.68 for the combined treatment. These values are influenced by the observed difference in latency period, differences in type of tumors and differences in survival times between the various groups [Lopes Cardozo et al., 1984; Zurcher et al., 1983; 1987]. Cyclophosphamide treatment frequently resulted in the development of multiple, malignant nerve sheath tumors i.e. in 66 % of the animals, however if cyclophosphamide was combined with TBI the incidence was lower i.e. 31 % compared to a 2 % incidence in untreated age-matched controls.

A typical non-neoplastic lesion, induced by cyclophosphamide treatment was incisor dysplasia. The resulting feeding problem could be overcome by powdering the food pellets. TBI as a single treatment resulted, compared to control animals, in an increased occurrence of especially mesenchymal tumors. The animals that received TBI only, showed a considerably longer survival time than the combined TBI/cyclophosphamide and the cyclophosphamide only group. The most likely explanation is the early appearance of nervous sheath tumors following the treatment with cyclophosphamide. So far it is not clear whether a similar type of tumor specifically develops in patients treated with high dose cyclophosphamide.

1.3.5 Elimination of leukemia cells from the autologous marrow graft

a. Biophysical separation methods

A number of techniques are available for the elimination of leukemic cells which are contaminating autologous marrow grafts, some of which were studied in the BNML leukemia model (Table 1-4B) [Hagenbeek & Martens, 1983c]. The various methods, which included discontinuous density centrifugation, velocity sedimentation at 1 g, free flow cell electrophoresis, were explored with regard to the possibilities and limitations for separating leukemic cells from normal hemopoietic stem cells within the context of treatment of acute leukemia with autologous bone marrow transplantation during the remission phase [Hagenbeek & Martens, 1981f; 1981g; 1983c; Valet et al., 1979].

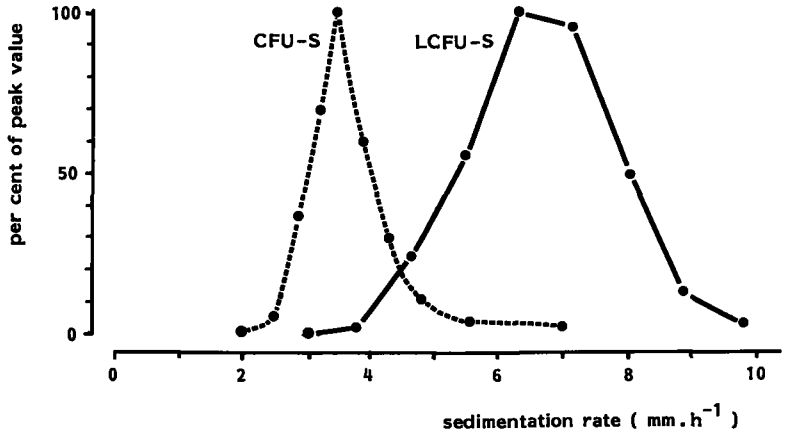


Figure 1-27:
Sedimentation rate profiles of normal hemopoietic stem cells (CFU-S) compared to clonogenic leukemic cells (LCFU-S).

The average density of BNML cells appeared to be higher than 1.071 g.cm^{-3} which is much higher than what is observed for normal stem cells i.e. 1.065-1.071 g.cm^{-3}. Depending on the leukemic cell type in human acute leukemia the density of the leukemic cells ranges between 1.050 and 1.080 g.cm^{-3}. In case of a pro-myelocytic leukemia the average density is in the order of 1.070-1.085 g.cm^{-3}, which implies that the density of BNML cells is in the expected range.

The sedimentation rate of the clonogenic BNML cells (LCFU-S) was found to be twice as high as that of hemopoietic stem cells (CFU-S) i.e. 6-8 versus 3-4 mm per hour (Figure 1-27). Combining density- and velocity sedimentation separation, proved to be superior to each of them separate: complete elimination of leukemic cells from mixtures containing 0.5 % leukemic cells could be achieved [Hagenbeek & Martens, 1981g]. However, a significant loss of normal stem cells occurred.
Another method tested was free-flow cell electrophoresis. The complete overlap of hemopoietic stem cells and leukemic clonogenic cells was clearly changed after pretreatment of the cells with neuraminidase (Figure 1-28). The BNML apparently expresses high amounts of removable sialic acid residues on the cell surface.

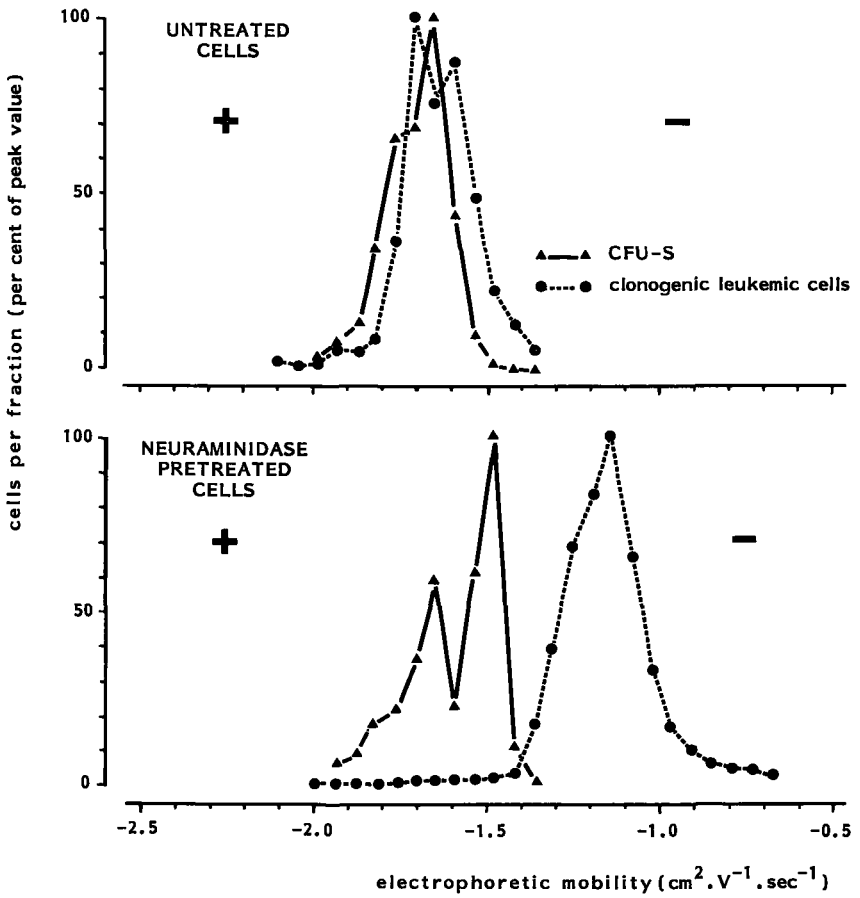


Figure 1-28:
Electrophoretic mobility of normal hemopoietic cells (CFU-S) and clonogenic leukemic cells (LCFU-S).
Top panel: control profiles
Bottom panel: after pre-treatment with neuraminidase.
+: positive pole of the electrical field
-: negative pole of the electrical field.

Subsequent free-flow cell electrophoresis results in an 98 % removal of clonogenic leukemic cells from the fractions which contain almost all hemopoietic stem cells.

b. In vitro chemotherapy

Another approach to in vitro elimination of residual leukemic cells has been exposure to chemotherapeutic agents based on a supposed difference in sensitivity between leukemic cells and pluripotent stem cells to active metabolites of cyclophosphamide, e.g. 4-hydroxycyclophosphamide, 4-hydroperoxycyclophosphamide and the more stable compound maphosphamide (ASTA-Z-7557); all three are closely related compounds. A number of studies was initiated in the BNML which predicted a possible successful outcome of such an approach [Sharkis et al., 1980; Hagenbeek & Martens, 1983c; 1984c]. The studies in the rat indicated that the most primitive stem cells (characterized by colony formation on day 12 in the spleen-colony-assay) were the least sensitive cell type [Martens et al., 1986; Table 1-7; Chapter 3]. The clonogenic leukemic cells were very sensitive. The sensitivity of the committed normal progenitor cell which forms colonies in vitro, the CFU-C, was found to be in the same range as the CFU-S (Figure 1-29).

TABLE 1-7

SURVIVAL OF DAY-8 AND DAY-12 CFU-S IN RAT BONE MARROW 24 HOURS AFTER TREATMENT WITH CYCLOPHOSPHAMIDE OR MAPHOSPHAMIDE

drug	experiment	day-8 CFU-S*	day-12 CFU-S*
cyclophosphamide	1	0.47 %	4.7 %
100 mg.kg^{-1} i.v.	2	0.11 %	9.4 %
	3	0.56 %	11.5 %
	mean	**0.38 %**	**8.5 %**
maphosphamide	1	0.32 %	11.4 %
(ASTA-Z-7557)	2	0.09 %	5.3 %
140 mg.kg^{-1} i.v.	3	0.07 %	8.1 %
	mean	**0.16 %**	**8.3 %**
maphosphosphamide	1	0.37 %	3.6 %
(ASTA-Z-7654)	2	0.31 %	9.5 %
154 mg.kg^{-1} i.v.			
	mean	**0.34 %**	**6.6 %**

N.B.: drugs are used at equimolar doses.
*: spleen colony assays were performed in mice.

In the rat model it was found that mixtures containing up to 1 % leukemic cells could be successfully "purged" with maintenance of the capacity to repopulate lethally irradiated rats. However, in human bone marrow no differences were observed between the sensitivity of human clonogenic AML cells using the PHA-leukocyte feeder layer technique and normal bone marrow progenitors [Kluijn et al., 1984a; 1984b]. Furthermore it was reported that after the incubation of autologous remission marrow grafts, the CFU-C, as well as the CFU-GEMM (a more primitive multipotent bone marrow precursor cell), were not detectable any more.

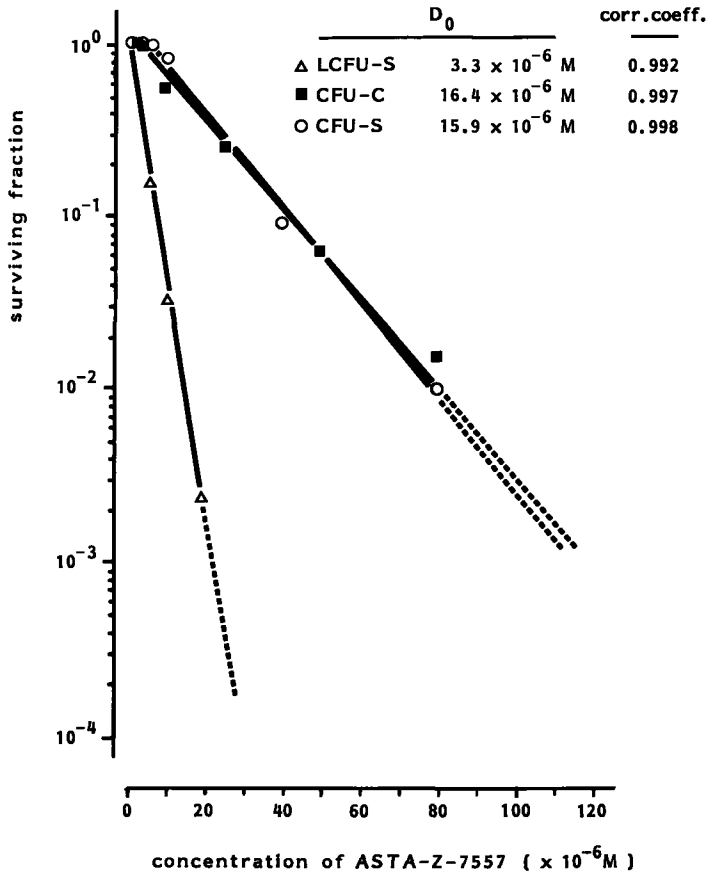


Figure 1-29:
Sensitivity of CFU-S, CFU-C and LCFU-S to in vitro treatment with Maphosphamide (ASTA-Z-7557).
D_0: dosage resulting in a surviving fraction of 0.37.

Nevertheless the bone marrow function was fully restored upon ABMT in these patients although with time delay [Kaizer et al., 1985]. Apparently, the in vitro colony assay does not permit the growth of the cell type responsible for the restoration of the bone marrow function in transplanted patients.

In the rat it appeared to be the most primitive CFU-S subtype (not detectable in in vitro assays), which showed the maximal survival during the purging procedure [Martens & Hagenbeek, 1986; Chapter 3]. This is fully in agreement with the clinical observation [Rowley et al., 1985; Gordon et al., 1985].

Another approach to in vitro treatment of BNML cells was the incubation with cholera toxin which resulted in a 5 log cell kill of the leukemic cells, while normal progenitor cells were resistant to the short term exposure [Lanotte et al., 1986].

Since it could not be shown in clinical studies, that the relapse rate was lower after purging, the necessity for in vitro bone marrow purging remains questionable. Extrapolation of the BNML data to the human situation indicates that the major source for a relapse after ABMT remains residual leukemia in the patient after the ablative conditioning treatment. The calculation of the "ED 50" for the human situation in case of re-infusion of autologous grafts for the treatment of acute leukemia was done based on the results of BNML studies. It was concluded that the minimal number of leukemia cells that causes a leukemia relapse in man will vary between 10^4 and 10^6 [Hagenbeek & Martens 1985c; 1986c; 1987c; Schultz et al., 1988]

1.3.6 Treatment after autologous bone marrow transplantation

The period after the initial remission-induction treatment, when there are still limited numbers of leukemic cells left, is defined as the phase of "Minimal Residual Disease" (MRD). To design optimal treatment during the MRD phase it is of importance, that more is known on the characteristics of MRD itself such as where are the residual cells located?; How is their growth influenced by the environment?; What is the cell kinetic status of the residual cells? etc.

When leukemic cells are present in very low numbers, e.g. below the detection level, they can only be studied indirectly. Because of the limits imposed by the detection methods, the only endpoint that can be used is the survival time. In the lower range region i.e. below 100 cells it was found that the survival times tends to increase [Van Bekkum et al., 1978]. This might imply that the phase of constant exponential growth, which is observed in the BNML during the intermediate stage of leukemia development, is preceeded by a phase in which the growth is much more slowly. The relationship between the growth fraction,

cell cycle times and cell loss fraction is not known for the MRD situation; the net cell production during the first stage of leukemia development is apparently low. For this stage of MRD in the BNML, it was tested whether the leukemic cells would behave similarly as in exponential growth phase upon treatment with Ara-C. Animals in a stage of MRD (i.e. 48 hours after injection of 10,000 leukemic cells) were injected with two doses of Ara-C, either 12 or 24 hours apart [Hagenbeek & Martens, 1980b; 1980c]. It was found that leukemic cells were recruited and subsequently killed by the 12 hour interval treatment indicated by a considerable prolongation of the survival time. In the 24 hour scheme the second injection is given at the time that hardly any cells are in the susceptible S-phase but rather in the G_1/G_0 phase. Indeed, the latter regimen resulted in a significantly shorter survival time (i.e., 6 days). This pattern of response was comparable to the recruitment/synchronization phenomenon observed in animals that were treated during full blown leukemia (i.e. at day 13 after injection of 10^7 BNML cells). At that stage leukemic cells are characterized by a cell cycle of 14 hours, a growth fraction of 0.4 and about 25 % of the cells in S-phase [Hagenbeek et al., 1977b]. In MRD, leukemic cells can also be recruited with a similar schedule. Hence, it seems likely that during MRD many leukemic cells are out of cycle, which requires timed-sequential treatment schedules to eliminate residual cells.

Experiments in which the fate of 100 potentially clonogenic cells was studied after injection directly into specific organs, indicated that growth of residual disease is also influenced by the local microenvironment. In some organs, i.e. spleen and bone marrow, leukemia growth was faster while in others, i.e. lungs and scrotum, it was much slower. The survival time varied from 35 to 100 days which is considerable [Hagenbeek & Martens, 1980c].

Another indication of the influence of the microenvironment on the proliferation of leukemic cells was derived from experiments in which non-leukemic animals were subjected to the ablative treatment with high dose cyclophosphamide and total body irradiation followed by isologous BMT. After a rest period of 25 days low numbers of leukemia cells were injected. A significant longer survival time was observed in this group compared to nonpretreated controls [Hagenbeek & Martens, 1985c].

Leukemia relapses in man occasionally occur after remission periods of upto 5 years or more. This long latency period suggests that other factors besides cell cycle time and proliferative fraction are involved. Some of these factors e.g. immunological processes were studied in the BNML.

BCG

The effect of BCG treatment on the growth of leukemic cells was studied at a relatively low tumor load, i.e., less than 10,000 cells because it is known, that immunotherapy is not effective in advanced disease. When the immunological status of the animals was manipulated by means of aspecific immunostimulation with BCG fourteen days prior to the challenge with leukemic cells, subsequent leukemia growth was inhibited. The ED 50 value was increased from 25 to 1000 BNML cells [Hagenbeek & Martens, 1983b]. Larger numbers of BNML cells could however not effectively be eliminated. Immunosuppression by means of sublethal total body irradiation did not affect the survival. When given after, though not before BCG, cyclophosphamide abolished this effect.
After the induction of a state of minimal residual disease, (by non-curative treatment consisting of cyclophosphamide plus total body irradiation followed by bone marrow transplantation), post-transplantation treatment with BCG resulted in effective cure in 100 % of the animals. From the prolongation of the survival time of the BMT control group it could be concluded that BCG treatment prevented the outgrowth of 10-100 surviving leukemic cells. In this respect macrophages were thought to play an important role because the blocking of their activity with high molecular weight dextran abolished the BCG effect.

Interferon

Rat specific interferon α_2 as well as interferon γ, were tested for a possible application in the treatment of minimal residual disease in the BNML model. Only an insignificant effect on the survival was obtained [Hagenbeek et al., 1983d].

Cytostatic agents

Various drugs have been employed for treatment of BNML rats during the phase of MRD in an attempt to test their curative potential. In such a situation the tumor load is significantly lower than it is during the remission-induction phase. Nevertheless it was found that, in order to prevent recurrent disease, it was essential to use high drug doses. One of the drugs that were tested for MRD treatment was cyclophosphamide. Remission was induced either by cyclophosphamide or Ara-C. In 100% of the animals cures were obtained [Hagenbeek & Martens, 1983a]. This was predicted by calculations of the tumor load at various stages of the disease and the log cell kill known to be induced by these two cytostatic agents.

Cures were also observed in animals that received remission-induction treatment with Dinaline followed by MRD treatment with Dinaline [Hagenbeek et al., 1988].

A similar approach with repeated peptichemio treatment resulted in a high cure rate, i.e. 70 % (Table 1-5). The dosages employed in these post-transplant chemotherapy studies were chosen such that the functioning of the marrow graft was not jeopardized.

1.4 RESIDUAL DISEASE IN THE BNML MODEL

1.4.1 Detection of minimal residual disease (MRD)

Minimal residual disease (MRD) is an arbitrary term which is used in leukemia for the phase of the disease in which the leukemic cell frequency has dropped below the detection level (Figure 1-18). When standard methods for cytological and cytochemical analysis are used to study residual disease in leukemia, the lower detection level is in the order of 5 %: below this level, leukemia can not be diagnosed with certainty. Obviously, the sensitivity of the method employed determines the lower limit at which leukemic cells can be selectively recognized and quantified. In recent years various new techniques have been developed and tested for use in selective recognition of leukemic cells and thus for application in the study of MRD.

Leukemic cells can be recognized at three different levels i.e. at the cellular level, the chromosomal level and the DNA level. Although specific monoclonal antibodies are not available, leukemic cells can be discriminated from normal cells by means of differences in the density of certain antigens on the cell surface or by co-existence of two or three antigens in an unusual combination. In such cases, sophisticated multiparameter analysis with e.g. a combination of different monoclonal antibodies and flow cytometry will allow the detection in the order of 1 residual AML cell among 10,000 to 100,000 normal cells [Martens et al., 1985; Hagenbeek & Martens 1984b, 1985a; 1985b, 1986b; Sullivan et al., 1986; Visser et al., 1986]. With methods based on immunofluorescence microscopy , similar values have been reported for acute lymphoblastic leukemia (ALL) [Van Dongen et al., 1986; Schultz et al., 1988b]

At the chromosomal level the majority of the leukemias are characterized by specific chromosomal aberrations. These abnormalities are highly specific for the leukemic cell population and would facilitate discriminative recognition of the residual malignant cell population. One prerequisite is that recurrent leukemia is characterized by the same chromosomal anomaly as the original. However, the standard cytogenetic analysis procedure is very laborious and limits the amount of mitotic spreads which can be analyzed.

Another problem is the quantification of cells with aberrant chromosomes, because only those cells which have entered mitosis during the mitotic arrest period can be examined. Furthermore, the procedure for the isolation of the mitotic cells always involves a certain period of cell culturing during which the production of leukemic cells cannot be monitored. This is another complication for deducing the leukemic cell frequency. In MRD phase only a fraction of the mitotic cells belongs to the leukemic cell population; the rest represents the dividing fraction of the normal cells. Because the non-dividing cells cannot be classified as being normal or leukemic, it is not possible to determine the mitotic fraction for both cell types and hence the total numbers cannot be calculated. Therefore, cytogenetic detection of MRD in leukemia may give a "yes" or "no" answer but quantification of residual leukemic cells will be very difficult. A solution to this problem might be the technique for "premature chromosome condensation", because it does not require cycling activity of the cells. The applicability of this method, in combination with flow karyotyping, is presently being investigated.

The BNML leukemia is also characterized by specific chromosomal aberrations (see section 1.2.4). Under which conditions chromosomal analysis may contribute to improved detection and quantification of residual disease is presently being explored with flow karyotyping [Arkesteijn et al., 1987; 1988]. At the DNA level a number of new approaches are explored in the BNML model for their applicability in detecting MRD. One of these methods i.e. the restriction fragment length polymorphism (RFLP) method which would allow the detection of 1 residual cell per 100-500 normal cells [Wright et al., 1987; Zehnbauer et al., 1986]. Better results are obtained with a method based on the polymerase chain reaction (PCR) which allows the detection of 1 residual cell per 10,000 normal cells [Lee et al., 1987]. Other approaches are in situ hybridization with chromosome specific probes, detection of tumor cell specific mRNA with in situ hybridization as well as measuring the levels of the corresponding protein product.
To which extent these new methods will be of value for the detection of minimal residual disease in leukemia and other malignancies remains to be established.

Nevertheless, for any of these methods used for the detection of residual leukemia in bone marrow biopsies or aspirates, the problem remains that the results have to be translated to the total marrow compartment.
Therefore a number of questions have to be answered:
-can leukemic cells be detected selectively ?
-what is the lower limit of detection?
-to which extent can the increase or decrease of the leukemic cells be quantified during treatment?
-are leukemic cells homogeneously distributed before and after chemotherapy?
-how does this relate to the calculation of the total tumor load?

Several of these issues were studied in the BNML model [Martens et al., 1987b]. The results and the conclusions are discussed in the Chapters 5, 6 and 7 of this thesis.

1.4.2 Development of drug resistance in residual disease.

The majority of patients with acute leukemia enter the remission phase after the initial chemotherapy treatment. However, in spite of continued consolidation treatment with chemotherapeutic agents, the size of the leukemic cell population can not be controlled. This implies that somehow cells are no longer sensitive to the action of the drugs which are being used. Either the cells have become resistant in the course of the treatment or they already had a natural resistance and were selected by the treatment. The frequency of this natural resistance is thought to be in the other of 1 per 10^6 [Skipper, 1986].

For certain drugs the basis for resistance is known, such as the dihydrofolate reductase (DHFR) gene amplification in case of methotrexate resistance and the increased expression of the transmembrane glycoprotein GP170 for the drugs associated with "multiple drug resistance", i.e. the group of anthracyclines and the vinca alkaloids. However, this does not mean that the mechanisms are completely understood. Often the drug metabolism follows a complex pathway in which resistance may occur at several levels by e.g. the induction of specific enzymes. Although certain types of resistance can be studied in vitro, animal models are very important for studying this major problem in todays cancer treatment [Kallman, 1987; Schultz et al., 1988a].
Successful attempts were made in the BNML model to generate sublines resistant to either daunomycin, cytosine arabinoside or cyclophosphamide, the drugs being most frequently used for the treatment of leukemia (see 1.3).

1.4.2 a. Cytosine arabinoside

The sensitivity of the BNML to Ara-C has been well documented (see section 1.3.1). Various investigators reported the development of cell lines, of human as well as animal origin, resistant to Ara-C, both in vitro as well as in vivo.
An in vivo totally resistant subline could be established in the BNML [Hagenbeek et al., 1987], which opened new possibilities in the investigation of the mechanism(s) behind the phenomenon of Ara-C resistance. The BNML line was transferred 32 times in total, while, during each passage, the leukemic animals were exposed to Ara-C. Treatment of the finally resistant cell line with Ara-C does not result in an increase in the survival time, while a similar Ara-C treatment applied to the parent BNML line will result in a 16 day increase in survival time. The cells from the resistant line are characterized by a 58-fold decrease in the deoxycytidine (dCyd) kinase level as well as an 18-fold reduction

in the dCyd deaminase level. In resistant AML patients, two distinct groups were identified [Colly et al., 1987]. One group was characterized by a low dCyd kinase and a low to normal dCyd deaminase; the other was characterized by a high dCyd deaminase and a normal dCyd kinase level. Thus the BNML/Ara-C resistant line can serve as a model for studying the first mentioned Ara-C resistant human AML sub-group.

TABLE 1-8

CYTOGENETIC ANALYSIS OF THE BNML PARENT LINE AND THE DRUG RESISTANT VARIANTS

cell line	chromosome aberrations							
BNML parent	1p+	2p+		8q+	-9	?12q-		20q+
cyclo resistant+	1p+	2p+q+		8q+	-9	?12q-		20q+
cyclo resistant-	1p+	2p+	6p+	inv(7)8q+	-9	?12q-	18q-	20q+
Ara-C resistant+	1p+	2p+		8q+	-9	?12q- 13q+		20q+

Cyclo: cyclophosphamide
Ara-C: cytosine-arabinoside.
"+" resistant variant.
"-" sensitive variant derived from the resistant line.

The Ara-C resistant BNML variant was cytogenetically analyzed (Table 1-8). The chromosomal aberrations characteristic of the BNML parent line, are all retained. However, in 13 % of the mitotic spreads, an extra band on chromosome 13q was observed (13q+). Because 100 % of the cells have become resistant to the action of Ara-c it is unlikely that this chromosomal aberration is related to the mechanism behind the Ara-C resistance.

1.4.2 b. Daunomycin

A single dose of daunomycin of 7.5 $mg.kg^{-1}$ in the BNML rat has little effect on the survival time [Colly, 1980]. In spite of the fact that large amounts of tumor cells are eliminated from the spleen (surviving fraction 1.1%) and the liver (surviving fraction 2.7%) (Table 1-9), many leukemic cells, in the order of 50%, survive in the bone marrow. The degree to which the bone marrow acts as a pharmacological sanctuary is related to the total tumor load [Nooter et al., 1986]. The continuous treatment of BNML rats, with daunomycin during subsequent transplantations finally resulted in a leukemia subline which showed a

considerable degree of resistance to the drug illustrated by an increased survival of leukemic cells in liver (63.7 %) and spleen (18.7 %) (Table 9). A total of 43 times a dose of 7.5 $mg.kg^{-1}$ was applied during 46 subsequent passages. To what extent this resistance is related to multiple drug resistance (MDR) remains to be established. Preliminary data indicate that daunomycin resistance in BNML cells is not related to the MDR phenotype as judged by unaltered kinetics of uptake of daunomycin measured with flow cytometry (Dr. K. Nooter, Radiobiological Institute TNO, Rijswijk, The Netherlands, personal communication).

TABLE 1-9

COMPARISON OF THE RESPONSE OF THE DAUNOMYCIN RESISTANT AND THE PARENT BNML LINE TO TREATMENT WITH DAUNOMYCIN

	surviving fraction of LCFU-S* in	
	spleen	liver
BNML parent line	1.1 %	2.7 %
daunomycin resistant	18.7%	63.7 %

daunomycin : 7.5 $mg.kg^{-1}$ i.v. on day 12 after 10^7 leukemic cells i.v.
LCFU-S: Leukemic Colony Forming Unit Spleen assay

However, Brox et al., demonstrated increased amounts of GP170 at the cell surface after 8 exposures of subsequent BNML passages to daunomycine. This glycoprotein is considered to be related to MDR type resistance [Brox et al., 1985]. However, no evidence for daunomycin resistance in terms of improved cell survival was reported.

In either case the daunomycin resistant BNML subline offers a good model for studying this phenomenon.

1.4.2 c. Cyclophosphamide

The BNML was found to be sensitive to treatment with cyclophosphamide as well as cyclophosphamide metabolites, in vivo as well as in vitro (see sections 1.3.3 and 1.3.4). There is a great difference in the survival between hemopoietic stem cells and clonogenic leukemic cells after treatment with cyclophosphamide (section 1.3.3). Therefore leukemic animals could repeatedly be treated with

cyclophosphamide in an attempt to generate resistant BNML cells. After the first signs of the development of resistance in animals sacrificed at specific intervals, the leukemia was transferred to new recipients in which exposure to cyclophosphamide was continued. After 17 exposures to cyclophosphamide doses of 100 mg.kg^{-1} each (total dose: 1.7 gram.kg^{-1}), a very high resistance was observed. In total the leukemia line was transplanted 17 times.

For the closely related drug Ifosphamide, complete cross-resistance was observed (Table 1-10). Surprisingly, cross-resistance was not complete for the activated form of cyclophosphamide i.e. maphosphamide (Table 1-10). For both drugs the final active substance phosphoramide mustard is formed at the end of the metabolic pathway [Connors et al., 1974; Domeyer & Sladek, 1980]. An explanation for the lack of full cross-resistance may be found by differences in the pharmacokinetics of the already activated maphosphamide.

TABLE 1-10

COMPARISON OF THE RESPONSE OF THE CYCLOPHOSPHAMIDE RESISTANT BNML LINE AND THE PARENT BNML LINE TO TREATMENT WITH CYCLOPHOSPHAMIDE AND RELATED DRUGS

drug	dose (mg.kg^{-1})	cell line	ILS* (days)
cyclophosphamide	100	parent	22
		resistant +	0
		resistant -	24
ifosphamide	200	parent	17
		resistant +	0
maphosphamide	154	parent	14
		resistant +	3.5

i.v. drug treatment: on day 12 after 10^7 leukemic cells i.v.
*ILS: increase in life span of 4 days corresponds with 1 log cell kill

Because the enzyme aldehyde-dehydrogenase (ALDH) plays an important role in the detoxification of cyclophosphamide [Sladek et al., 1985] a possible role for this enzyme in the resistant BNML variant was investigated. Blocking of the activity of the enzyme with disulfiram has been reported to restore the response to cyclophosphamide [Kohn et al., 1987]. Limited studies performed in the BNML so far, indicated that pretreatment with disulfiram resulted not only in an increased toxicity to normal tissues but also in a partly restored sensitivity to cyclophosphamide treatment (Table 1-11). Whether or not altered levels of aldehyde-dehydrogenase are responsible for this, is being investigated. Preliminary data indicate that in a resistant BNML subline developed at the Johns

Hopkins University, Baltimore, USA, the ALDH level is significantly increased (Dr. J. Hilton, personal communication).

TABLE 1-11

EFFECT OF PRETREATMENT OF THE CYCLOPHOSPHAMIDE RESISTANT BNML LINE AND THE PARENT BNML LINE WITH THE ALDEHYDE-DEHYDROGENASE INHIBITOR DISULFIRAM

drug	dose ($mg.kg^{-1}$)	cell line	ILS*(days)
exp 1:cyclophosphamide	100	parent	18
		resistant	8
exp 2:cyclophosphamide	50	parent	6.5
		resistant	5.5

cyclophosphamide: on day 12 after 10^7 leukemic cells i.v.
ILS: increase in life span: 4 days corresponds with 1 log cell kill
disulfiram: 5 times 500 $mg.kg^{-1}.day^{-1}$ per os on days 8-12

To study whether the observed resistance was reversible, the cyclophosphamide resistant BNML variant was continuously transplanted without cyclophosphamide pressure. At certain time intervals resistance was tested and confirmed until the 23rd transplantation, which was very sensitive again for cyclophosphamide treatment, indicating that the resistance had completely disappeared. At the cytogenetic level interesting phenomena occurred. The resistant subline was found to have an additional chromosomal marker compared to the parent BNML line, i.e. on the q-part of the marker chromosome 2p+ an extra stretch of DNA was found yielding a 2p+q+ marker chromosome. The subline characterized by the loss of the resistance to cyclophosphamide, had lost this marker chromosome and gained some additional aberrations.

The cytogenetic analysis (Dr. A. Hagemeijer, Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands) of the parent BNML cell line and the drug resistant variants are shown in Table 1-8.

To what extent the chromosomal aberrations can be related to changes at the DNA level, to provide a basis for the observed resistance, is presently being investigated. It might well be that the 2p+q+ marker chromosome involves amplification of the gene coding for ALDH which subsequently can lead to increased cellular enzyme levels. The first hypothesis would be that the loss of

resistance may also be related to the loss of this extra 2q+. However, it can as yet not be excluded that other chromosomal aberrations in the cell line that became again sensitive are involved.

In summary, the basic requirements in terms of cyclophosphamide resistant and sensitive cell line variants are now available in the BNML model.

1.5 OUTLINE OF THIS THESIS

The relevance of the BNML model for studying a great variety of different aspects of leukemia is reflected by the large number of research institutions which are employing this model for comparative leukemia studies.

Part of the investigations which were performed during recent years in the BNML model at the Radiobiological Institute TNO are presented in Chapters 2 to 7 and focus on two specific aspects of leukemia growth, i.e.:
-what is the fate of the normal hemopoietic stem cells during leukemia development and
-what are the characteristics of residual leukemia cells after the induction of a "complete remission".

The materials and methods which were used are described in Chapter 2.

The method for the detection and quantification of pluripotent hemopoietic stem cells in the rat, already briefly described in section 1.2.3, was further explored and is discussed in Chapter 3. The fate of the pluripotent stem cell during leukemia growth and its subsequent regrowth after chemotherapy is discussed in Chapter 4.

The leukemic cell population was extensively studied by means of flow cytometry in combination with monoclonal antibody (MCA) labeling. The characterization of a MCA for selective recognition of the leukemic cell population by means of flow cytometry is described in Chapter 5. The relevance of this approach with regard to leukemic cell population growth kinetics, is discussed in Chapter 6. These studies focused on the lower detection limit for leukemia cells during the so-called phase of "minimal residual disease" (MRD).

The implications for "staging of the disease" based on limited aspirations or biopsies are discussed in Chapter 7, with emphasis on the heterogeneous distribution of MRD over the bone marrow compartment. The relation between the tumor load and the efficacy of treatment, the subsequent implications for the distribution of leukemia over the marrow compartment, some aspects of local regrowth and subsequent spread of relapsing leukemia are discussed as well in Chapter 7.

A detailed discussion of the data is included in the various Chapters. For that reason the general discussion in Chapter 8 is mainly focused on the overall conclusions that have emerged from the studies. An attempt is made to put them into perspective in relation to the problems encountered in studying human leukemia.

Even when animal models have many properties in common with the human disease, as is the case for the BNML and human AML, they have their limitations with respect to the extrapolation to the clinical situation. This also holds for the BNML, so that conclusions should only be drawn with great caution.

For the human disease as well as for the animal model, the characteristics first have to be determined. The similarities and dissimilarities have to be identified before it is possible to define the specific question to be studied in the animal model. Nevertheless, determining the limitations inherent to the animal model contributes to the understanding of the complex processes involved in leukemia in man.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental animals

Rats as well as mice were used in the studies that are described in this thesis. Rats were used for studying leukemia or for measuring the hemopoietic stem cells in a spleen-colony-assay. The rats were from the barrier derived inbred Brown Norway (BN) strain BN/BiRij, produced in the Rijswijk breeding colony. Male rats between 13 and 16 weeks of age were used (mean body weight 260 grams).

Mice were only used for measuring the hemopoietic stem cells in a spleen-colony-assay. They were barrier derived F_1 mice ($C_{57}Bl$ x C_3H) and were also produced in the Rijswijk breeding colony.

2.2 The rat leukemia model

The BN acute myelocytic leukemia (BNML), which was induced in a female BN rat by treatment with 9,10-dimethyl-1,2-benzanthracene, shows striking similarities with human AML (1,2). Upon cellular transfer the leukemia shows a reproducible growth pattern. Some of its major characteristics are: a) 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: colony forming unit spleen); c) the presence of clonogenic leukemic cells (in vivo, LCFU-S: leukemic colony forming unit spleen; in vitro clonogenic assays); and d) response to chemotherapy as in human AML. An additional advantage of this model is that normal hemopoietic stem cells and leukemic clonogenic stem cells can be discriminated by modified spleen colony assays.
(See Chapter 1, for a more detailed description of the model).

2.3 Preparation of cell suspensions

Cell suspensions were made from various organs e.g. spleen, liver and bone marrow. After weighing the spleen and the liver part of them were minced with scissors and suspended through a nylon sieve in order to obtain a monocellular suspension. The physiological solution which was used for making the cell suspension was Hanks' Hepes Buffered Balanced Salt Solution (HHBBSS, 283 mOsm; pH 6.8). The cell content was determined by counting a sample (after dilution and staining with Türks' solution), with a Bürker-type hemocytometer. Because the weight of the total organ as well as the fraction which was suspended is known, the total cell content per organ could be deduced.

Bone marrow cell suspensions were usually derived from the femoral bone, which was first carved with a scalpel blade and subsequently broken in two halves. Both halves were flushed 3 times with approximately 2 ml HHBBSS in order to collect the total femoral marrow content. Cell numbers were counted as

described before. Because the total volume of the cell suspension was known, the total cell content per femoral bone could be deduced.
For investigating residual leukemia in a situation of minimal residual disease (MRD) a different procedure was followed for the collection of marrow from femoral bone sections or from the ribs. For this purpose animals were sacrificed at different stages of undisturbed leukemia development or during the phase of MRD, i.e., between 17 and 20 days after treatment with chemotherapy.

The bone marrow was collected as follows: ribs were cut on both sides while care was taken to prevent the loss of bone marrow. The sternal side of the rib was inserted in silicon tubing through which HHBBSS was flushed. In this way the total marrow could be collected from individual ribs.

Transverse sections of the femur were made by sawing the bone in several slices of 2-3 mm each, using a co-axial driven circular sawing blade (diameter 2.0 cm; thickness 0.05 mm). Cross-contamination of the samples was prevented by cleaning the blade thoroughly in between. The marrow was flushed from the bone segment by repeated pipetting of 1 ml of HHBBSS with an Eppendorf type pipet. Histological examination of bone segments after marrow collection indicated that virtually all cells had been removed.

2.4 Spleen-colony-assays (SCA)

Rat-to-rat: Barrier derived rats from the BN/BiRij inbred strain were lethally irradiated with a dose of 8.5 Gy total body irradiation (Philips-Muller 300; 300 KV X-rays; dose rate 0.34 $Gy.min^{-1}$). Per data point three groups of 7 rats each were injected with three different cell doses intravenously. The number of cells per animal was chosen such, that 5-30 colonies were expected to appear on the recipients spleen. Animals were sacrificed at various time intervals and the number of macroscopically visible spleen colonies was determined. For each injected cell dose the mean corresponding spleen colony count was calculated. The three mean values were averaged and the total CFU-S number was determined.

Rat-to-mouse: Barrier derived F_1 mice ($C_{57}Bl$ x C_3H) were lethally irradiated (9.5 Gy gamma-rays; 1.15 $Gy.min^{-1}$; Cesium source, Gammacel 220, Atomic Energy of Canada). They were injected with three different cell doses intravenously. The animals were sacrificed at various time intervals and the number of macroscopically visible spleen colonies was counted. For each injected cell dose the mean corresponding number was calculated. The three mean values were avaraged and the total CFU-S number was determined. (See Chapter 1 for a detailed description of the assays).

2.5 In vitro colony-forming cell assay (CFU-C assay)

A methylcellulose culturing method, which has been described for mouse bone marrow CFU-C [Wagemaker & Visser, 1980], was modified to allow colony formation by rat bone marrow CFU-C. Briefly, bone marrow cells were cultured in an enriched modification of Dulbecco's medium, supplemented with 5% FCS (Flow Laboratories), 1.0 % bovine serum albumin (BSA, Sigma), 3 x 10^{-5} M egg lecithin (Merck), 3.5 x 10^{-6} M human transferrin saturated with $FeCl_3$ (as modified from Guilbert & Iscove, 1976) and 10^{-4} M 2-mercaptoethanol (Merck), using 0.8 % (wt/v) methylcellulose (Methocel A4 premium; Dow Chemicals Co) as viscous agent. The cultures were stimulated by adding 10 % (v/v) medium conditioned with pokeweed mitogen-stimulated rat spleen cells (RSCM). The colonies were scored after 7 days of culture at 37°C in an atmosphere of 5 % CO_2 in air at 100 % humidity.

RSCM was produced by incubating rat spleen cells for 7 days in RPMI-1640 (Gibco), supplemented with FCS (5 % v/v), 100 µg/ml streptomycin, 10 $U.ml^{-1}$ penicillin, 10^{-4} M 2-mercaptoethanol, and 6.7 $\mu g.ml^{-1}$ of pokeweed mitogen solution (Johnson & Metcalf, 1977). After an incubation period of 7 days at 37°C in an atmosphere of 5 % CO_2 and 100 % humidity, the cells suspension was centrifuged for 30 minutes at 400 g to remove the cells. The supernatant was harvested and stored at -20°C until use.

A morphological analysis showed that the colonies were composed of granulocytes and macrophages.

2.6 Treatment of animals with cyclophosphamide and related drugs

In vivo:
In part of the studies, normal as well as leukemic animals were treated with cyclophosphamide or related compounds. Cyclophosphamide was routinely given (in a variety of different dosages) i.p., although in certain experiments i.v. injection was used. The other compounds i.e. ASTA-Z-7557 (maphosphamide; cyclohexylamine salt; 140 $mg.kg^{-1}$) or ASTA-Z-7654 (maphosphamide; lysine salt; 154 $mg.kg^{-1}$) were given i.v. These drugs were kindly provided by Dr. P. Hilgard; Asta Werke, Bielefeld, West-Germany).

In vitro:
Bone marrow cell suspensions, prepared as described, were incubated in HHBBSS supplemented with 5 % fetal calf serum (FCS) containing ASTA-Z-7557 at two different doses for 30 minutes at 37°C. After incubation the cell suspensions were centrifuged at 400 g, washed once with HHBBSS and diluted to the desired cell concentration. CFU-S survival was determined using the

described SCA in mice and calculated relative to a control cell suspension, which was incubated in the presence of FCS without ASTA-Z-7557.

2.7 The Rm124 Monoclonal antibody

This monoclonal antibody (MCA) was produced by Drs H. Kaizer and R.J. Johnson, Johns Hopkins University, Baltimore, MD. Its affinity for normal and leukemic cells is discussed in Chapter 5. Briefly, the MCA-Rm124 reacts with an antigen which is present on leukemic cells as well as on normal granulocytes although in different amounts. Differences in labeling intensity can therefore be used to discriminate between both cell types.

Determinations of leukemic cell numbers by using the MCA-Rm124 and flow cytometry correlated with data that were obtained with standard bio-assays. This approach proved to be applicable for the detection of MRD in leukemia [Chapters 6 and 7].

2.8 Complement-dependent cytotoxicity assay

Leukemic as well as normal bone marrow cells were suspended in medium consisting of HHBBSS supplemented with inactivated fetal calf serum (FCS, 5% v/v); 5 x 10^6 cells were pelleted and resuspended in various serially diluted MCA-Rm124 concentrations. The cells were incubated at 0°C, washed once and than 250 µl guinea pig complement was added. After incubation at 37°C for 30 minutes, the cells were centrifuged and washed once, resuspended in medium and diluted to the desired cell concentration. The effect of Rm124 on the survival of CFU-S, LCFU-S and CFU-C was determined by using the assays as described.

2.9 Immunofluorescence labeling of cells

For labeling with the MCA-Rm124, cells were centrifuged and resuspended in HHBBSS supplemented with inactivated fetal calf serum (FCS, Flow Laboratories; 5% v/v) and sodium azide (0.01% v/v). For fluorescence labeling samples of $2x10^6$ cells were pelleted and labeled with ascites fluid containing the FITC conjugated MCA-Rm124 (100 µl) at a 1/243 dilution at 0°C for 45 minutes. After 30 minutes of labeling 25 µl of Propidium Iodide (PI, Calbiochem, San Diego, CA; 20 $\mu g.\mu l^{-1}$) were added. PI can easily enter dead cells, in contrast to viable cells, and will subsequently bind to the DNA. This approach enabled the exclusion of the dead cells. This is of importance as dead cells show an aspecific binding of the MCA, and thus influence the accuracy of

the measurements. After careful washing, the cells were resuspended and processed on a modified fluorescence activated cell sorter (FACS-II).

2.10 Flow cytometry

The labeled cells were analyzed on a modified FACS II (Becton and Dickinson, Sunnyvale, CA), with an argon ion laser tuned at 488 nm (0.5 Watt). Forward light scatter signals (FLS) were used for triggering the measurements. Perpendicular to the laserbeam the light was collected on a Dichroic Mirror DM570 in order to separate "green" FITC fluorescence (<570 nm) and "red" PI fluorescence (>570 nm). The FITC fluorescence was then measured through a broad band multicavity interference filter (520-550 nm transmission, Pomfret, Stamford, CT) and a 520 nm cut-off filter (Ditric, Mannheim, FRG) by an S-20 type photomultiplier. A logarithmic amplifier (Nozaki, T., Stanford, CA) was used for the FITC fluorescence signals. PI fluorescence was measured through a 620 nm long-pass filter by means of an S-11 type photomultiplier. PI signals were linearly amplified.

On the basis of PI fluorescence the viable cells were identified (negative "red" fluorescence) and "gated out" electronically. The Rm124/FITC fluorescence of 50,000-300,000 cells was measured as described and stored as a histogram in a Hewlett Packard HP87 personal computer. The leukemic cell frequency was determined by measuring the relative area under the curve.

2.11 DNA analysis

For kinetic studies, either all cells or a subpopulation selected on the based on their immunofluorescence properties, were sorted into a DNA staining solution containing Propidium Iodide (10 $\mu g.ml^{-1}$, CalBiochem), Triton-X-100 (1% solution) and RNase (1 $mg.ml^{-1}$, Serva, Heidelberg, FRG.), according to the method described by Tailor [1980]. Cells were measured on the same day with the laser beam tuned at 488 nm. To ensure stability of the system, saline (0.9% NaCl) was used as a sheath fluid [Martens et al., 1981]. Fluorescence intensity above 620 nm was measured to generate a DNA distribution histogram. The fraction of cells in the various cell cycle compartments (G_1/G), S, (G_2/M) were determined according to the DNA computer analysis program as described by Schultz et al. [1985].

2.12 Reproducibility of flow cytometry measurements

To study the accuracy and the reproducibility of the flow cytometry measurements of leukemic cells when present in low frequencies such as in

MRD, artificial mixtures of leukemic and normal cells ranging from 10% to 0.01% leukemic cells (1/10-1/10,000) were labeled with the MCA-Rm124 and measured on the flow cytometer (Table 2-1). Mixtures containing 10% to 0.01% leukemic cells were prepared in quadruplicate and processed for flow cytometry as described. Each sample was measured four times to allow statistical analysis of the accuracy and reproducibility of the method. Only for the lowest leukemic frequency samples (0.01%) the standard error showed a tendency to rise, however within acceptable limits. An example of a typical, representative fluorescence histogram for a 1/10,000 mixture, is shown in Figure 1-7. The leukemic cell frequency is derived from the area under the curve in relation to the total cell number.

TABLE 2-1

REPRODUCIBILITY OF FACS MEASUREMENTS IN DETECTION OF MINIMAL RESIDUAL DISEASE USING THE Rm124 MONOCLONAL ANTIBODY

*artificial mixture	**mean leukemic cell frequency measured	standard deviation
0.01	0.0182	29 %
0.1	0.1000	10 %
1	0.6324	7.8 %
10	5.946	1.7 %

* % leukemic cells per total normal marrow cells

** Rm124-FITC positive cells corrected for background frequency signals derived from normal bone marrow

NB: -per mixture 4 samples were prepared
-each sample was measured 4 times, each time 150,000-300,000 cells were analyzed. Standard deviation is from the mean of 4 experiments.
-FACS: fluorescence activated cell sorter

CHAPTER 3

HETEROGENEITY WITHIN THE SPLEEN COLONY FORMING CELL POPULATION IN RAT BONE MARROW

Reprinted with minor modifications from
Martens, A.C.M., van Bekkum, D.W. and Hagenbeek, A.:

Heterogeneity within the spleen-colony-forming cell population in rat bone marrow.

Exp. Hematol. 14 [1986] 714-718.

Permission was obtained from the editor.

3.1 INTRODUCTION

Pluripotent hemopoietic stem cells of mice [Till & McCulloch, 1961] as well as rats [Comas & Byrd, 1966; Dunn, 1973] can be detected and quantified by injection of cell suspensions into lethally irradiated litterateur and scoring the number of colonies that will develop on the surface of the spleen. Each colony is a product of a single cell with colony forming ability.

For the mouse it has been shown, that on different days, different subpopulations of colony forming cells (CFU-S) are detected [Magli et al., 1982]. So far two subtypes have been distinguished; one producing colonies around day 8, the other producing colonies around day 12 in a mouse-to-mouse spleen colony assay (SCA). The day 8 CFU-S can be distinguished from the day 12 CFU-S by differences in antigen membrane composition [Monnette & Stockel, 1981; Harris et al., 1984; Visser et al., 1984], biophysical characteristics [Visser et al., 1984] and response to chemotherapy [Hodgson & Bradley, 1979; Porcellini et al., 1984]. Furthermore the day 8 CFU-S has lost the capacity to produce thymic lymphocytes [Mulder et al., 1985].

The standard rat-to-rat spleen colony assay for detection of CFU-S in rat bone marrow yields colonies at days 11-12 [Goldschneider et al., 1980; Castangnola et al., 1981]. The mouse however, can also be used as a recipient to study rat CFU-S. Mice are routinely sacrificed around day 9 [Rauchwerger et al., 1973; Van Bekkum, 1977]. Although in both species rat bone marrow cells are injected, a difference in the time of colony appearance is observed. Generally it is assumed that in both assays the same colony forming cell is detected. However, in view of the mouse CFU-S heterogeneity, the rat-to-mouse SCA had to be re-evaluated.

Because rat and mouse hemopoietic stem cells show similarities in many of their characteristics it is feasible to suppose a heterogeneity within the rat CFU-S population. In this paper experimental evidence is presented to support this hypothesis.

3.2 RESULTS

Mice and rats were lethally irradiated and injected with normal rat bone marrow. The mice were killed from day 6 onwards, the rats from day 10 onwards and the number of macroscopically visible spleen colonies was determined. In both species colonies developed; in the mouse they were visible as early as day 6 but in the rat not before day 11 (Figure 3-1). It is obvious that in the mouse the colony number is not constant throughout the observation period.

In the rat spleen the number of colonies reaches a plateau and is constant from day 12 through 14. The colony number is nearly at the same frequency as observed in the mouse spleen during the corresponding time interval.

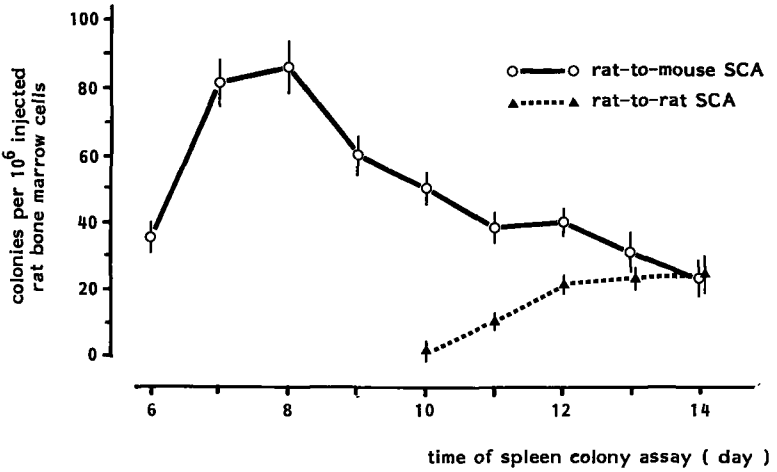


Figure 3-1:
Number of colonies on the spleens of lethally irradiated mice and rats after injection of normal rat bone marrow cells.
SCA: spleen colony assay.
Data were obtained from two separate experiments.
Bars represent the standard error of the mean.

To determine the reproducibility of the observations, in total ten separate experiments were performed to determine the spleen colony numbers on mouse spleens on day 8 and day 12 after injection of rat bone marrow. For the day 8 CFU-S determination, the standard injected cell doses were $4x10^5$, $2x10^5$ or $8x10^4$ and for the day 12 determination the standard injected cell doses were $8x10^5$, $4x10^5$ or $2x10^5$. The mean day 8 CFU-S content per rat femur was 8200 with a standard error of the mean (SEM) of 682; and the day 12 CFU-S content was 3482 with a SEM of 296. The mean ratio of the day 8 and the day 12 CFU-S of these experiments was 2.38 with a SEM of 0.13.

Part of the evidence for heterogeneity in the CFU-S population in mouse bone marrow was derived from experiments showing a different response to chemotherapy of the two CFU-S subtypes [Hogson & Bradley, 1979; Martens et al., 1986]. For the rat a similar approach was chosen. Normal rat bone marrow was subjected to in vitro treatment with maphosphamide (ASTA-Z-7557, an in vitro active cyclophosphamide derivative) at two different drug dose groups

(Figure 3-2). A SCA was performed in mice. At a dose of $40x10^{-6}M$ the difference in sensitivity between the day 8 and the day 12 CFU-S is significant (p-value <0.001). The day 12 CFU-S appeared to be the least sensitive.

In vivo treatment of rats with the parent drug cyclophosphamide and two forms of maphosphamide, ASTA-Z-7654 and ASTA-Z-7557, resulted in a similar observation. At 24 hours post treatment, employing the rat-to-mouse SCA, a 20 to 50-times lower

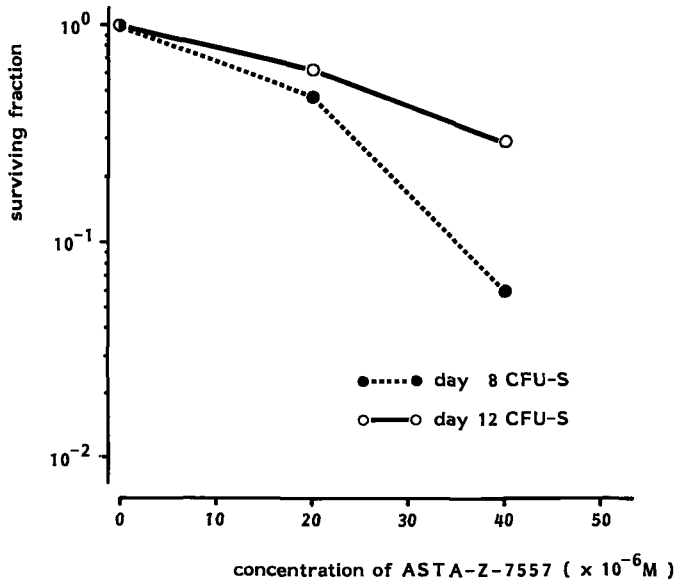


Figure 3-2:
Response of day 8 CFU-S and day 12 CFU-S in normal rat bone marrow to in vitro treatment with maphosphamide (ASTA-Z-7557).
The CFU-S were determined in a rat-to-mouse spleen colony assay.

surviving fraction of day 8 CFU-S compared to the day 12 CFU-S was found. All three drugs gave similar results (Table 3-1).

After in vivo cyclophosphamide treatment, the regrowth patterns of day 8 and day 12 CFU-S were found to be clearly different (Figure 3-3). During the exponential growth phase the day 8 CFU-S showed a population doubling time (Td) of 0.85 days in contrast to the day 12 CFU-S which showed a population doubling time of 1.65 days. For day 8 CFU-S as well as day 12 CFU-S, pre-treatment levels are reached at the same day, e.g. at day 7 after treatment. The production of total nucleated cells in the marrow is following the CFU-S production with a 3 day time delay, but occurs at a fast rate.

TABLE 3-1

SURVIVING FRACTION OF DAY-8, AND DAY-12 CFUS IN NORMAL RAT BONE MARROW 24 HOURS AFTER TREATMENT WITH CYCLOPHOSPHAMIDE COMPOUNDS

drug	surviving fraction of day-8 CFU-S*	surviving fraction of day-12 CFU-S*	ratio day8/12
a. Cyclophosphamide	3.8×10^{-3}	8.5×10^{-2}	22.4
SEM	1.4×10^{-3}	2.0×10^{-2}	
b. ASTA-Z-7557	1.6×10^{-3}	8.3×10^{-2}	51.7
SEM	0.8×10^{-3}	1.7×10^{-2}	
c. ASTA-Z-7654	3.4×10^{-3}	6.6×10^{-2}	19.3
SEM	0.3×10^{-3}	2.9×10^{-2}	

* spleen colony assay performed in mice
data are derived from three independent experiments
SEM: Standard Error of the Mean
a. 100 $mg.kg^{-1}$; b. 140 $mg.kg^{-1}$; c. 154 $mg.kg^{-1}$

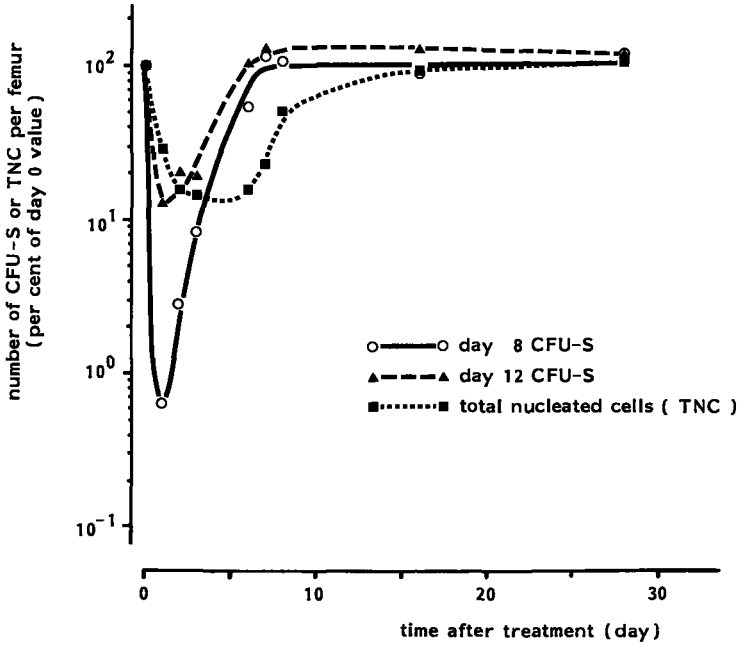


Figure 3-3:
Regrowth kinetics of day 8 CFU-S, day 12 CFU-S and total nucleated cells in the femoral bone marrow of normal BN rats after in vivo treatment with cyclophosphamide (100 $mg.kg^{-1}$ i.p.).
The CFU-S were determined in a rat-to-mouse spleen colony assay.

The existence of heterogeneity of the CFU-S population was also studied in the BNML rat leukemia model. During the development of leukemia the number of CFU-S in the bone marrow decreases while in the leukemic spleen there is an increase in the number of CFU-S at the same time. At a relevant time point during this process, i.e. at day 14 after transfer of 10^7 BNML cells, the number and ratio of day 8 and day 12 CFU-S for both the spleen and the bone marrow CFU-S population were determined (rat-to-mouse SCA). In the bone marrow of leukemic rats the total number of both types of CFU-S is much lower than in the femur of normal controls (Table 3-2). However the day 8/day 12 ratio is not significantly different (2.08 versus 2.38). In the spleen there is a drastic increase of mainly the day 8 CFU-S type. The increase in day 12 CFU-S numbers is relatively less impressive. This results in a ratio of day 8 and day 12 CFU-S of 4.49 which is clearly different than the ratio in normal rat bone marrow (2.38).

TABLE 3-2

CHANGES IN THE NUMBER AND THE RATIO OF DAY 8/DAY 12 CFU-S IN RAT FEMUR AND SPLEEN DURING THE DEVELOPMENT OF LEUKEMIA (BN acute myelocytic leukemia)

group	day-8 CFU-S[a]	day-12 CFU-S[a]	ratio day8/day12
control rats:			
femur[b]	8200	3482	2.38
SEM	862	296	0.13
spleen[c]	ND	ND	-
leukemic rats:[d]			
femur	4907	2355	2.08
spleen	101336	24550	4.49

a: spleen colony assays were performed in mice
b: control femur data are mean values of 10 independent experiments
c: normal spleen contains in total 640 day 9 CFU-S
d: day 14 after 10^7 BNML cells i.v.
SEM: Standard Error of the Mean.
ND: not determined.

3.3. DISCUSSION

After injection of rat bone marrow into lethally irradiated mice certain cell types are lodging in the spleen. They will form colonies that eventually are visible as nodules on the surface of the spleen (Figure 3-1), however the number is not constant during the observation period. At the maximum (day 7-8) 2 to 3 times more colonies are observed than at the plateau phase around day 12 to 14. Because the number is not constant it is of questionable value to relate the number of spleen colonies to one particular single stem cell type. Heterogeneity among the colony forming cell population has to be considered. However such a conclusion would not immediately be drawn from the rat-to-rat SCA data. In the rat spleen the number of colonies after injection of rat bone marrow increases and reaches a plateau, which would be expected if only one type of cell was forming the colonies in this type of assay.

Based on the rat-to-mouse SCA data the hypothesis was postulated that heterogeneity within the CFU-S population of the rat is responsible for the type of colony formation that is observed. Part of the CFU-S are forming colonies early (around day 8); other CFU-S are forming colonies late (around day 12). A ratio of day 8/day 12 CFU-S of 2.4 was observed repeatedly (Table 3-2).

It was attempted to induce changes in this ratio by treatment with cytostatic drugs. In vitro incubation of rat bone marrow with maphosphamide resulted in a difference in the surviving fractions of the day 8 and the day 12 CFU-S (Figure 3-2) as detected in the rat-to-mouse SCA. For mouse bone marrow a similar observation has been reported [Porcellini et al., 1984]. With regards to the use of three cyclophosphamide derivatives it should be noted, that these drugs show differences in the initial part of the metabolism: therefore differences in CFU-S survival might be expected and could be related to different CFU-S subtypes. In vivo treatment of rats with cyclophosphamide or maphosphamide resulted after 24 hours in a 20 to 50-fold difference in the surviving fraction between day 8 and day 12 CFU-S (Table 3-1), indicating a different sensitivity of the two CFU-S types. Furthermore, the regrowth kinetics of day 8 and day 12 CFU-S after their initial reduction is not identical (Figure 3-3). Although the reduction in the number of day 12 CFU-S is far less compared to the day 8 CFU-S reduction, an equal amount of time is needed to return to pre-treatment levels. This is due to clear differences in population doubling times, e.g. 0.85 days for day 8 CFU-S and 1.65 days for the day 12 CFU-S.

In mouse hemopoiesis it has been suggested that the day 12 CFU-S is a more immature stem cell [Burton et al., 1982; Chertkov & Drize, 1984] which has the capacity of selfreplication as well as producing day 8 CFU-S. For the rat no data are available yet to draw such a conclusion. The big difference in population doubling time found after cyclophosphamide treatment may be an indication that

part of the day 12 CFU-S become day 8 CFU-S. However, studies to measure the production of day 8 and day 12 CFU-S within the spleen colonies have to be performed before drawing any definite conclusions.

Another argument to support the hypothesis of heterogeneity within the rat CFU-S population is derived from experiments performed in the rat leukemia model. A typical feature of this model is a severe suppression of normal hemopoiesis in the bone marrow. The leukemia becomes clinically manifest around day 13-14 after 10^7 BNML cells i.v., which is characterized by an increase in spleen- and liver weight and peripheral leukocytosis. The CFU-S numbers in the bone marrow drop quickly to low levels (determined by a day 9 CFU-S SCA in mice) [Van Bekkum et al., 1978]. Meanwhile the number of CFU-S in the leukemic spleen increases rapidly. At this stage it was decided to investigate the number and the ratio of day 8 CFU-S and day 12 CFU-S in leukemic bone marrow and leukemic spleen. The observed change in the day 8/day 12 CFU-S ratio of bone marrow CFU-S population in leukemic rats is only minor compared to the big change observed in the spleen (2.1 and 4.5 respectively; Table 3-2). In the leukemic animal the CFU-S have not been exposed to cytostatic drug treatment. Drug-induced intracellular defects as an underlying cause for a different behaviour of the CFU-S in the SCA can therefore be excluded. Apparently, the microenvironment in the leukemic spleen is capable of supporting hemopoiesis. The change in the ratio of day 8 and day 12 CFU-S might be due to an increasing demand of blood cell progenitors. This leads then to an increased recruitment of resting stem cell in to cycle. The day 8 CFU-S subtype in the mouse is found to be correlated with the state of cycling of hemopoietic stem cells [Visser et al., 1984; Mulder et al., 1985]. The micro-environment in which the CFU-S are detected is the same in all instances (the mouse spleen). Observed differences have to be related therefore to differences in the composition of the injected cell suspensions.

The experimental data presented point to the existence of at least two different spleen colony forming cells within the hemopoietic stem cell population of the rat. However, the observation can only be done using the mouse spleen for CFU-S detection. The rat spleen only seems to allow the detection of one type of rat CFU-S. Whether this type of CFU-S should be related to the day 8 or the day 12 CFU-S in the rat-to-mouse system is uncertain. More experiments are needed before any definite conclusion which CFU-S type on the mouse spleen corresponds with which type of CFU-S on the rat spleen after injection of rat stem cells. One might consider experiments with CFU-S populations with an altered ratio of day 8 and day 12 CFU-S, which might be obtained by using adequate cell separation methods as has been reported for the mouse [Visser et al., 1986].

A second possibility would be the incubation of rat bone marrow cells with IL3, which results in the mouse in the generation of almost 100 % day-8 CFU-S

[Mulder et al., 1986]. When the SCA's are performed simultaneously in rats and in mice it might be possible to draw definite conclusions.

The observation that the hemopoietic stem cell population in both the mouse and the rat is heterogeneous, suggests that this is a general phenomenon in mammalian hemopoiesis.

CHAPTER 4

KINETICS OF NORMAL HEMOPOIETIC STEM CELLS DURING LEUKEMIA GROWTH BEFORE AND AFTER INDUCTION OF A COMPLETE REMISSION.

STUDIES IN A RAT MODEL FOR ACUTE MYELOCYTIC LEUKEMIA (BNML).

Reprinted with minor modifications from:
Martens, A.C.M. and Hagenbeek, A.:

Kinetics of normal hemopoietic stem cells during leukemia growth before and after induction of a complete remission. Studies in a rat model for acute myelocytic leukemia (BNML)

Leuk. Res. 11 [1987] 453-459

Permission was obtained from the editor.

4.1 INTRODUCTION

A characteristic of the leukemic process in the human is the severe suppression of bone marrow hemopoiesis. Anemia, granulocytopenia and thrombocytopenia are the first clinical symptoms of the disease in many cases. This decrease of mature cells is caused by a decrease in the number of committed precursor cells of various types in the bone marrow [Van Bekkum & Van Oosterom, 1976]. A direct inhibitory effect of leukemic cells on the growth of normal hemopoietic cells has been suggested by some authors [Morris et al., 1975; Quesenberry et al., 1978] as the underlying mechanism. Others have found evidence that the leukemic cells are producing humoral factors that negatively influence the growth of normal cells [Olofsson et al., 1984]. A third mechanism has been proposed by Prins & van Bekkum [1981], supported by experimental evidence, that there is competition for favourable sites in the bone marrow between the pluripotent hemopoietic stem cells and the clonogenic leukemic cells. This leads to replacement of the stem cells by leukemic cells.

Recognition of the pluripotent hemopoietic stem cell in man is still a major problem but in animal models the characteristics of the pluripotent hemopoietic stem cells are well described. The Brown Norway acute myelocytic leukemia model fulfils the requirements for a good model for human acute leukemia [Van Bekkum & Hagenbeek, 1977]. The main advantage is that the leukemia is growing rather slowly allowing the development of failure of normal hemopoiesis including the decrease in the number of pluripotent stem cells. This results in the manifestation of a severe suppression of hemopoiesis in the bone marrow. Of particular advantage is the fact that both the pluripotent hemopoietic stem cell and the clonogenic leukemic cell of the rat can be selectively detected and quantified by means of modified spleen colony assays (SCA).

This paper describes the kinetics of the normal stem population in the bone marrow and in the spleen during the development of leukemia and how it is affected by the treatment with a so-called "priming dose" or a therapeutic dose of cyclophosphamide with emphasis on endogenous repopulation, migration and remigration of stem cells. A "priming dose" (PRD) of cyclophosphamide is a low dose treatment which increases the tolerance towards a subsequent treatment e.g. total body irradiation. PRD treatment was reported to have a beneficial effect on lung epithelial in the mouse [Collis et al., 1980], on intestinal tract epithelial of sheep [Millar et al., 1978], as well as on CFU-S numbers in the bone marrow [Hagenbeek & Martens, 1984a]. Because of the latter activity, the effect of PRD treatment on the CFU-S population in leukemia, was studied in the BNML model.

4.2 RESULTS

The changes of the hemopoietic stem cell (CFU-S) contents in rat bone marrow and spleen during the growth of 10^7 leukemic cells injected on day 0 are shown in Figure 4-1. The number of CFU-S decreased from day 10 onwards, while the CFU-S numbers in the spleen began to rise from day 8 onwards.

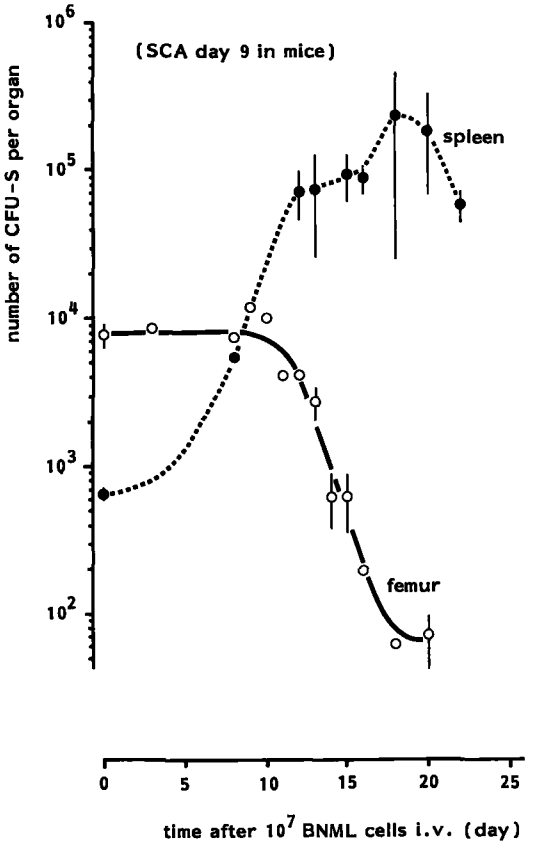


Figure 4-1:
The fate of the hemopoietic stem cell in the femoral bone marrow and in the spleen during the development of the BN acute myelocytic leukemia (BNML).
(SCA in mice on day 9.)
Bars represent standard errors of the mean.

During the decrease of the CFU-S number in the bone marrow to about 1 % of the normal value, the number of CFU-S in the spleen steadily increased, reached a maximum value between day 18 and 20. At the peak value the spleen contained about 100 times the control value of CFU-S, but since the size of the spleen

increased a factor of 10 (due to the progression of leukemia), the relative frequency of the CFU-S was only 10 times increased. However, when the leukemia progressed further, the CFU-S number in the spleen started to decrease concurrently towards the terminal stage of the disease.

Leukemic animals were treated on day 13 with the relatively low "priming dose" (PRD) of cyclophosphamide (10 mg.kg^{-1} i.p.). Compared to control animals, hardly any effect on the total nucleated cell number per femur was observed (Figure 4-2A). The Mean Cell Volume (MCV) of leukemic BNML cells is 16 micrometer which is more than the average MCV of heterogenous normal bone marrow. This might explain why there is a reduction in the total number of nucleated cells in the femur during the progression of the leukemia.

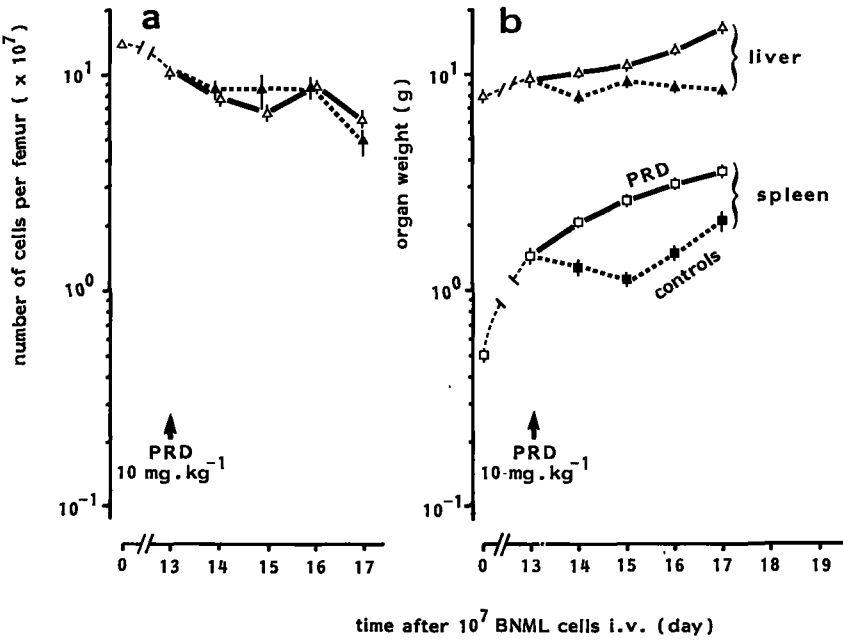


Figure 4-2:
The effect of a "priming dose" (PRD) of cyclophosphamide of 10 mg.kg^{-1} i.p. on the tumor load of leukemic rats (BN acute myelocytic leukemia) on day 13 after 10^7 BNML cells i.v.
Panel a: effect on the cellularity of the femur.
Panel b: effect on the weight of spleen and liver
Bars represent standard errors of the mean.

The leukemic cell load in spleen and liver was monitored by means of organ weights as there is a strict correlation between the organ weight and its leukemic cell content [Van Bekkum & Hagenbeek 1977]. In the spleen, the PRD treatment induced a growth delay of two days. In the liver the PRD treatment caused an arrest of growth for the rest of the observation period (Figure 4-2B). PRD treatment with cyclophosphamide did not influence the survival time (Table 4-1).

TABLE 4-1

THE INFLUENCE OF A SMALL "PRIMING DOSE " (PRD) OF CYCLOPHOSPHAMIDE ON THE GROWTH OF THE BN ACUTE MYELOCYTIC LEUKEMIA

Exp no.	Median Survival Time (days) Control group	PRD group
1	23	20
2	22	21
3	21	23
4	20	20

Day 0: 10^7 BNML cells i.v.;
Day 13: PRD treatment: 10 $mg.kg^{-1}$ i.p.(n=8)

The effect of a PRD treatment on the CFU-S numbers in both the bone marrow and the spleen is evident (Figure 4-3). In the spleen the PRD treatment resulted in lower numbers of CFU-S on day 14 compared to the control spleen, which in fact, reached a peak value on day 14. The peak value of the PRD group was reached on day 16. The differences between the spleens of the two groups however, were not statistically significant.

The decline of the CFU-S numbers in the bone marrow, as it is observed in the control group (Figure 4-3) after day 13, is drastically changed by the PRD treatment. On day 15 and 16 about 5-fold higher CFU-S numbers are found in the femoral marrow. From day 16 onwards the CFU-S numbers follow the decline typical for the BNML. The pattern of decreasing CFU-S numbers in the bone marrow was however reversed for two days.

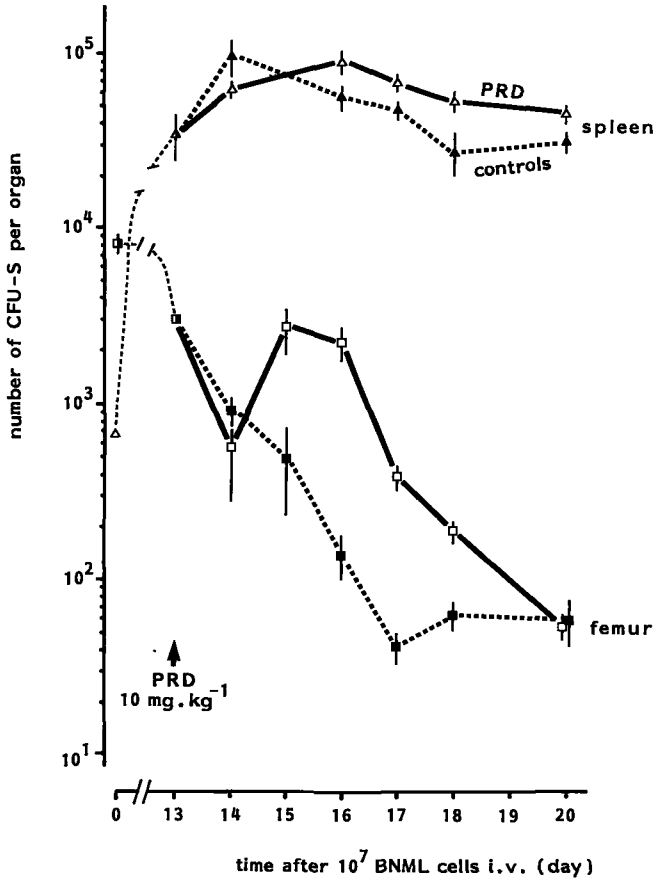


Figure 4-3:
The effect of a "priming dose" (PRD) of cyclophosphamide of 10 mg.kg^{-1} i.p. on the number of CFU-S in the femoral bone marrow and in the spleen of leukemic rats on day 13 after 10^7 BNML cells i.v.
Bars represent standard errors of the mean.

The effect of a therapeutic dose of cyclophosphamide (100 mg.kg^{-1} i.p. on day 13) on the CFU-S population was also investigated. In each experiment a leukemic control group was included in order to verify that it corresponded with the normal pattern of CFU-S decrease, as shown in Figure 4-1. As all control determinations were within the confidence limits, control curves were not included in all the figures. The cyclophosphamide treatment led to an immediate, sharp reduction in the number of CFU-S in both the spleen and the bone marrow (Figure 4-4). Immediately thereafter, a rapid CFU-S increase was observed in both organs reaching pretreatment values at day 20. It is interesting that spleen CFU-S numbers did not increase further after the femur CFU-S reached normal values.

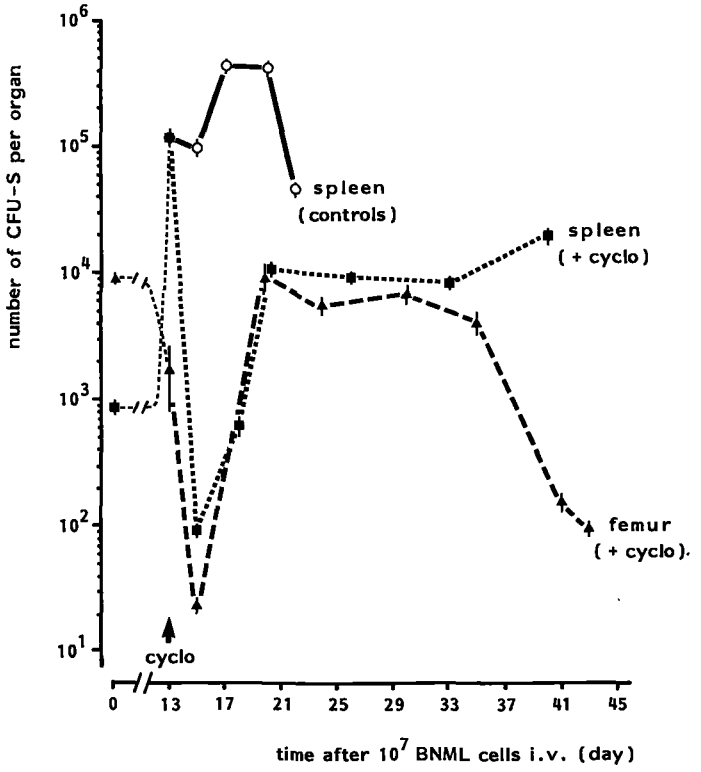


Figure 4-4:
Kinetics of CFU-S in the femoral bone marrow and in the spleen of leukemic rats (BN acute myelocytic leukemia) after remission-induction treatment with cyclophosphamide (100 mg.kg^{-1} i.p.) on day 13 after 10^7 BNML cells i.v.
Bars represent standard errors of the mean. Cyclo: cyclophosphamide 100 mg.kg^{-1} i.p.

To determine whether the CFU-S which are repopulating the marrow after treatment are coming from the spleen or from the endogenously surviving CFU-S in the bone marrow, the following experiment was designed. Leukemic animals were treated on day 13 with cyclophosphamide (100 mg.kg^{-1} i.p.). Animals were splenectomized within half an hour after drug treatment making it unlikely that CFU-S surviving cyclophosphamide treatment could migrate from the spleen to the bone marrow and were compared to non-splenectomized controls. The results are shown in Figure 4-5 and Figure 4-6.

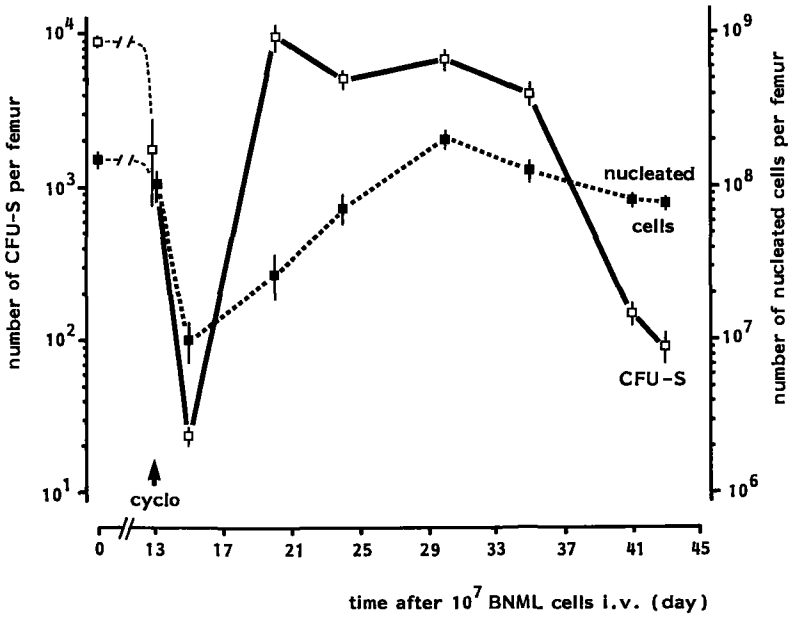


Figure 4-5:
Repopulation of CFU-S and total nucleated cells in the femoral bone marrow in the BN acute myelocytic leukemia after remission- induction treatment using cyclophosphamide (100 mg.kg^{-1} i.p.) on day 13 after 10^7 BNML cells i.v.
Bars represent standard errors of the mean. Cyclo: cyclophosphamide 100 mg.kg^{-1} i.p.

The number of CFU-S in the bone marrow of the group which was splenectomized showed the same rapid recovery at day 20, compared with the non-splenectomized group. When however the total nucleated cell production in the femoral marrow was compared, differences were found i.e. in the splenectomized animals a doubling time of the total nucleated cell population of 2.4 days was found, while in the non-splenectomized group the doubling time was at the lower rate of 3.5 days. Although the peak value was reached on the same day i.e. day 29 (Figure 4-5 and 4-6), on days 21 and 25 respectively, a two fold difference in the total nucleated cell content per femur, was observed.

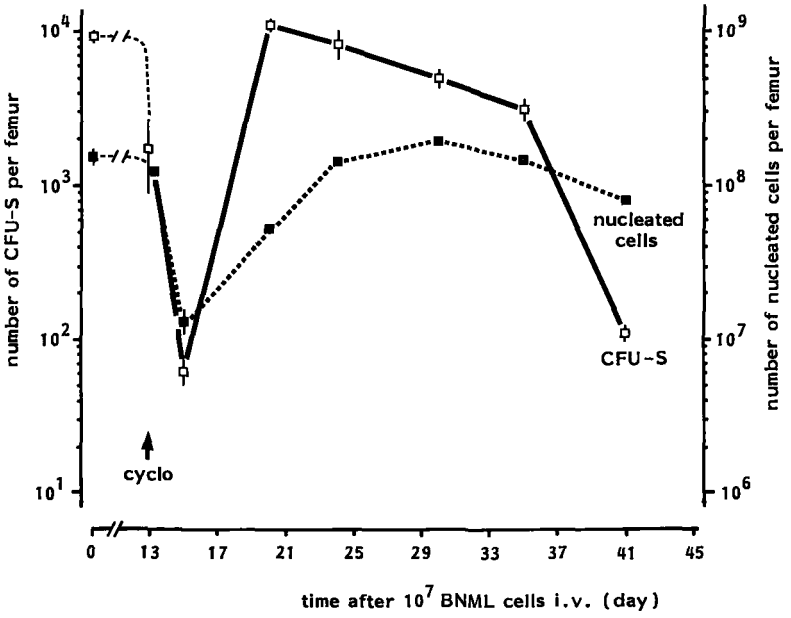


Figure 4-6:
Repopulation of CFU-S and total nucleated cells in the femoral bone marrow in the BN acute myelocytic leukemia after remission-induction treatment using cyclophosphamide (100 mg.kg^{-1}) on day 13 after 10^7 BNML cells i.v., followed by splenectomy.
Bars represent standard errors of the mean.

4.3 DISCUSSION

During the progression of the BNML leukemia the number of stem cells (CFU-S) in the bone marrow decreased (Figure 4-1). Previously it was shown that leukemic cells of myeloid nature preferentially lodge near the endosteal sites in femoral bone marrow [Prins & Van Bekkum, 1981]. In contrast, lymphatic leukemic cells spread at random in the bone marrow cavity. As the hemopoietic stem cells in the bone marrow are thought to be localized near the endosteal region [Shakney et al., 1975; Lord & Schofield, 1979], it was postulated that clonogenic leukemic cells and normal CFU-S are competing for these sites that are apparently most favourable for proliferation [Schofield et al., 1980].

When in the BNML leukemic rat the bone marrow compartment was almost completely infiltrated by leukemic cells and the CFU-S numbers in the bone marrow were rapidly decreasing (day 13 after 10^7 BNML cells), the spleen showed extramedullary hemopoiesis (Figure 4-1). Treatment with a low dose of cyclophosphamide at this stage (10 mg.kg^{-1} i.p.) resulted in a small reduction of

the leukemic cell load (Figure 4-2). This was confirmed by the observation that PRD treatment did not influence the survival time (Table 4-1).

Compared to clonogenic leukemic cells, CFU-S are relatively spared by cyclophosphamide or cyclophosphamide metabolites [Hagenbeek & Martens, 1983; 1984]. The kinetics of the CFU-S re-population in both the femoral bone marrow as well as in the spleen were strongly influenced (Figure 4-3). During the first day after treatment no essential differences in bone marrow CFU-S numbers between controls and the treated group were observed. However a drastic increase in CFU-S numbers was observed on the two following days. In the spleen the differences were much less impressive. Apparently the mild cytotoxic treatment created spaces in the marrow, by eliminating leukemic cells, which were subsequently occupied by the surviving CFU-S. However, the leukemic cell population rapidly recovered from the low-dose treatment and the competition for space started again. As a result the newly formed CFU-S left the marrow cavity or died.

The delay in the rise of the spleen CFU-S population suggests that the splenic CFU-S is mainly dependent on an influx from the bone marrow. Experiments in which therapeutic doses of cyclophosphamide were used revealed data that support this explanation. Treatment of leukemic rats with cyclophosphamide at a dose of 100 $mg.kg^{-1}$ results in a surviving fraction of the leukemic cell load of 10^{-5} to 10^{-6} [Hagenbeek & Martens, 1982]. Compared to this, the CFU-S survival in femur and spleen is much better e.g. 3 % survival in the femur and 0.1% in the spleen (Figure 4-4). This 30 fold higher decrease of splenic CFU-S may be related to the fact that in the spleen the fraction of CFU-S in cycle is higher than in the bone marrow [Hagenbeek & Martens, 1981e] which might make them more susceptible to the action of cyclophosphamide.

With regards to the origin of the CFU-S which are repopulating the bone marrow it should be noted that after treatment with 100 $mg.kg^{-1}$ cyclophosphamide, the whole spleen contained 100 CFU-S which equaled the number of CFU-S in one femur after the treatment. One femur,however, represents only 2.5% of the total bone marrow compartment [Sonneveld, 1980]. It is therefore unlikely that surviving CFU-S in the spleen contributed in a significant way to the restoration of normal hemopoiesis as they are far outnumbered by the surviving CFU-S in the entire bone marrow compartment (which equals 40 times 1 femur). This was further studied by splenectomizing the rats shortly after treatment. No essential differences in the regrowth pattern of the CFU-S in the femur between both groups were observed (Figures 4-5 and 4-6). The rapid increase of CFU-S in the bone marrow in the splenectomized group, however, was accompanied by a faster production of mature nucleated cells, i.e. with a population doubling time of 2.4 days compared to 3.5 days for the non-splenectomized group. This suggests that in the non-splenectomized animals, part

of the CFU-S or their offspring entered the circulation and homed in the spleen to continue proliferation and/or maturation.

In both the bone marrow and the spleen the number of CFU-S increased rapidly (Figure 4-4). It is, however, interesting to note that the increase in the spleen stopped at the moment that the femoral marrow compartment reached preleukemic values. For the entire duration of the remission period the number of CFU-S in the bone marrow stayed at this level. In the spleen the relatively low level was also maintained. It should be noted that the spleen is capable of producing much higher numbers of CFU-S as is illustrated in untreated control leukemic animals on days 12 through 20 after the injection of 10^7 leukemic cells (Figure 4-1). Nevertheless, the number of CFU-S in the spleen is not rising until the number of CFU-S in the bone marrow starts to fall. This leads to the conclusion that the spleen is not an active production site of CFU-S but merely an organ with a supporting function activated upon increased demands, that has to rely on an influx of CFU-S from the bone marrow.

The probability of self-renewal of CFU-S in the spleen may be very low [Schofield & Lajtha, 1983]. The function of the spleen in supporting hemopoiesis on demand, is therefore probably mainly restricted to facilitating growth of committed precursor cells beyond the stage of the CFU-S and to allow cells to mature. The marrow, however, remains the major production site of pluripotent hemopoietic stem cells.

CHAPTER 5

CHARACTERISTICS OF A MONOCLONAL ANTIBODY (Rm124) AGAINST ACUTE MYELOCYTIC LEUKEMIA CELLS

Reprinted with minor modifications from:
Martens, A.C.M., Johnson, R.J., Kaizer, H. and Hagenbeek, A.:

Characteristics of a monoclonal antibody (Rm124)
against acute myelocytic leukemia cells.

Exp. Hematol. 12 [1984] 667-671.

Permission was obtained from the editor.

5.1 INTRODUCTION

Many attempts have been made in the past to raise antisera against leukemia cells of different type and origin. The objective was the development of antisera that would specifically recognize leukemia-associated antigens. Preferentially, the antigen should not be present on any of the normal cells. If these antisera should become available, application for diagnosis and in vivo or in vitro immunotherapy would be obvious. With the development by Brown et al. [1975] of a conventionally raised antiserum against acute lymphoblastic leukemia, the situation looked promising. Afterwards, a number of monoclonal antibodies [Knapp et al., 1982; Ritz et al., 1980] that detected the same antigen (gp100-CALLA) were raised by other investigators. Based on recent reports [Braun et al., 1983; Greaves et al., 1983; Hokland et al., 1983], which indicate that some normal cells cross-react with the "anti-CALLA" sera, the tumor specificity of the antigen that it detects is questioned. Similar observations have been reported with respect to antisera to acute myelocytic leukemia. By means of the hybridoma technique, a number of monoclonal antibodies that react with myeloid/monocytoid leukemia cells such as MY-1 [Strauss et al., 1983], B5 [Billing et al., 1982], MMA [Hanjan et al., 1982], OKM-1 [Breard et al., 1980], D5 [Majaic et al., 1981], D5D6 and C5H10 [Linker-Israeli et al., 1981], and others have been produced. So far, all of these MCAs show to a greater or lesser extent cross reactivity with normal cells in bone marrow or blood. If, however, differences exist between the labeling intensity of leukemic and normal cells, there may still be a basis for discriminating between the two. In that case, this might enable the detection of both "minimal residual disease" after remission-induction chemotherapy and at early relapse. In addition, this would offer possibilities for the elimination of residual leukemia cells while leaving unharmed the hemopoietic stem cells in autologous bone marrow grafts. This report describes the characteristics of a MCA raised against cells from a rat leukemia model (BNML). Our findings suggest that, under certain conditions, absolute specificity is not required to obtain the desired results.

5.2 RESULTS

5.2.1 Antibody-binding studies

The reactivity patterns of BNML leukemic cells and normal rat bone marrow cells, after exposure to serial dilutions of the MCA-Rm124, are shown in Figure 5-1. As a measure for the average density of the antigen that is detected by this antibody, the peak position of the fluorescence intensity profile is taken. Examples of the fluorescence intensity profiles are shown in Figure 5.2. The data shown in Figure 5.1 indicate a great difference in the peak position between leukemic cells and normal cells after labeling with the MCA-Rm124. Normal

bone marrow, however, is a mixture of different cell types and can be divided largely into erythrocytes, lymphocytes, granulocytes, and blast cells. These cell types can be discriminated with the FACS-II cell sorter on the basis of differences in their forward and perpendicular light-scatter properties [Visser et al., 1980].

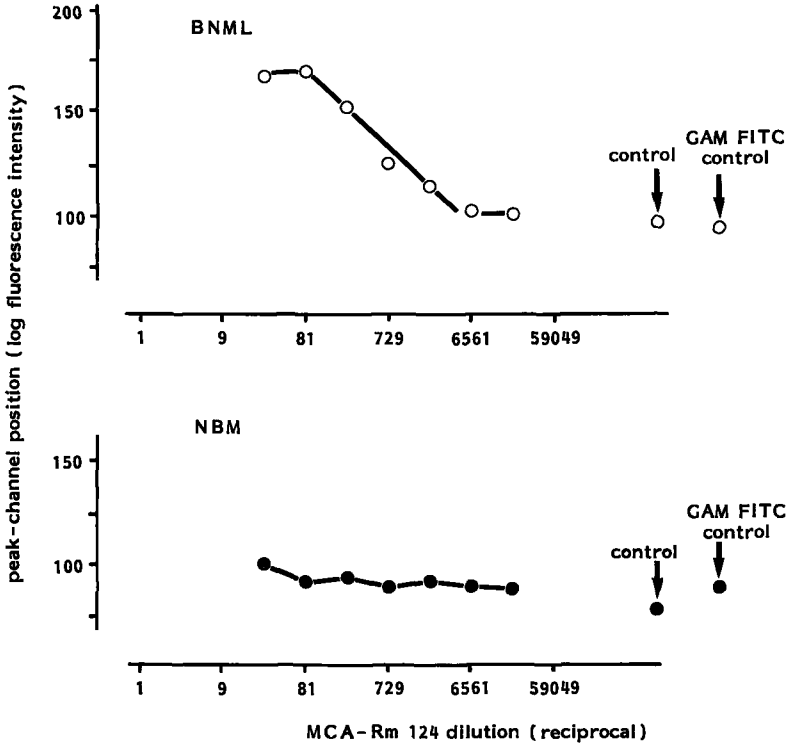


Figure 5-1:
Fluorescence intensity of BNML cells as compared with normal bone marrow cells after labeling with the MCA-Rm124.
x-axis: serial dilution of the antibody
y-axis: peak position of (logarithmically amplified) fluorescence-intensity histograms. The units are arbitrary and relate to channel numbers.
For each datum point, at least 20,000 cells were processed with the FACS II cell sorter.

When the fluorescence intensity profiles of these individual cell types are determined, striking differences are observed (Figure 5-2). Lymphocytes and the blast cells appear to belong to the negative fraction. A subpopulation of granulocytes, however, revealed increased antibody labelling. Leukemic cells represent the highest and most homogeneously labelled cell population. These data suggest that the hemopoietic progenitor cells that are found in the blast cell population in low frequencies [Visser et al., 1980] have a low density of the antigen that is recognized by the MCA-Rm124

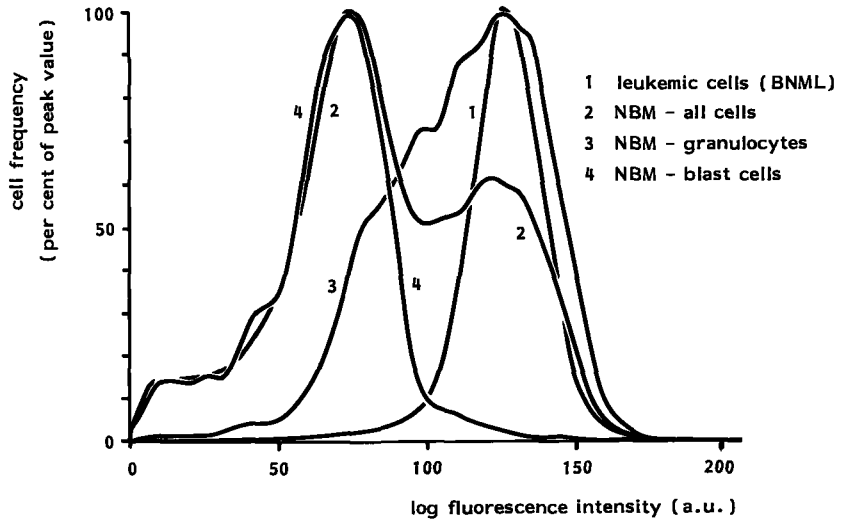


Figure 5-2:
Fluorescence intensity of BNML cells as compared with various cell types from normal rat marrow after labeling with the MCA-Rm124.
x-axis: fluorescence intensity (logarithmically amplified); units are arbitrary and relate to channels
y-axis: cell frequency normalized to the peak value.; NBM: normal bone marrow cells

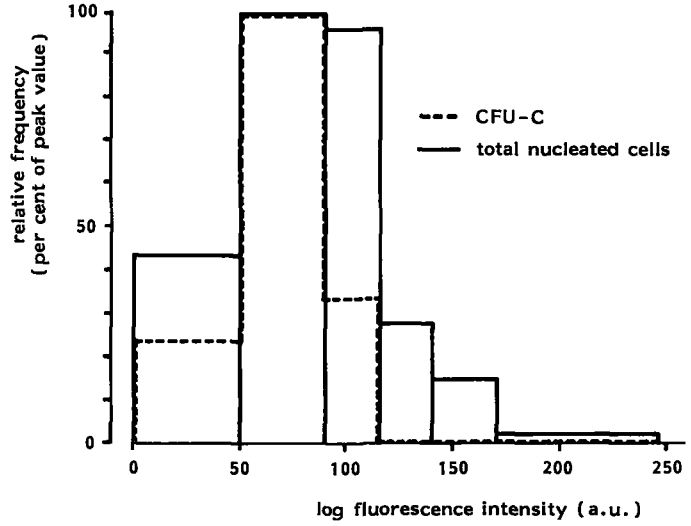


Figure 5-3:
Relative fluorescence-intensity distribution of total nucleated cells and CFU-C from normal rat bone marrow after labeling with the MCA-Rm124 followed by FACS-II cell sorting.
x-axis: fluorescence intensity (logarithmically amplified); units are arbitrary and relate to channels
y-axis: relative frequency of total cells or CFU-C, normalized to the peak values.

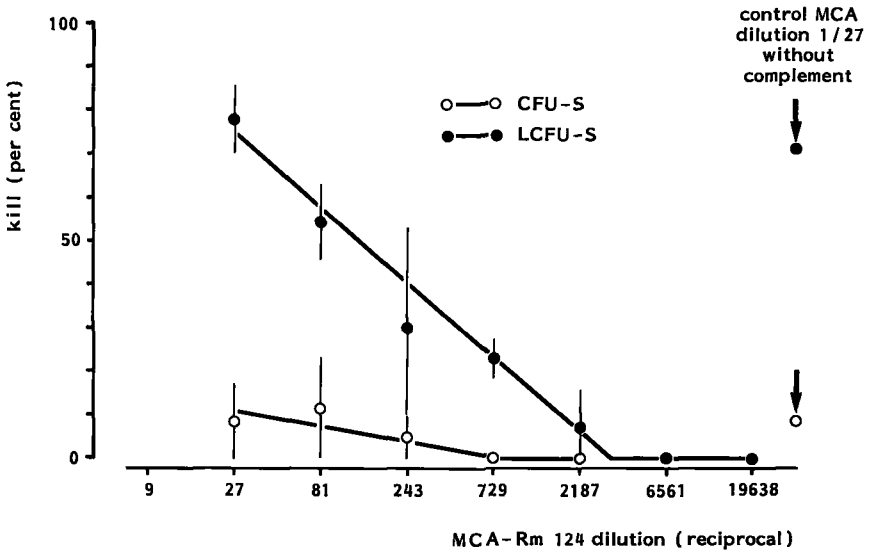


Figure 5-4:
Cytotoxicity of the MCA-Rm124 towards clonogenic leukemic cells (LCFU-S) and to hemopoietic stem cells (CFU-S).
x-axis: serial dilution of antibody.
y-axis: the relative kill in LCFU-S or CFU-S compared with control cell suspensions
CFU-S: mean values of two separate experiments; LCFU-S: mean values of three separate experiments.
Bars represent standard errors of the mean.

This was tested by sorting of normal marrow cells based on differences in fluorescence intensity and culturing CFU-C from the various fractions obtained. The results are shown in Figure 5.3. The peak of the CFU-C distribution profile coincides with that of the total nucleated cell fluorescence intensity profile. In the higher fluorescence intensity range, no CFU-C are found.
This indicates that using this method no MCA-Rm124 binding to CFU-C is observed. Apparently, the antigen to which it is binding is not present on the CFU-C. The binding of the MCA to the hemopoietic stem cell (CFU-S) was tested in the complement-dependent cytotoxicity assay.

5.2.2 Complement-dependent cytotoxicity assays

The reaction of the MCA-Rm124 toward the CFU-S and LCFU-S was tested with a cytotoxicity assay as described earlier. The results are shown in Figure 5.4. At the dilutions studied, the MCA exerted a very low cytocidal effect on the CFU-S. It was toxic for the LCFU-S. A 50% kill was found at a dilution of 1:140. In a control group where cells were incubated with the MCA and inactivated fetal calf serum added instead of complement, 70% of the LCFU-S were killed. This indicates that, for LCFU-S, the cytotoxicity of the MCA is not complement dependent.

5.3 DISCUSSION

The MCA-Rm124 detects an antigen that is present on cells from the BNML rat leukemia model. All leukemia cells are labeled, as indicated by the immunofluorescence studies (Figure 5-2). However, some cross-reaction with a fraction of the granulocyte population is observed. Therefore, the antigen that is recognized is certainly not a unique leukemia-associated one, but rather a myeloid-linked differentiation antigen. Apparently, primitive myeloid precursor cells do not bind the MCA-Rm124 which is rather unique as compared with many other MCAs, which - besides myelocytic leukemia cells - bind to a variety of other normal blood/bone marrow cells, including CFU-C [Billing et al., 1982; Breard et al., 1980; Hanjan et al., 1982; Majaic et al., 1981; Strauss et al., 1983].

A differential killing of LCFU-S with, at the same time, sparing of the hemopoietic stem cells is demonstrated by the cytotoxicity studies. A 100% kill of LCFU-S may be envisaged by using undiluted antibody-containing ascites fluid, based on the extrapolation of the antibody-dilution-cytotoxicity-response relationship (Figure 5-4). This is of importance in view of eliminating residual leukemia cells from autologous bone marrow grafts. In that situation, the frequency of the leukemic cell will be much lower than in this study.

Another application of this MCA is the detection of residual leukemia for diagnostic purposes during the remission period after leukemia treatment. Under the conditions described in this study, difficulties with respect to specific recognition of the BNML cells may be encountered due to cross reactivity with a subpopulation of granulocytes. These granulocytes, however, can be removed selectively by using a two-step density gradient. Due to differences in the densities of granulocytes and leukemia cells, removal of granulocytes from the leukemic cell fraction can be envisaged [Metcalf et al., 1971]. Labeling of this purified cell fraction with the MCA-Rm124 will enable the detection and quantification of residual leukemia cells. Preliminary studies indicate that the lower detection limit at present will be in the order of one leukemic cell per 10,000 normal cells [Hagenbeek and Martens, 1984c]

CHAPTER 6

DETECTION OF MINIMAL DISEASE IN ACUTE LEUKEMIA USING FLOW CYTOMETRY: STUDIES IN A RAT MODEL FOR ACUTE MYELOCYTIC LEUKEMIA

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Martens, A.C.M. and Hagenbeek, A.

Detection of minimal disease in acute leukemia
using flow cytometry: studies in a rat model
for human acute leukemia.

Cytometry 6 [1985] 342-347.
Permission was obtained from the Editor

6.1 INTRODUCTION

A critical question in the treatment of human leukemia is the fate of the residual leukemic cell population once remission has been achieved by induction chemotherapy. Since the majority of leukemia patients develop a relapse within a period of 1 to 2 years [Lister & Rohatiner, 1982] it can be concluded that a substantial number of leukemic cells survive the treatment. Thus, it is of utmost importance to be able to recognize and to quantify the number of surviving cells.

The classical hematological methods do not allow the detection of less than 5% leukemic cells in the bone marrow. The monoclonal antibody (MCA) methodology, however, offers new possibilities for recognizing small numbers of leukemic cells. Using a limited number of MCAs all leukemia subtypes can be recognized and distinguished, but for studying the residual tumor load, the cross reactivity of many monoclonal antibodies with normal cells is a major drawback. As leukemic cells are characterized by their arrest in a certain maturation-differentiation stage, they will generally express the phenotype of their normal counterpart. One of the disappointing experiences in this respect was with the anti-CALLA MCAs. CALLA positive cells have been found in the bone marrow of normal individuals [Braun et al., 1983; Greaves et al., 1983], fetal hematopoietic tissues [Hokland et al., 1983] and also in non-hematopoietic tissues [Metzgar et al., 1981]. Although this means that absolute specificity is not achieved, differences in labeling intensities (i.e. antigen densities) between normal and leukemic cells might offer a possibility to discriminate between the two cell populations.

The study presented in this Chapter was performed in the BN acute myelocytic leukemia, which is a realistic model for studying human acute myelocytic leukemia (AML) and has been described in detail before [Hagenbeek & Van Bekkum, 1977; Van Bekkum & Hagenbeek, 1977; Chapter 1]

A monoclonal antibody (Rm124), which enables the discrimination between normal and leukemic cells, based on differences in labeling intensities that can be visualized with flow cytometry, has been produced [Johnson & Shin, 1984]. The lower detection limit for leukemic cells using this MCA is 1 per 10,000, as described earlier [Hagenbeek & Martens, 1984]. The studies to be described were performed with a commercially available flow cytometer. The latter has restrictions when searching for "rare" cells. The maximum number of cells that one can analyse per second with the desired accuracy is in the order of 10,000. Another limitation is the occurrence of artifact background signals which are in the order of 1-4 per 10^5 [Visser et al., 1984].

With this observation in mind, we focussed in this study on the detection of leukemia in the range of "minimal residual disease" (MRD) i.e. in the range of 0.01 % leukemia up to full blown disease. Three major organs were studied for sequential quantification of MRD in order to provide data for leukemic cell

population kinetics. Cell kinetic studies in MRD were performed by combining cell surface labeling, sorting of the leukemic subpopulation and subsequently determining the DNA content of the leukemic cells using the propidium iodide method.

6.2 RESULTS AND DISCUSSION

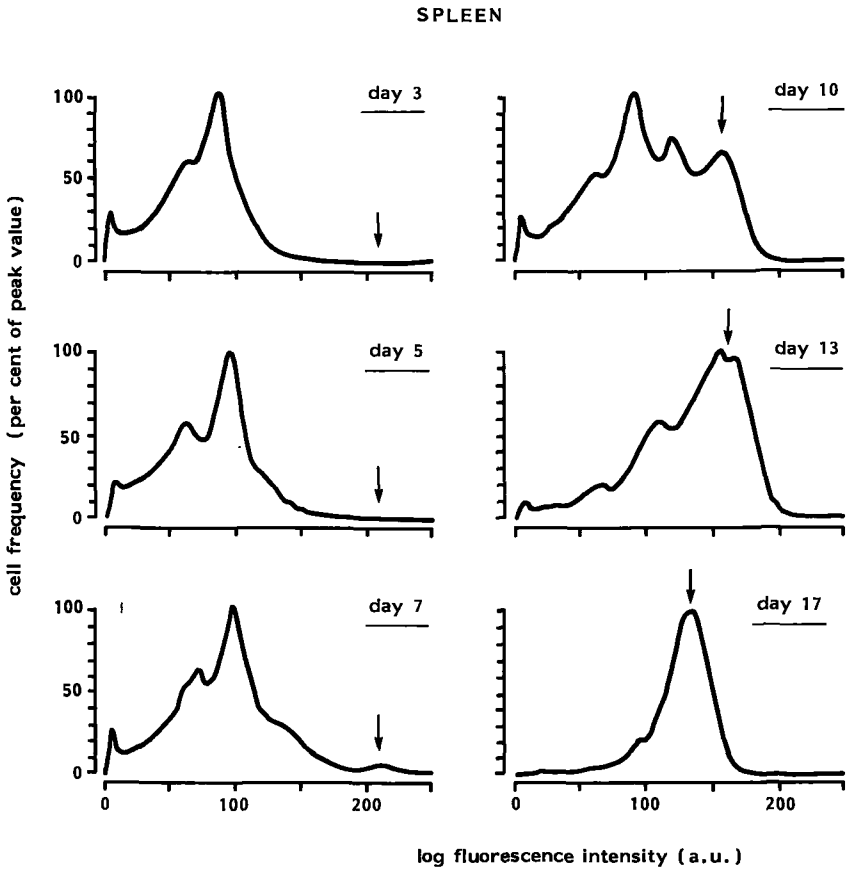

Figure 6-1:
Fluorescence-intensity distribution of MCA-Rm124 labeled spleen cells on various days after i.v. inoculation of 10^7 BNML cells.
For each day one typical histogram out of three is shown.
x-axis: Fluorescence intensity (logarithmically amplified); units are arbitrary and relate to channel numbers.
The arrows point to the leukemic subpopulation
y-axis: Cell frequency as per cent of peak value.

The number of leukemic cells in the spleen, liver and bone marrow were monitored with the MCA-Rm124 on various days after inoculation of 10^7BNML cells i.v. From these organs cell suspensions were prepared as described. Portions of the cell suspensions were labeled with the MCA-Rm124 for flow cytometric analysis.

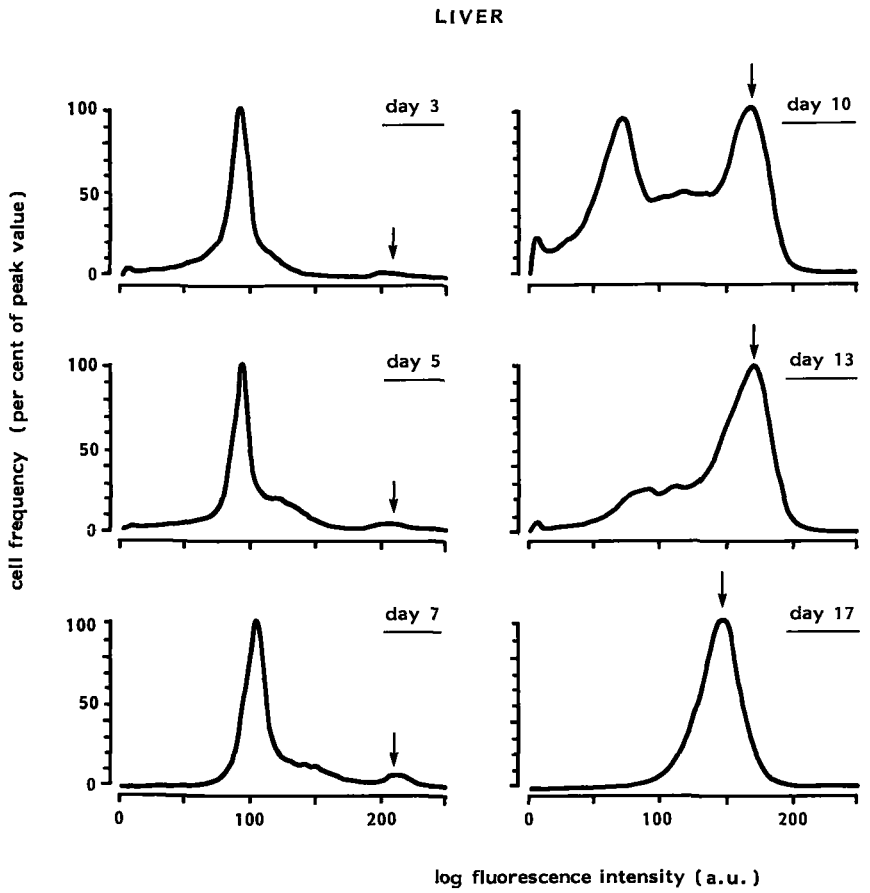

Figure 6-2:
Fluorescence-intensity distribution of MCA-Rm124 labeled liver cells on various days after i.v. inoculation of 10^7 BNML cells.
x-axis: Fluorescence intensity (logarithmically amplified); units are arbitrary units and relate to channel numbers.
The arrows point to the leukemic subpopulation
For each day one typical histogram out of three is shown.
y-axis: Cell frequency as per cent of peak value.

In the case of the femoral bone marrow, cells were also injected into normal rats for an in vivo bioassay to determine the number of LCFU-S. In this way a correlation could be made between the flow cytometry derived data and the bioassay derived data. The flow cytometric detection of the leukemic subpopulation in the spleen, the liver and the bone marrow is shown in Figures 6-1, 6-2 and 6-3, respectively.

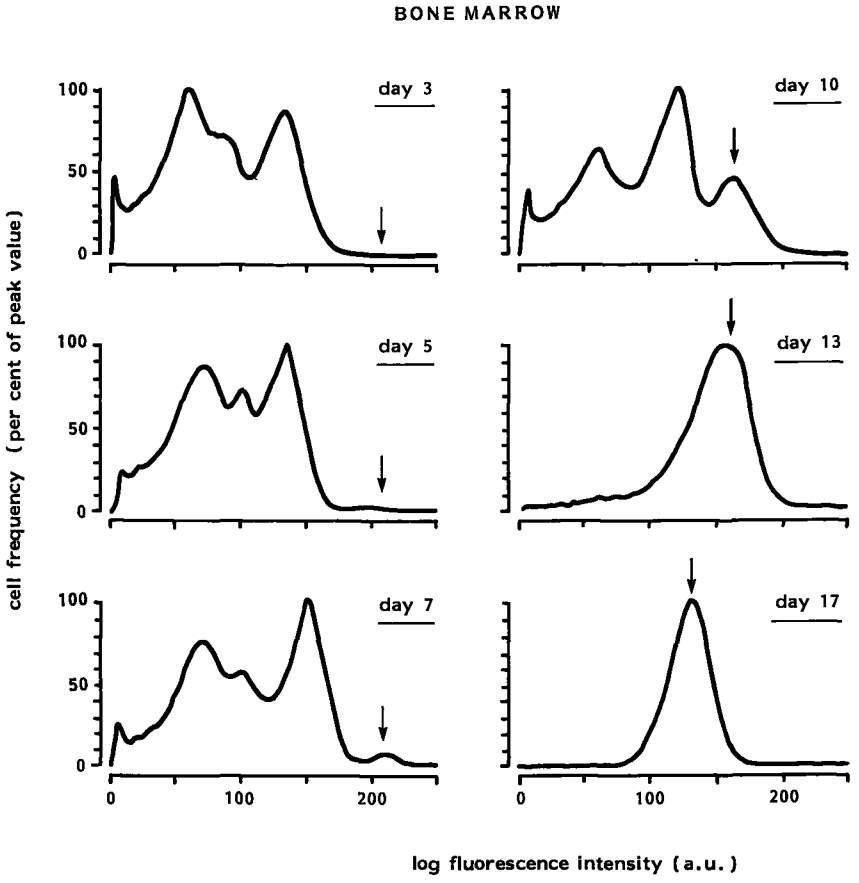

Figure 6-3:
Fluorescence-intensity distribution of MCA-Rm124 labeled bone marrow cells on various days after the i.v. inoculation of 10^7 BNML cells.
x-axis: Fluorescence intensity (logarithmically amplified); units are arbitrary units and relate to channel numbers.
The arrows point to the leukemic subpopulation
For each day one typical histogram out of three is shown.
y-axis: Cell frequency as per cent of peak value.

Already on day 3 after the i.v. injection of 10^7 leukemic cells a leukemic subpopulation can be detected in all three organs studied, although it only becomes visible in the fluorescence intensity histograms after expressing the cell frequencies on a logarithmic scale (Figure 6-4). During the subsequent days, the leukemic cell population steadily increases in size in all three organs investigated. In the initial studies, in which the labeling pattern of the MCA-Rm 124 was determined, cells that appeared in this separate peak having high fluorescence properties were sorted. Morphologic examination after May-Grünwald staining indicated that they were indeed leukemic cells.

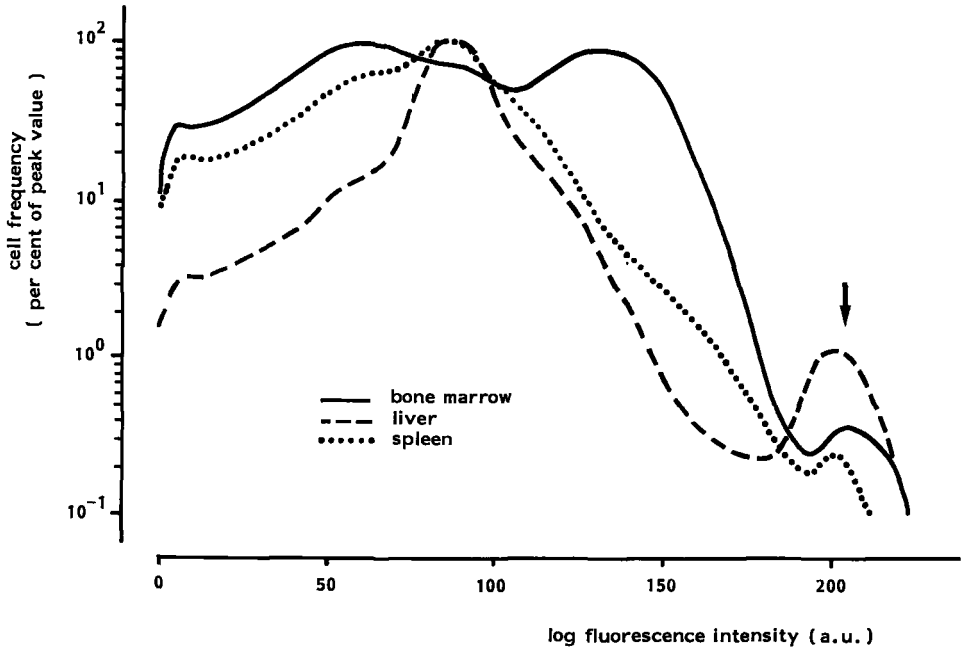

Figure 6-4:
Fluorescence-intensity distributions of bone marrow, spleen and liver on day 3 after the i.v. inoculation of 10^7 BNML cells.
x-axis: Fluorescence intensity (logarithmically amplified): units are arbitrary and relate to channel numbers. The arrow points to the leukemic subpopulation
y-axis: Cell frequency as per cent peak value on a logarithmic scale.
For each organ one typical histogram out of three is shown.

The leukemic cell frequencies were determined with the MCA-Rm124 and used to enumerate the total organ content. The growth curve for the leukemic cell population in the bone marrow is shown in Figure 6-5 and is compared with a growth curve which was obtained on the basis of bioassay derived data and

yielded similar results. The increase in leukemic cell numbers is linear when expressed on a logarithmic scale.

The growth of the leukemic subpopulation in the liver was determined in the same way. The growth curves of leukemic cells in bone marrow and liver are compared in Figure 6-6. It is obvious that the population doubling times for these two organs are not identical. Therefore, it should be concluded that the increase in the number of cells is dependent on various organ-related factors. It is quite possible that the percentages of cells in cycle is not the same for the two organs; there might be differences in the cell cycle times (although that is not the most likely explanation) or in the cell loss rate between the two organs. The continuous influx and efflux of cells between organs is probably also of importance [Hagenbeek & Martens, 1979]. The possibilities offered by the MCA and flow cytometry will enable the study of these processes.

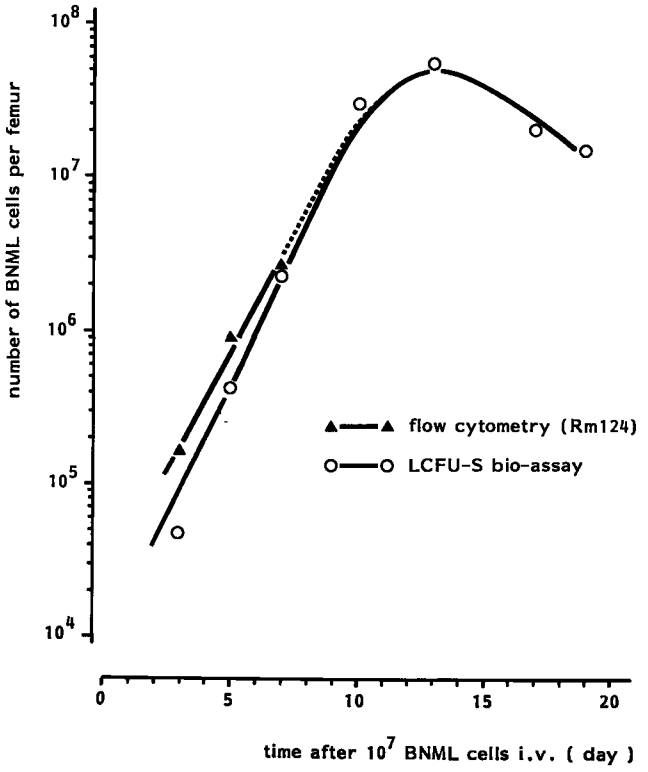

Figure 6-5:
Growth kinetics of the BN acute myelocytic leukemia in the femur.
Comparison of growth curves obtained with the MCA-Rm124 or with the bio-assay.

Because the leukemic cells could be detected by using the MCA-Rm124, the next step was the sorting of cells and subsequently processing them for DNA analysis. The advantage of this approach is clearly illustrated in Figure 6-7. Normally, it would not be possible to obtain cell kinetic data of liver cell suspensions using flow cytometric methods due to the fact that a certain fraction of liver cells contain multiple nuclei. Sorting of the MCA-positive fraction of cells and processing them for DNA analysis as described, yielded DNA histograms that enabled the cell kinetic study of the leukemic subpopulation (Figure 6-7, lower right). Similar type of analysis can be performed for any other organ provided that the MCA can discriminate between the leukemic cells and the normal cells. At present, studies are being undertaken to investigate the lower detection limit for leukemic cells which is of importance for studying the growth of the leukemic cell population after the application of chemotherapy and/or radiotherapy.

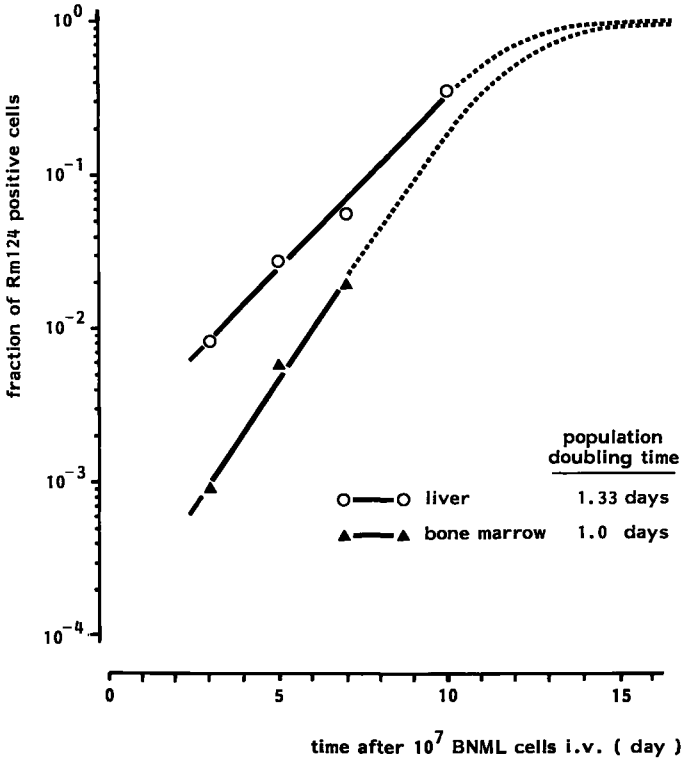

Figure 6-6:
Growth kinetics of the BN acute myelocytic leukemia in the bone marrow and liver monitored with the MCA-Rm124.
MCA-Rm124 data are obtained from separate analysis of 3 leukemic rats.

This will more strictly be in the area of "minimal residual disease" and it is of utmost importance to answer questions such as: Can leukemic cells, previously exposed to chemotherapy still be recognized under the same conditions as in unperturbed leukemia? How are cells in regenerating bone marrow reacting with the MCA? Is MRD equally distributed over the target tissues for leukemia regrowth or can a selective localization be identified? These preclinical animal studies form a basis for clinical explorations. In the latter, the situation is more complex. There is a need for specific antisera, which are as yet not available for the acute myelocytic leukemias and only for the minority of the lymphocytic leukemias. In summary, the animal model data indicate that when adequate MCAs are used in combination with flow cytometry, normal and leukemic cells can be discriminated on the basis of their fluorescence intensity properties. The MCA-Rm124 can therefore be used for selectively recognizing the leukemic subpopulation when present in low frequencies. Serial measurements using this method will enable the study

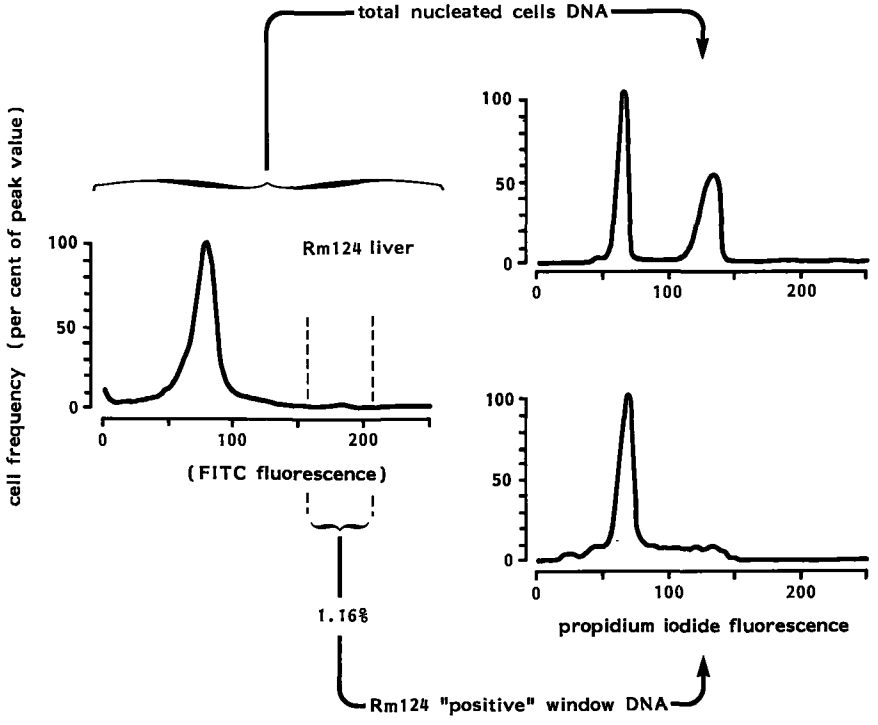

Figure 6-7:
DNA analysis of BN acute myelocytic leukemia cells in the liver (1 % of total)
Cell suspensions were labeled with MCA-Rm124 and the leukemic fraction (having the highest fluorescence) was sorted and stained for DNA analysis. The unsorted cell suspension was also stained for DNA analysis (top right-handside) and is compared with the sorted leukemic subpopulation (bottom right-hand side).

of the population kinetics in "minimal residual disease" because flow cytometry data were found to correlate with conventional, expensive bioassays. By performing DNA analysis of MCA labeled, FACS sorted leukemic cells, information on the distribution of leukemic cells over the cell cycle and hence estimation of the growth fraction during "minimal residual disease" can be obtained.

CHAPTER 7

THE STUDY OF THE LEUKEMIC CELL DISTRIBUTION BEFORE AND AFTER THE INDUCTION OF A PHASE OF MINIMAL RESIDUAL DISEASE

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"Nonhomogeneous distribution of leukemia in the bone marrow during minimal residual disease"

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7.1. INTRODUCTION

Although the remission-induction rate for acute leukemia reaches 70-80 % at present, most patients will relapse within 2 years [Keating et al., 1986]. This indicates, that a substantial number of leukemic cells survive treatment. Standard cytological methods for the detection of residual leukemia cells are failing once the leukemic cell frequency in bone marrow or blood drops below the detection level of 1-5%, because at this level leukemic cells can no longer be distinguished from normal blast cells. At this detection limit, a large residual leukemic cell load i.e. $1\text{-}5 \times 10^{10}$ cells may be left in the bone marrow. If the use of modern analysis methods would result in lowering the detection limit, a second limitation with regard to the detection of the residual disease will be the sampling method.

The distribution of residual leukemic cells over the body in minimal disease is totally unknown. Decreasing the detection level for residual leukemia cells is a prerequisite for studying the so-called phase of "Minimal Residual Disease" (MRD). Furthermore, the earliest detection of an imminent relapse is of utmost importance because reinduction treatment will be more successful when the tumor load is small.

Among various methods that have been explored for the detection of residual leukemic cells are: DNA aneuploidy using flow cytometry [Barlogie et al., 1986]; cytogenetics [Dewald et al., 1985; Hagemeijer et al,. 1979; 1986]; immunological methods and cell culture methods [Estrov et al., 1986]. For lymphocytic leukemia only immunological methods were successful for lowering the detection level [Janossy et al., 1980; Ryan et al., 1984; Van Dongen et al., 1986]. For acute myelocytic leukemia, however, the combination of several antibodies [Griffin et al.,1983] or antibodies and lectins is essential to enable the discrimination of leukemic cells from their normal counterparts [Delwel et al., 1986].

Recently developed detection methods, based on recombinant DNA techniques allow, under defined conditions, a demonstration of the presence of a leukemic cell subpopulation. For example, for the method based on the restriction length polymorphism fragments (RLPF), it has been reported that a leukemic cell frequency in the order of 0.2 % could be detected [Wright et al., 1987; Yoffe et al., 1987; Zehnbauer et al., 1986]. For this technique, gene rearrangements are used as clonal markers.

Another technique, based on DNA sequence amplification with the polymerase chain reaction (PCR), was used to detect residual disease in case of cytogenetically well-described follicular lymphoma. Residual lymphoma cells were detectable at frequencies as low as 1 per 100,000 [Lee et al., 1987]. The procedure is however, based on analyzing DNA extracted from cells, therefore the residual cells are not identified as such. Although the method in itself is very powerful, it will have to be modified to allow in situ detection of the specific

DNA sequences at the cellular level before it can be used for the enumeration of residual cells.

In the study of human leukemia, the possibilities for determining the effect of varying the dose of cytostatic drugs, split-dose treatment, treatment interval variation, as well as the possible influence of the tumor load at the time of treatment, are in general limited. In fact, in patients the only possibility is the recording of the "disease free" survival time. It requires large, randomized clinical studies to get answers to the questions raised above.

In this respect, animal models e.g. the BNML, offer a unique opportunity to perform studies which can never be undertaken in human patients [see also Chapter 1]. In experimental animals there are no restrictions with regard to the collection of material or the size of the sample one wishes to investigate; i.e. each bone or any other organ can be studied in detail at any time, to determine the leukemic cell fraction.

In the BNML model it is possible, by using a MCA in combination with multiparameter flow cytometry, to specifically recognize leukemic cells at frequencies as low as 1 per 10^4- 10^5 normal cells [Martens & Hagenbeek, 1985; Chapters 5 and 6; Hagenbeek & Martens, 1985; Visser et al., 1986]. Other, independent methods are available as well for the determination of leukemic cell numbers [see Chapter 1]. One of these is based on the relation between the number of tumor cells present in the animal and the survival time: a factor of 10 reduction in leukemic cell load results in a 4 day prolongation of the survival time. In this way, the overall tumor reducing capacity of certain treatment schedules can be deduced from the prolongation of the life span.

Various factors influence the response to treatment as measured by the leukemic cell distribution during the MRD phase. A number of assumptions arose from clinical and experimental experience, concerning the response to treatment and the subsequent distribution of leukemic cells within the bone marrow compartment . The assumptions which were investigated in the BNML model are listed below:

-a certain drug dose will result in a certain fraction of leukemic cell kill;
-leukemic cells are randomly distributed over the different bones from the marrow compartment before as well as after chemotherapy;
-the leukemic cells are randomly distributed within the marrow cavity of a particular bone;
-the site of marrow puncture (i.e. the type or size of the bone) which is studied for MRD is of minor importance for the interpretation of the data.

In the first part of the study the leukemic cell distribution in the bone marrow was studied by a survey of "extensive marrow sampling. In this approach a variety of bones from leukemic animals were studied, i.e., including both femora, tibiae, humeri and scapulae, while a random choice was made of 3 or 4 specimens from the costae, vertebrae and sternum sections. Animals were

investigated before as well as after the induction of a remission. For the latter purpose the leukemic rats were treated with cyclophosphamide (100 mg.kg^{-1} i.p.) at the time of full blown leukemia, i.e. at day 13 after the i.v. inoculation of 10^7 BNML cells. At 14 to 16 days after treatment, the animals were sacrificed and bone marrow was collected for leukemic cell frequency determination. This interval was based on previously determined tumor load reduction and re-growth after cyclophosphamide treatment [Schultz et al., 1986b]. It was assumed that at this stage the leukemic cell frequency would again have reached the level required for MCA and flow cytometry analysis (schematically shown in Figure 7-1).

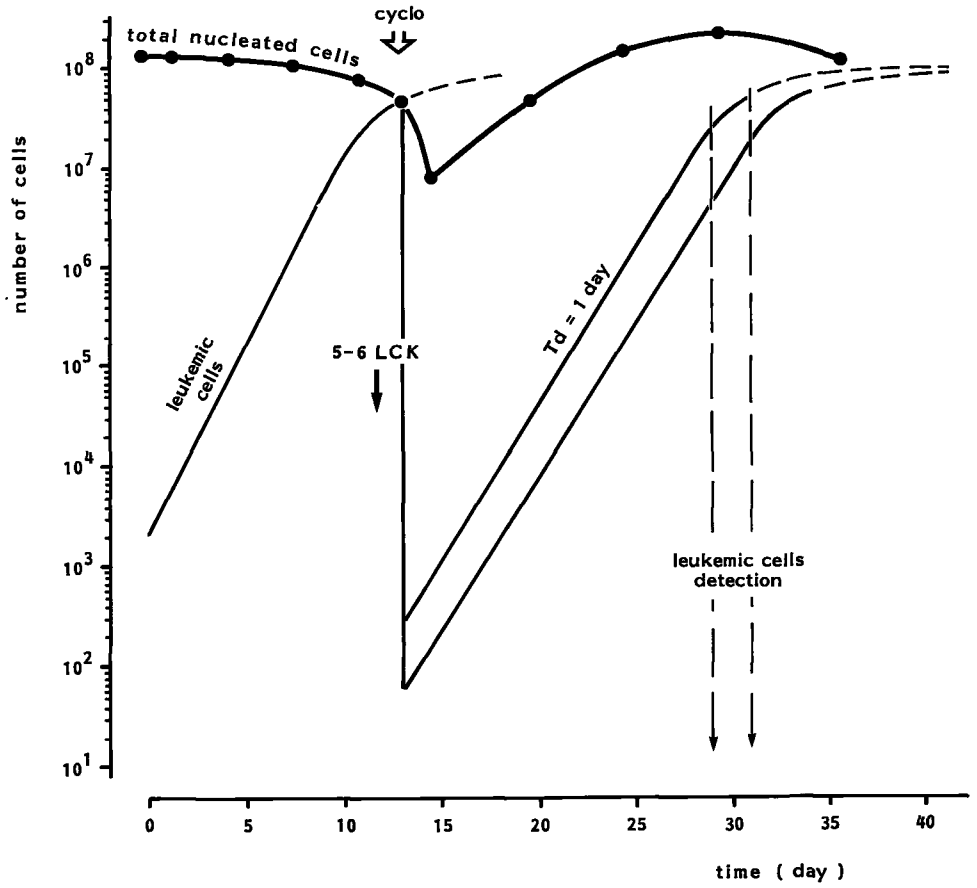

Figure 7-1:
Experimental design for the study of residual leukemia.
The cyclophosphamide treatment on day 13 induces a 5-6 log cell kill. The doubling time of the leukemic cell population after treatment is 1 day.

This initial analysis was designed to determine whether the pattern of distribution of leukemic cells over the various bone marrow compartments during recurrent leukemia is similar to that during leukemia development following the i.v. injection of leukemic cells. This appeared not to be the case. Firstly, the variation in the leukemic cell frequencies between various bones from the same rat was extremely large in recurrent leukemia. Secondly, "small bones" were more frequently discrepant than "large bones", which is to be expected when treatment causes the same fraction of leukemic cell kill in all marrow compartments.

More detailed studies of the difference between recurrent leukemia development and primary leukemia development were then carried out in rats focussing on a larger number of single "small" bones, namely the ribs and on a representative "large" bone i.e. the femur. The ribs were chosen because they provide a large number of bones of equal size and the leukemic cell population in the different ribs can therefore be expected to respond similarly to therapy. To study the migration of cells within the marrow cavity, the complete femoral bone was cut into 15 to 19 sections of 1 mm each using a circular saw [see Chapter 2 for details].

Again, animals were investigated before and after treatment with cyclophosphamide. They were treated at various stages of leukemia development and with different doses of cyclophosphamide. Also the time interval between treatment and sampling during MRD varied. The intervals were chosen using the kinetic scheme depicted in Figure 7-1. In the course of the investigation it seemed that the animals, which were treated earlier in leukemia development, had a higher than expected increase in survival time, which only could be explained by assuming a higher than expected fraction of cell kill. For that reason the relation between the tumor load and the fraction of cell kill was studied more closely by treatment of leukemic animals with varying doses of cyclophosphamide at various stages of leukemia development.

The animals which were to be studied during MRD were treated with cyclophosphamide at day 8, day 11 or day 14 after 10^7BNML cells i.v. The leukemic cell frequency and distribution at these stages of primary leukemia development (i.e. before treatment) was also determined.

The total leukemic cell content of a rat femur is 1×10^8 cells. In theory this means that up to an 8 log leukemic cell kill (LCK) can be measured, provided that it can be demonstrated that no leukemic cells survive the treatment.

Ribs contain on the average 6×10^6 cells each and vary little in size so that the influence of bone compartment size differences can be excluded. In theory, upto a 6-7 log cell kill can be measured in the ribs.

For the interpretation of the rib data it was necessary to determine whether the same growth parameters found for the femoral bone marrow applied to leukemic cell growth in the ribs. The growth parameters for the leukemic cell population in femoral bone marrow, before as well as after treatment with

cyclophosphamide, had already been determined in a previous study by means of a computerized leukemia growth simulation analysis program [Schultz et al., 1986b]. After fitting simulated growth curves to experimental data points, it was found that leukemia growth can be described by an initial phase of exponential growth, later turning into a Gompertz curve.

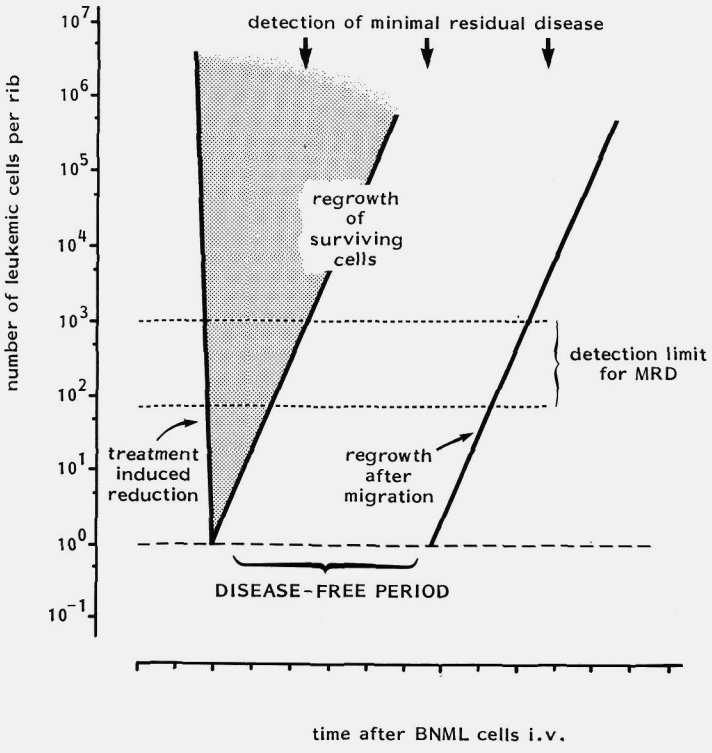

Figure 7-2:
Experimental design for studying the regrowth and/or re-migration of BNML cells in the rib marrow compartment.
When the frequency of the residual leukemic cells, investigated during MRD, is found within the shaded area, the extrapolation backwards to the day of treatment will yield the surviving fraction and will indicate that the leukemia in the rib in question contains "primary relapse growth". If however, extrapolation reaches the "1 cell per rib level" beyond the day of treatment, the "disease-free interval" can be deduced. The leukemic cells which are found in the rib represent a "secondary relapse site". The length of the "disease free interval" in the ribs depends on the degree of tumor load reduction as well as on the initiation of leukemic cell migration.

The leukemic cell frequencies, which were determined during the leukemia recurrence phase, were extrapolated backwards (with the computer simulation program) which is schematically illustrated in Figure 7-2. In this way either the surviving fraction of leukemic cells for each investigated rib bone could be deduced, or in case that a certain marrow compartment was cleared from leukemic cells, the "disease-free period" could be determined.

7.2 RESULTS FROM A SURVEY OF EXTENSIVE MARROW SAMPLING

7.2.1 Variability in leukemic cell frequency before treatment

Four animals were sacrificed, two on day 7 and two on day 10 after injection of 10^7 BNML cells i.v. At that moment the clinical signs of leukemia become noticeable (increase in spleen and liver weights and pronounced infiltration of the bone marrow by the leukemic cells).

Marrow cell suspensions were prepared of the following bones: both femora, tibiae, humeri, scapulae and limited numbers (3 to 4) of the costae, vertebrae and sternum sections. The leukemic cell frequencies in the different samples ranged from 4×10^{-3} to 5×10^{-2} on day 7 and from 7×10^{-2} to 4×10^{-1} on day 10 after leukemic cell transfer. Obviously, the increase from day 7 to day 10 is a result of leukemia growth. The ratio between the highest and lowest measured values of leukemic cell frequencies in the various bones was used as a parameter indicating the range between the two extreme values and is defined as the "maximal ratio value" (MRV).

The MRV determined for all the animals, were in a narrow range: 6.6-11.7 for day 7 and 3.7-4.4 for day 10 (Table 7-1). The leukemic cell frequencies for each of the various individual bones are shown in Figure 7-3 a.

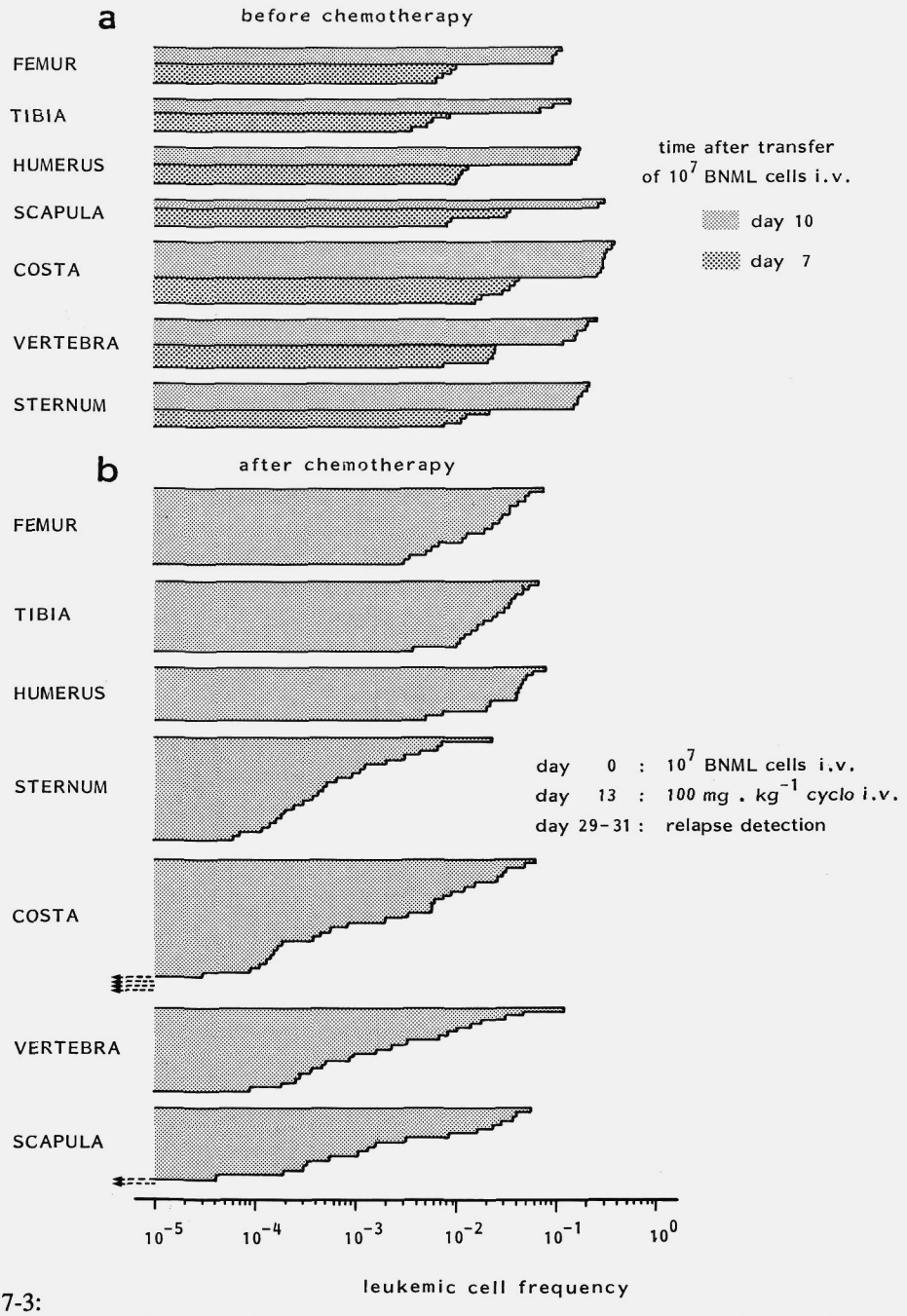

Figure 7-3:
The frequency of leukemic cells in various bone marrow specimens before chemotherapy (Panel a) and during the phase of Minimal Residual Disease (Panel b) thereafter.
The arrows (costa and scapula) indicate that no leukemic cells were detected.

TABLE 7-1

RATIOS OF MEASURED MAXIMAL AND MINIMAL LEUKEMIC CELL FREQUENCIES IN VARIOUS BONES OF LEUKEMIC RATS BEFORE CYCLOPHOSPHAMIDE CHEMOTHERAPY

rat number	day after 10^7 BNML	MRV*
1	7	11.7
2	7	6.6
3	10	3.7
4	10	4.4

*The examined bones were: femur, tibia, humerus, scapula, vertebra, costa and sternum, up to a total of 16-19 bones per rat.
MRV: maximal ratio value (i.e. the highest/lowest frequency ratio)

TABLE 7-2

EFFECTIVENESS OF VARIOUS DOSES OF CYCLOPHOSPHAMIDE IN RELATION TO THE DAY OF TREATMENT (corresponding to different tumor loads)

dose ($mg.kg^{-1}$)	log cell kill after treatment on day 5	8	9	11	13
60	3.6 (1)	2.6 (1)	-	-	3.1 (2)
80	-	-	-	-	3.9 (2)
100	8 (1)	5.0 (1)	5.3 (1)	-	5.4 (10)
120	-	-	-	6.0 (1)	5.8 (3)
140	8 + (1)	9 (1)	-	-	5.4 (1)
160	8 + (1)	9 + (1)	-	8.0 (1)	6.8 (1)

-: not determined
+: a proportion of animals is cured
The tumorload on day 5: 10^8; on day 8: 10^9
(..): number of experiments, each experimental group consisted of 8-10 rats.

7.2.2 Induction of a remission by treatment with cyclophosphamide: dose response curves for the BNML

The injection of 10^7 BNML cells i.v. into normal BN rats will result in a survival time of approximately 21-24 days. The animals die spontaneously with disseminated leukemia and have a greatly enlarged spleen (increase from 0.5 to 3-4 grams) and liver (from 8 to 14-18 grams). Meanwhile the normal bone marrow cells have nearly completely been replaced by leukemic cells. At various stages during leukemia development (corresponding with different tumor loads) dose-response relationships for cyclophosphamide were determined. Animals were treated with doses ranging from 60 to 160 $mg.kg^{-1}$ i.p. on days 5, 8, 9, 11 or 13 after 10^7 leukemic cells. The results of a large number of separate experiments are shown in Table 7-2.

The parameter used to quantify the effectiveness of the treatment was the prolongation of the survival time: as has been established before, an increase of 4 days in the survival time corresponds with a 10-fold overall reduction in tumor load expressed as 1 log cell kill (LCK). For each treated group of animals the median survival time was compared with the median survival time of the non-treated control group. The difference in survival time (in days) was divided by 4 yielding the "log cell kill" (LCK). In this way, the fraction of cell kill induced by a certain cyclophosphamide dose at a given stage of leukemia development was determined. It became clear that treatment of earlier stages of leukemia resulted in a relatively higher fraction of cell kill than observed with the same drug dose given at later stages of leukemia. Hence, a certain treatment is more effective when the tumor load is smaller.

7.2.3 Variability in leukemic cell frequency during the MRD phase

Nine animals were investigated during the supposed phase of minimal residual disease (MRD), which was created as follows. Leukemic animals were treated at day 13-14 with a single injection of cyclophosphamide (100 $mg.kg^{-1}$ i.p.). An increase in the survival time of 20-24 days, corresponding with a 5 to 6 log leukemic cell kill, was expected (Table 7-2). It was speculated that the regrowing leukemic cells would again be detectable (i.e. above the detection limit of 1 leukemic cell per 10,000 normal cells) about 12 days after treatment (schematically illustrated in Figure 7-1). Sampling of the bone marrow was performed a few days later i.e. 16-18 days after treatment, presumably at the time when sufficient leukemic cells would be present for quantitative estimates. For each animal the leukemic cell frequencies in the various bones were measured and the maximal ratio value (MRV) was calculated (Table 7-3). From one rat to another, large differences between the lowest and the highest MRV

were found: the lowest MRV being 36 (rat 7), the highest MRV over 13,000 (rat 4). All other animals were in between these extremes.

All measured values for the various bones of the nine rats are shown in Figure 7-3b. The largest variation was observed in the group of "smaller bones" such as sternum, costae, vertebrae and scapulae. For these bones the highest leukemic cell frequency which was observed was 1.3×10^{-1}, the lowest observed frequency was below the detection limit of 1×10^{-5}.

The high variability between bones in the "extensive marrow sampling" study, led to the assumption that the size of the marrow compartment was playing an essential role with regard to response to treatment and subsequent outgrowth of residual cells. Because the study of all individual bones from each of the animals would be too laborious, it was decided, guided by the data obtained so far, to make an arbitrary distinction between "large" and "small" type bones. Therefore, the second part of the study i.e. "selected marrow site sampling" comprising the femoral bone and the ribs, which were considered as being representative for the "large bone type" and the "small bone type", respectively, were studied in detail.

TABLE 7-3

MAXIMAL RATIO VALUES (MRV) FOR LEUKEMIC CELLS IN VARIOUS BONES DURING THE PHASE OF MINIMAL RESIDUAL DISEASE AFTER CYCLOPHOSPHAMIDE CHEMOTHERAPY

rat number	MRV	
1	160	
2	> 3290	(823)
3	> 9840	(1100)
4	>13000	(4360)
5	> 2500	(281)
6	84	
7	36	
8	340	
9	205	

Cyclophosphamide: 100 mg/kg i.p. at day 13-14 after 10^7 BNML i.v.
From each rat 16-19 bones were investigated: 50,000-300,000 cells per sample were analyzed.
MRV: maximal ratio value (i.e. the highest/lowest frequency ratio)
>: In case that in 1 or more samples in a serie no leukemic cells were found, the calculation of the MRV was performed using the detection limit of 10^{-5} as the lowest frequency.
(..):the calculated ratio using the lowest frequency of leukemic cells which could be accurately measured in the series.

SELECTED MARROW SAMPLING

Leukemic animals were investigated or treated with cyclophosphamide at various time intervals after i.v. inoculation of 10^7 leukemic cells. These cells were derived either from leukemic animals (type A) or after thawing cell suspensions which had been directly stored in liquid nitrogen (type B). It was found that animals which were injected with cells from the liquid nitrogen stock, showed a longer survival time than the animals which were injected with equal numbers of fresh leukemic cells. The cell numbers which were found in the ribs and femora of both groups, i.e. type A and type B both on day 11 and day 14 were compared by fitting computer simulated growth curves to the data points. It appeared that the leukemic growth curve in the animals injected with the "frozen" cells, had shifted 3 days backwards in time compared to the animals which were injected with fresh leukemic cells. Such a shift can only be explained by assuming that the viability of cryopreserved cells was in the order of 15 to 20% of that of fresh cells. The only difference between the two groups is therefore the number of injected "effective" leukemic cells. In order to enable direct comparison of the results obtained with the cryopreserved and the fresh inoculum, the former were converted to values for fresh cells. This conversion yielded that the 11th and 14th day after injection of cryopreserved cells corresponds with the 8th day and the 11th day after injection of fresh cells. Thus all animals have been standardized accordingly for days 8, 11 and 14 after inoculation.

7.2.4 Variability in leukemic cell frequency before treatment.

Animals were investigated on days 8 (n=2), on day 11 (n=4) and on day 14 (n=2) after inoculation with 10^7 BNML cells. Each time, the leukemic cell frequencies were determined in most of the ribs as well as in serial sections of a complete femur. The results are shown in Figure 7-4 (a-d). The corresponding MRV for ribs and femur sections are shown in Table 7-4. They range between 1.2 and 4.0 for ribs and between 1.7 and 6.4 for the femoral marrow sections, which is comparable to the values found in the "extensive marrow sampling" study (Table 7-1).

The growth curve for the leukemic cell population in the ribs was compared with the previously determined and analyzed growth curves for BNML cells in the femoral marrow [Schultz et al., 1986]. A functional relationship between the BNML population size, N, and time, t, was found by fitting growth curves to the data points for rib as well as femoral marrow, using a non-linear least square computer algorithm characterized by an initial exponential growth phase changing into a Gompertz curve. During the exponential phase the leukemic cell population doubling time was identical for the femur as well as for the ribs i.e. 0.78 days. The time point at which the exponential phase changed into a Gompertzian growth curve was almost equal

i.e., at 8.6 days for the femur and at 8.5 days for the ribs, as illustrated in Figure 7-5.

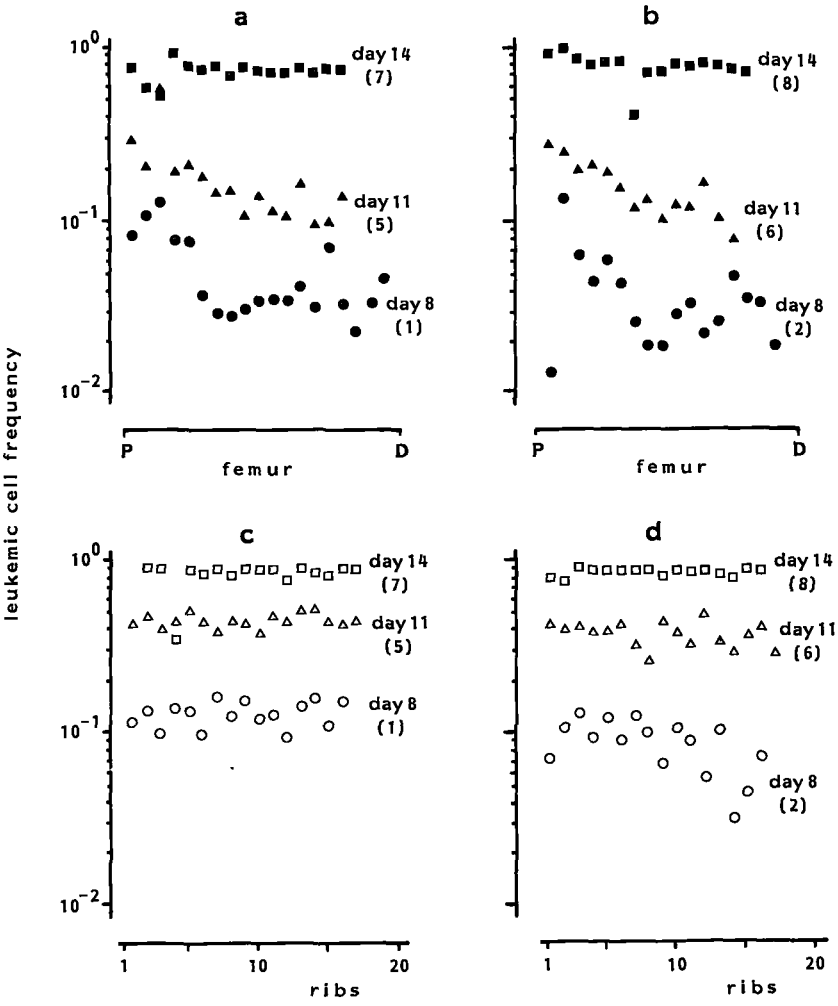

Figure 7-4:
The leukemic cell frequencies in femur and ribs at three different time points during undisturbed leukemia (panels a and b) are shown as well as all ribs from the same individually analyzed rats (panels c and d).
From one femur, between 14 and 19 sections were obtained and analyzed. On the abscissa the femur sections: P corresponds with the proximal end of the femur and D corresponds with the distal end. The rat numbers are in between brackets. Rats 3 and 4 analyzed on day 11, were comparable to rats 5 and 6 and are not shown.
Leukemic cells frequencies found in the rib are plotted randomly.

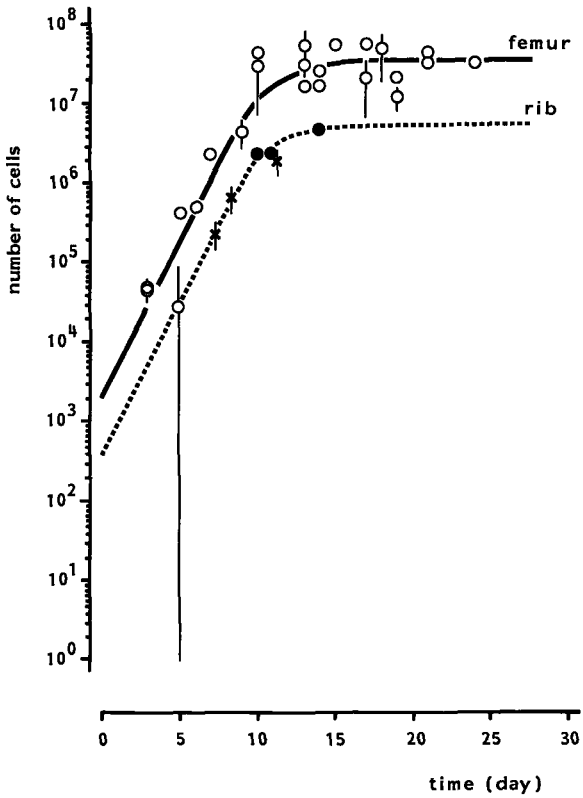

Figure 7-5:
Growth curves of BNML cells in femur and in ribs after i.v. inoculation of 10^7 BNML cells on day 0.
Shown are: -datapoints with standard deviations.
The femur growth curve was derived from pooled data from 5 independent experiments (bio-assay data); The ribs growth curve was obtained by pooling the data of 2 independent experiments (flow cytometry data); Fitted growth curves (least squares method) consist of an exponential phase (doubling times: femur 0.78 days; ribs 0.78 days) which at day 8.6 (femur) and 8.5 (ribs) respectively, changes into Gompertzian growth with a retardation constant of 0.44 d^{-1} (femur) and 0.43 d^{-1} (ribs).

This implies that for extrapolation backwards of the leukemic cell frequencies in ribs, the same parameters can be applied as have been determined for femoral marrow. Therefore the regrowth parameter values for leukemic cells in femoral bone marrow after cyclophosphamide treatment from a previous study [Schultz et al., 1986] were used to perform calculations with the data obtained in rib marrow after therapy.

TABLE 7-4

RATIOS OF MEASURED MAXIMAL AND MINIMAL LEUKEMIC CELL FREQUENCIES IN FEMUR SECTIONS AND IN RIBS BEFORE CHEMOTHERAPY

rat number	ribs sections	femur	day after 10^7 BNML cells iv
1	1.6	5.5	8
2	4.0	7.3	8
3	2.8	4.5	11
4	2.9	6.4	11
5	1.6	5.8	11
6	1.9	3.5	11
7	2.7	1.7	14
8	1.2	2.4	14

A typical observation was that in particular during the early and intermediate stage leukemia (day 8 and day 11), the average frequency of leukemic cells in rib marrow was considerably higher than in femoral marrow (about a factor of 3). The reason for this is as yet unknown, although this might reflect differences in the initial homing of the leukemic cells after the i.v. injection. In this respect it is interesting to note that the curves in Figure 7-5 provide leukemic cell content values for the average rib and for the femur, which differ a factor of 6, while on the basis of equal distribution of the injected cells, a factor of 20 was expected (being the size difference between a rib and the femur).

This difference between the expected and the observed ratios amounts to a factor of three, which is exactly the same as is found for the leukemic cell frequency in the two bones. The surprising agreement between the two values is subject to caution because of the variation in the growth curves. Nevertheless, they seem to provide support for the hypothesis that leukemic cells home roughly three times better to rib marrow than they do to femoral marrow.

7.2.5 Variability in leukemic cell frequency during MRD after treatment of early stage leukemia: Day 8

Four animals were treated at day 8 after 10^7 BNML cells i.v. with 100 $mg.kg^{-1}$ (rat 3, 7 and 9) or 140 $mg.kg^{-1}$ cyclophosphamide (rat: 1). On the basis of the dose response data it can be expected that this will result in a 6 and a 9 log cell kill (LCK) respectively (Table 7-2). At this stage the ribs contain on average 6.7×10^5 leukemic cells, which implies that the treatment should be sufficient to eliminate all leukemic cells. The leukemic cell frequencies in ribs and femoral

marrow sections were determined during the MRD phase i.e. between day 29 and 36 after treatment with cyclophosphamide. The results are shown in Figure 7-6. The MRV found for the animals treated with 100 mg.kg^{-1}, are 7 for rat 7 and 2.4 for rat 9 (Table 7-5). The third animal which received 100 mg.kg^{-1} (rat 3), was apparently studied too early in the MRD stage (i.e. day 29), since in all bone marrow suspensions from the ribs as well as from the femur sections the leukemic cell frequency was below the detection limit (data not shown). However, some leukemic cells must have survived in this animal because the analysis of the remaining femur, which was not cut into slices but flushed in total, yielded a leukemic cell frequency of 0.05%. If indeed these cells originated from a single leukemic focus, it must have contained 5 x 10^4 cells.

The animal which was treated with 140 mg.kg^{-1} (rat 8) on day 8 and analyzed on day 32, showed a pattern comparable to rats 7 and 9, i.e. uniformly distributed leukemia in ribs, with a MRV of 66. The majority of the ribs (15 out of 16) were however in a narrow range with a MRV of 10.

The measured leukemic cell frequencies were extrapolated backwards on the basis of the parameter values derived from the computer simulation analysis. For the day 8 treatment group, it was found that in all investigated ribs the 1 leukemic cell per rib level was reached between 9 and 20 days after treatment (Table 7-6).

TABLE 7-5

MAXIMAL RATIO VALUES (MRV) FOR LEUKEMIC CELL FREQUENCIES IN FEMUR SECTIONS AND IN RIBS DURING THE PHASE OF MINIMAL RESIDUAL DISEASE AFTER CHEMOTHERAPY

rat number	day of treatment	dose of cyclo*	day of sampling	ribs MRV	femur MRV
3	8	100	29	*	*
7	8	100	32	7	7
9	8	100	36	2.4	>30 (28)
8	8	140	32	>320	66
1	11	66	26	38	202
4	11	100	31	>220 (70)	>28,300 (1900)
5	11	140	31	> 84	*
6	11	140	38	35	350
2	14	100	31	40	4
10	14	100	24	80	not determined

From each sample 50,000-300,000 cells were analyzed.
MRV maximal ratio value (i.e. the highest/lowest frequency ratio)
> In case that in 1 or more samples of a series no leukemic cells were found, calculation of the ratio was performed using the detection limit of 10^{-5} as the lowest frequency.
(...) the MRV using the lowest leukemic cell frequency that could accurately be measured in one of the samples of the series.* all samples were below the detection limit

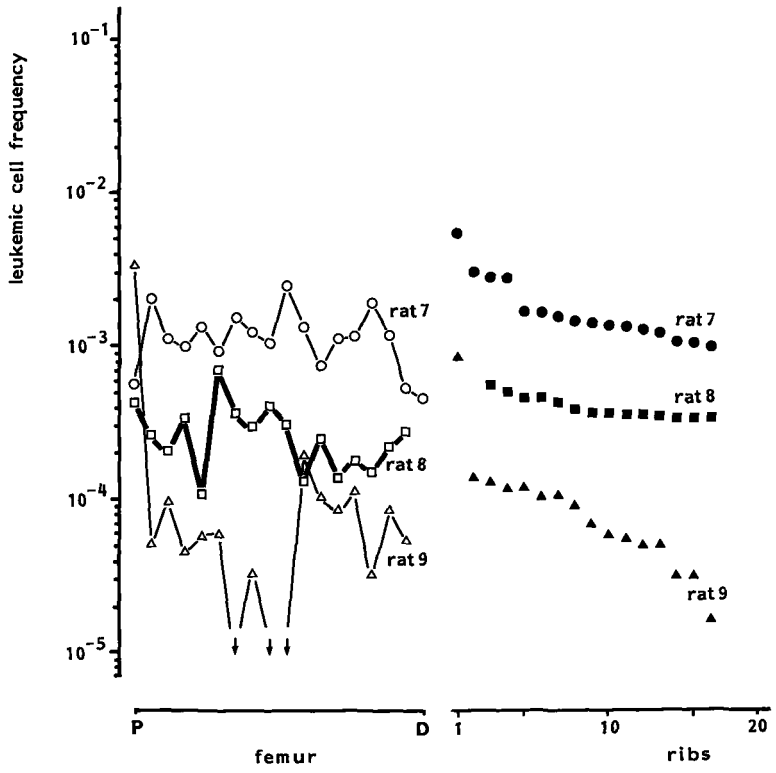

Figure 7-6:
The leukemic cell frequencies in femur and ribs determined during the phase of Minimal Residual Disease (MRD) after remission-induction therapy with cyclophosphamide on day 8 after 10^7 BNML cells i.v.
On the x-axis neighboring femur segments are shown at the left panel: "P" refers to the proximal side and "D" to the distal side of the bone. On the right hand side the rib data are shown: the values are ranked with the highest measured value plotted on the left. On the y-axis the frequency of the leukemic cell population is indicated.
The rat number is indicated between brackets.

In other words, the ribs of these animals were free of disease during some length of time which is in agreement with the assumption that the treatment provided, is more than sufficient to eradicate all leukemic cells which are present in the ribs at that time (see LCK Table 7-2).
The re-growth of leukemic cells in ribs is schematically shown in Figure 7-2.

TABLE 7-6

DISEASE FREE INTERVALS FOR RIBS OF RATS TREATED WITH CYCLOPHOSPHAMIDE AND INVESTIGATED DURING THE STAGE OF MINIMAL RESIDUAL DISEASE

rat number	treatment on day	cyclo dose ($mg.kg^{-1}$)	sampling day	ribs* free of leukemia per total	disease free interval (days)
3	8	100	29	13/13	>16
7	8	100	32	16/16	8.8-11.1
9	8	100	36	14/14	15.8-16.7
8	8	140	32	16/16	14.3-19.5
1	11	66	26	16/18	0.4- 2.0
4	11	100	31	12/12	6.0->12
5	11	140	31	12/12	4->12
6	11	140	38	12/12	16.8-21.9
2	14	100	31	2/13	1.0 & 1.5
10	14	100	31	10/19	0.2-3.4

* Immediately after treatment as derived by backwards extrapolation (Figure 7-5).

7.2.6 Variability in leukemic cell frequency during MRD after treatment of intermediate stage leukemia: Day 11

Four animals were treated with cyclophosphamide at day 11 after 10^7 BNML cells. They received 66 $mg.kg^{-1}$ (rat 1), 100 $mg.kg^{-1}$ (rat 4), or 140 $mg.kg^{-1}$ (rat 5 and 6). The animals were investigated during the MRD phase i.e. on days 26, 31, 31 and 38, respectively. The observed leukemic cell frequencies are shown in Figure 7-7. For rat 1, treated with 66 $mg.kg^{-1}$, the leukemic cell frequencies in 16/18 ribs were found within a narrow range (resulting in a MRV of 4). In the remaining 2/18 ribs high leukemic cell frequency values were found, yielding a MRV of 38 for this animal.

In the femur sections two regions with high frequencies were found, i.e. in the proximal as well as in the distal end of the femur. The area in between contained, homogenously distributed, residual cells at a 100-fold lower frequency: The MRV in this femur was 202.

Two animals, one treated with 100 $mg.kg^{-1}$ (rat 4), the other one with 140 $mg.kg^{-1}$ (rat 5), were investigated at day 31. This was too early, as could be concluded from the fact that the leukemic cell population in practically all ribs was below the detection limit. However, in both animals a few ribs were found to contain leukemic cells, yielding a MRV of 220 (rat 4) and 84 (rat 5) respectively.

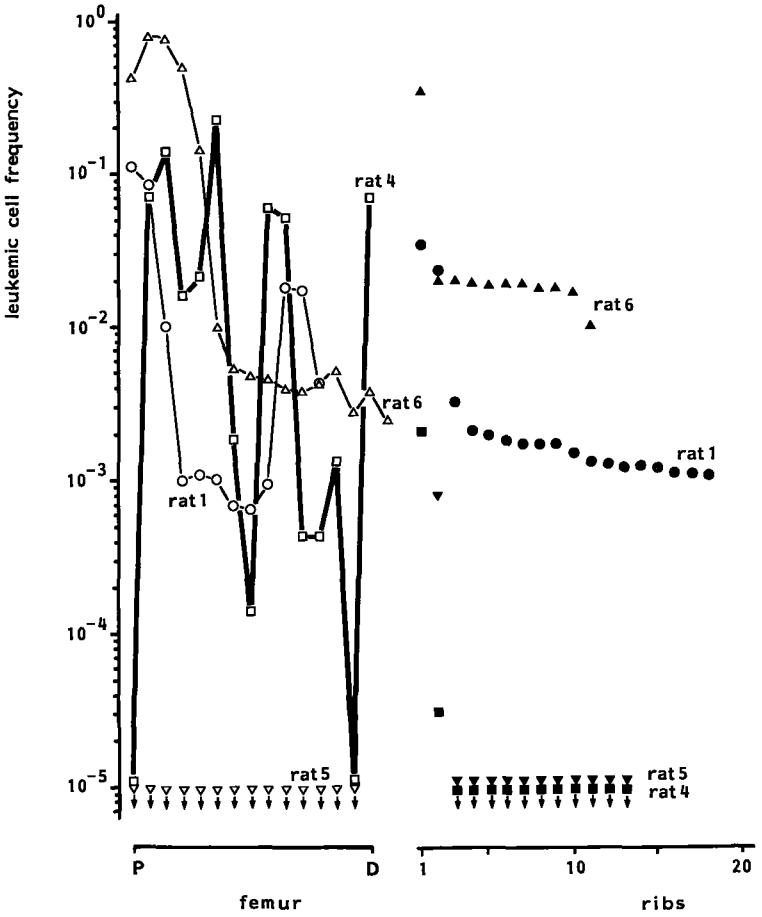

Figure 7-7:
The leukemic cell frequencies in femur and ribs determined during the phase of Minimal Residual Disease (MRD) after remission- induction therapy with cyclophosphamide on day 11 after 10^7 BNML cells i.v.
On the x-axis neighboring femur segments are shown at the left hand panel: "P" refers to the proximal side and "D" to the distal side of the bone. On the right panel the rib data are shown: the values are ranked with the highest measured value plotted on the left. On the y-axis the frequency of the leukemic cell population is indicated. The rat number is in between brackets.

In the femur sections of rat 4, focal regrowth areas could be identified next to areas with much lower values, with a MRV of more than 28,300.
In the femur sections of rat 5 no leukemic cells were found.

Rat 6 received 140 $mg.kg^{-1}$ on day 11 and was investigated at a later stage during MRD, i.e. day 38 (27 days after chemotherapy), which was the reason why leukemic cells were detectable in the rib marrow: 10/11 were within the same range (a MRV of 2.4) but 1/11 contained leukemic cells at a higher

frequency yielding a MRV of 35. The femur sections of this animal showed 1 focal regrowth area at the proximal side yielding a MRV of 350. The rest of the femoral cavity showed a homogeneous, low frequency pattern with a MRV of 3.

Backwards extrapolation of the rib data (from the "day 11 group") yielded a response pattern that was clearly dose related. The ribs from the animal treated with 66 mg.kg^{-1} (rat 1) had been free of disease only for 0-2 days after treatment (Table 7-6). For the animal receiving 100 mg.kg-1 (rat 4) these values were between 6 to >12 days (median >12 days) and for the two animals receiving 140 mg.kg-1 these values were between 7.4 to >12 days (median >12 days) and between 16.8 and 21.9 days, respectively.

7.2.7 Variability in leukemic cell frequency during MRD after treatment of late stage leukemia: Day 14

Only two animals were treated on day 14 and were investigated at day 31, which means only 17 days after cyclophosphamide treatment.

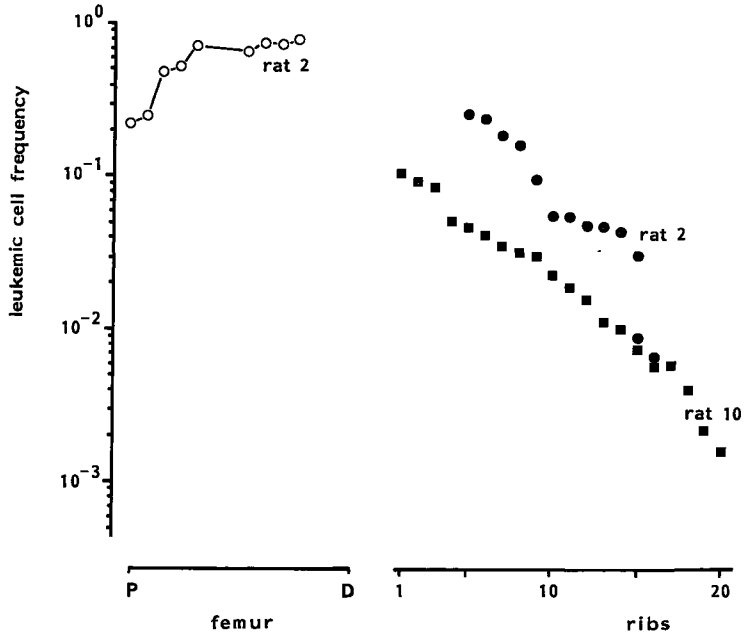

Figure 7-8:
The leukemic cell frequencies in femur and ribs determined during the phase of Minimal Residual Disease (MRD) after remission- induction therapy with cyclophosphamide on day 14 after 10^7 BNML cells i.v.
On the x-axis neighboring femur segments are shown at the left hand panel: "P" refers to the proximal side and "D" to the distal side of the bone. On the right panel the rib data are shown: the values are ranked with the highest measured value plotted on the left. On the y-axis the frequency of the leukemic cell population is indicated.The rat number is indicated between brackets.

The distribution of leukemic cells in the ribs yielded a comparable pattern for both animals (Figure 7-8). For rat 2 the MRV was 40 and for rat 10 it was 80.

The extrapolation data for the ribs from rat 2 indicated that there was no disease free period in 11/13 ribs (Table 7-6). The disease free periods in the 2 remaining ribs of rat number 2 were 1 and 1.5 days respectively.

For rat 10 no disease free period was observed 9/19 ribs. For the 10/19 ribs from rat number 10 the disease free periods ranged from 0.2 to 3.4 days. Only for rat 2, femur sections were investigated, which yielded a rather homogeneous pattern of distribution throughout the marrow with a MRV of 4 (Figure 7-8).

7.3 DISCUSSION

Leukemia is considered to be a rapidly spreading disease characterized by a homogeneous distribution. Therefore, equal leukemic cell frequencies would be expected at all bone marrow sites. Information on the behaviour of leukemic cells shortly after remission-induction is not available. Concerning leukemia regrowth it is generally assumed that leukemic cells spread rapidly, resulting in homogeneous distribution and proliferation over the entire bone marrow. There are only few reports in the literature describing the results of comparative studies of multiple punctures of individual patients. In some studies strongly "positive" bone marrow samples were found among many "negative" results from the same patients [Mathé et al., 1966; Hánn et al., 1977; Jacobs, 1977; Pollock, 1977]. However, classical methods for studying residual disease are not sensitive enough to exclude the presence of leukemic cells in the "negative" samples. It is problematic to develop an optimal strategy for sampling bone marrow for routine screening of patients during the period in which the leukemia is in remission. The current clinical practice is to analyse limited bone marrow samples at certain time intervals in order to identify a leukemia relapse. It is assumed that in relapse the observed leukemic cell frequency is representative for the total marrow compartment.

In the BNML model it was possible to study the influence of varying the stage of leukemia which is treated, the dose of cyclophosphamide, the type of marrow compartment and the time interval at which the residual, regrowing leukemic cell population is investigated. When the rat leukemia is compared with the human disease in order to serve as a model, the following has to be considered. Human leukemia is thought to originate from one malignant precursor cell. After an unknown period of proliferation, leukemic cells will spread through the circulation and home in other parts of the bone marrow as well as outside the bone marrow. This phase is comparable to metastasis formation in solid tumors. It is generally felt that the size of the leukemic cell population is very small when the cells begin to spread. This spreading phase of leukemic cells is mimicked by

the i.v. transfer of BNML cells in to normal rats. Hereafter, the proliferation of the randomly distributed leukemic cells results in a replacement of normal hemopoiesis and in an accumulation of leukemic cells leading to overt leukemia. It is this stage in the rat leukemia which is comparable with advanced disease in human patients. In the rat model remission-induction treatment has usually been studied during this particular stage of disease. If the treatment is effective, a phase of variable duration results, during which there are no signs or symptoms of leukemia. This phase is called "minimal residual disease" (MRD).

A number of general conclusions emerged from the study of MRD in the rat model. First of all it was found that the anti-leukemic effect of treatment with cyclophosphamide depended on the tumor load at the time of treatment. Early leukemia treatment yielded even upto a 2-4 log cell kill difference compared to the same treatment given at a later stage. This is in sharp contrast with the much quoted conclusion of Skipper, that a constant fraction of leukemic cell kill is obtained with fixed doses of chemotherapy [Skipper et al., 1964]. The latter assumption is also based on chemotherapy and radiotherapy studies which were performed in the L1210 mouse leukemia model.

With regard to the regrowth of the residual leukemic cells, the pilot study in the BNML model i.e. the survey of extensive marrow sampling, already indicated that unexpected phenomena occurred. The study was performed based on the assumption that after treatment, the surviving leukemic cells in each of the bone marrow compartments would rapidly redistribute in the animal including in the bone marrow. Therefore, at any given time interval after treatment, similar leukemic cell frequencies in each marrow compartment were expected. However, when the animals were investigated during the phase prior to the imminent relapse, a totally inhomogeneous distribution of the regrowing leukemia cells was observed. The analyses of the many marrow samples obtained from a variety of different bones from the various investigated animals, revealed large differences in leukemic cell frequency, up to a factor of 28,000 fold.

This variation was most pronouncedly found in the group of so-called smaller bones e.g. ribs, vertebrae and scapulae. It was obvious that the measured leukemic cell frequencies in one of the marrow samples does not automatically reflect the situation in the total marrow. Another conclusion was that the leukemic animals which were studied had to be analyzed on an individual basis.

The alternative hypothesis for early spread during MRD to explain the data, is that surviving leukemic cells do not immediately migrate during the MRD phase, but that residual cells show localized regrowth for some period of time. This was called "primary relapse growth". However, at some later stage during the MRD phase cells migrate to other sites, which can be concluded from the fact that in

all marrow sites sooner or later leukemic cells were found. This was called "secondary relapse growth".
Three types of regrowth can thus be found:
-"primary relapse growth", in case that many leukemic cells survived in a certain marrow compartment,
-"secondary relapse growth" in case that no leukemic cells survived and all residual cells were derived from migrated leukemic cells from a relapse site elsewhere, or
-a combination of the two.

All three situations have been encountered in the second part of the study, in which it was attempted to identify a general pattern in the leukemic regrowth process. Animals were treated with varying doses of cyclophosphamide at various stages of leukemia development, i.e. varying tumor load. Because large femoral bones and small rib bones were examined, a large range in tumor load per marrow compartment was covered. The number of investigated animals for each combination is rather limited. However, from each animal numerous numbers of marrow samples (up to 40 per animal) were analyzed. Therefore conclusions could be drawn.

The leukemic cell content in a particular marrow compartment after treatment is a function of the cell load before treatment and the dose of cyclophosphamide used. This directly affected the pattern of leukemia distribution during the MRD phase. Early treatment resulted in complete elimination of leukemic cells in all investigated marrow sites. However, few leukemic cells did survive treatment, because it is known that eventually the animals will die from recurrent leukemia. These few residual cells are forming the foci of "primary relapse growth". These foci proliferate locally until the population has reached a size of about 4×10^5 after which the leukemic cells start to migrate. Obviously, the exact size reached by the leukemic foci in human leukemia patients and the duration of the "disease free interval" are unknown. In the rat this interval was about 20 days, during which the animal had been systemically free of disease. Migration is likely to take place predominantly via the blood circulation. Therefore, the leukemic cell frequencies in ribs as well as in femoral bone marrow in the group of animals treated early in leukemia, showed such a high degree of homogeneity. This is typical of secondary relapse sites.

The complete opposite was observed in animals that received treatment late in leukemia development. Hardly no disease-free period was seen in the rib compartment indicating predominantly primary relapse sites.

The influence of higher leukemic cell loads at the time of treatment on the type of subsequent leukemia relapse is illustrated in animals which were treated at intermediate stage leukemia. As predicted by the tumorload-dose effect relationship, a higher fraction of leukemic cells survived.

In the "small" ribs, leukemia cells are not expected to survive in the animals treated with 100 $mg.kg^{-1}$ or more, in contrast to the femur where few surviving cells were expected. In the rib compartment only secondary relapse sites are observed, while in the femur sections a number of primary relapse sites were found indeed. The marrow cavity in between these leukemic foci was characterized by homogeneously distributed leukemic cells at a 2-3 log lower level. It is postulated that this reflects secondary relapse growth of cells that entered the marrow cavity via the blood circulation and does not reflect intra-medullary migration. In that case, a gradient in leukemic cell frequencies would be expected. Again, it is striking to see that there is a 3-fold difference between rib and femoral marrow with respect to the "homing" of migrating leukemic cells, identical to what is seen after i.v. injection of leukemic cells (i.e. 3 times higher values in the ribs).

The 2-3 log difference between the primary and secondary relapse sites within the marrow cavity, correlates with an 8-12 day period during which primary relapse foci developed and that migration had not yet occurred. Obviously, this implies that at the moment that animals were investigated, within a single animal, primary and secondary relapses are present, depending on the type of treatment in relation to the tumorload, but also on the time interval between treatment and investigation.Therefore, it will remain difficult to decide which type of relapse growth is observed. With respect to the estimation of the total tumor load a similar problem is encountered. If one has detected a secondary relapse site the tumor load will be underestimated but if a primary relapse focus is aspirated, the tumor load will be overestimated. The conclusion of the rat data should be that it is of importance to consider regrowth of leukemic cells during the first phase of MRD as a localized phenomenon and to focus on methods for the detection of these foci.

If the results of the rat apply to the human situation, the following should be considered. Provided that the methods for the detection of residual disease in leukemia patients will indeed be improved in future, residual leukemic cells might be detected at frequencies in the order of 1 per 100,000 to 1,000,000 normal cells. The most important and largest part of the tumor load to be detected, is located at the primary relapse sites. What are then the chances to detect them by using limited marrow sampling? The number of these foci will depend on the effectiveness of the treatment. Currently used therapy for leukemia is rather effective, indicated by the rather long periods of remission which are observed clinically. This may indicate that the number of surviving cells will be limited. Hence the number of primary relapse foci will be limited and the chances to detect them will be small. Especially when smaller marrow compartments are sampled it is most likely that the measured leukemic cells frequency represents a secondary relapse site which is characterized by homogeneous distribution of leukemic cells. The minimal tumorload can than be

calculated. Of more importance for the total tumorload, however, are the primary relapse sites. For that reason one should focus on detection of these. This means that other methods should be developed e.g. radiolabeling of MCA's or development of NMR techniques, for in situ detection of these residual leukemic foci.

CHAPTER 8

GENERAL DISCUSSION

8.1. The normal hemopoietic stem cell of the rat.

Two types of studies in the BNML model were highlighted in this thesis. They concern the fate of the two cell types that are of major importance in leukemia i.e. the normal pluripotent hemopoietic stem cell and the clonogenic leukemia cell.

The normal hemopoietic stem cell is detected in an indirect bio-assay, which is based on the formation of hemopoietic colonies on the surface of the spleen after injection of bone marrow cells into lethally irradiated recipients and is therefore defined as colony-forming-unit-spleen (CFU-S). The rat stem cell can be detected in a rat-to-rat SCA (spleen-colony assay) but alternatively also in a rat-to-mouse SCA [Van Bekkum et al., 1977a]. Routinely the spleen colonies were scored at day 9 identical to the original standard procedure in the mouse-to-mouse system. However, when the rat-to-mouse SCA was studied in more detail [Chapter 3], it appeared that in contrast to the mouse-to-mouse assay, the colony numbers varied on consecutive days i.e. the number of "early" appearing colonies on day 8 was 2.4 times higher than the number of "late" appearing colonies e.g. on day 12. In the rat-to-rat assay, colonies were not observed before day 11 and reached a plateau from day 12 onwards. The colony number was nearly at the same frequency as observed on the mouse spleen in the rat-to-mouse assay during the corresponding period. The study of the spleen colonies in the rat-to-rat assay beyond day 13 to 14 appeared to be difficult to perform on a routine basis: many animals were dying during this period due to aplasia in combination with infections following the total body irradiation (TBI) conditioning.

For mouse hemopoiesis is has been established that the primitive and (pluripotent) stem cells are characterized by "late" colony formation. Therefore, it would be very interesting to relate the day 8 and day 12 CFU-S type (detected in the rat-to-mouse assay) to a CFU-S type in the rat-to-rat assay. The ratio between day 8 and day 12 colonies, derived from normal rat bone marrow and detected in the rat-to-mouse assay, was investigated repeatedly and was found to be 2.4. Different ratios, however, were found in bone marrow after treatment with cyclophosphamide or its metabolites i.e. after in vitro treatment a ratio of day 8 and day 12 colonies of 4.5 was found, while 24 hours after treatment in vivo ratios between 19.3-51.7 were observed. A second example of a changed ratio was derived from assays performed on cell suspensions from the spleen and the bone marrow of leukemic animals during the later stages of leukemia. In the bone marrow no essential change was observed (i.e. a ratio of day 8 and day 12 CFU-S of 2.1) while in the spleen the ratio of day 8 and day 12 CFU-S was increased to 4.5. In the latter situation the stem cells had not been exposed to drugs thereby excluding the possibility that sublethal damage to part of the surviving stem cells could account for the differential appearance of the colonies.

Obviously, the question which type of cell is detected by the rat-to-rat SCA can be approached by using hemopoietic cell suspensions that yield widely different day 8 and day 12 ratios in the rat-to-mouse SCA and measuring the CFU-S numbers in both assays simultaneously. For this purpose two types of experiments could be performed. By using in vitro incubation of bone marrow cells with the stem cell specific growth factor IL3, it is envisaged that the majority of the stem cells will be of the day 8 CFU-S type, in analogy to the experience with mouse bone marrow [Mulder et al., 1986].

The second approach would be to use "pure" stem cells from rat bone marrow. For mouse stem cells it was found that by combining biophysical separation methods e.g. density gradient separation and flow sorting of stem cells after labeling with specific monoclonal antibodies and Rhodamine-123, an enrichment of a factor or 135-fold for day 12 type CFU-S and only 50 fold enrichment for the day 8 CFU-S could be obtained [Visser et al., 1984; Lord et al., 1986]. The ratio between day 8 and day 12 CFU-S of 1 which is found in normal mouse bone marrow is has changed to 3.

Methods for purification of the rat stem cell have been reported, [Goldschneider et al., 1980; Castagnola et al., 1981; McCarthy et al., 1985], however, since these experiment were performed using the rat-to-rat SCA only, they cannot be related to the rat-to-mouse SCA. Electronmicroscopical analysis of 100 % "pure" stem cell fractions indicated that based on morphological criteria, two distinct cell types could be identified [Goldschneider et al., 1980; Castagnola et al., 1981].

8.2 The fate of the hemopoietic stem cell during leukemia growth.

Normal hemopoiesis is seriously affected by the leukemic process. In this context, the assay for normal hemopoietic stem cells of the rat, made it possible to follow their fate during leukemia development. It was observed, that early during the development of leukemia the hemopoietic stem cells decrease rapidly in the marrow while concurrently a continuing increase is observed in the peripheral blood and in the spleen. On the basis of studies by Prins & Van Bekkum [Prins & Van Bekkum, 1981] it was postulated that in the marrow there is competition for space between the leukemic cells and the normal stem cells. The leukemic cells appear to lodge successfully near the endosteal region, close to the so-called "niches" which are thought to be important for the maintenance of the pluripotent nature of the hemopoietic stem cells [Schofield et al., 1985]. Differentiation and maturation of normal bone marrow cells is accompanied by migration from the endosteal region of the marrow towards the central areas. In this way a differentiation gradient is formed [Shackney et al., 1975].

Leukemic blast cell growth is differently in this respect. Once leukemic daughter cells are produced, they do not migrate immediately but stay at the production site. When marrow of leukemic animals is investigated early in leukemia

development, small leukemic foci are observed [Van Bekkum & Prins, 1981]. A typical characteristic of leukemic cells is that they fail to differentiate. It is hypothesized that these two phenomena are associated i.e. a differentiating cell will migrate (schematically illustrated in Figure 8-1) and a non-differentiating cell will not, which would be the reason why leukemic cell accumulate locally (schematically illustrated in Figure 8-2).

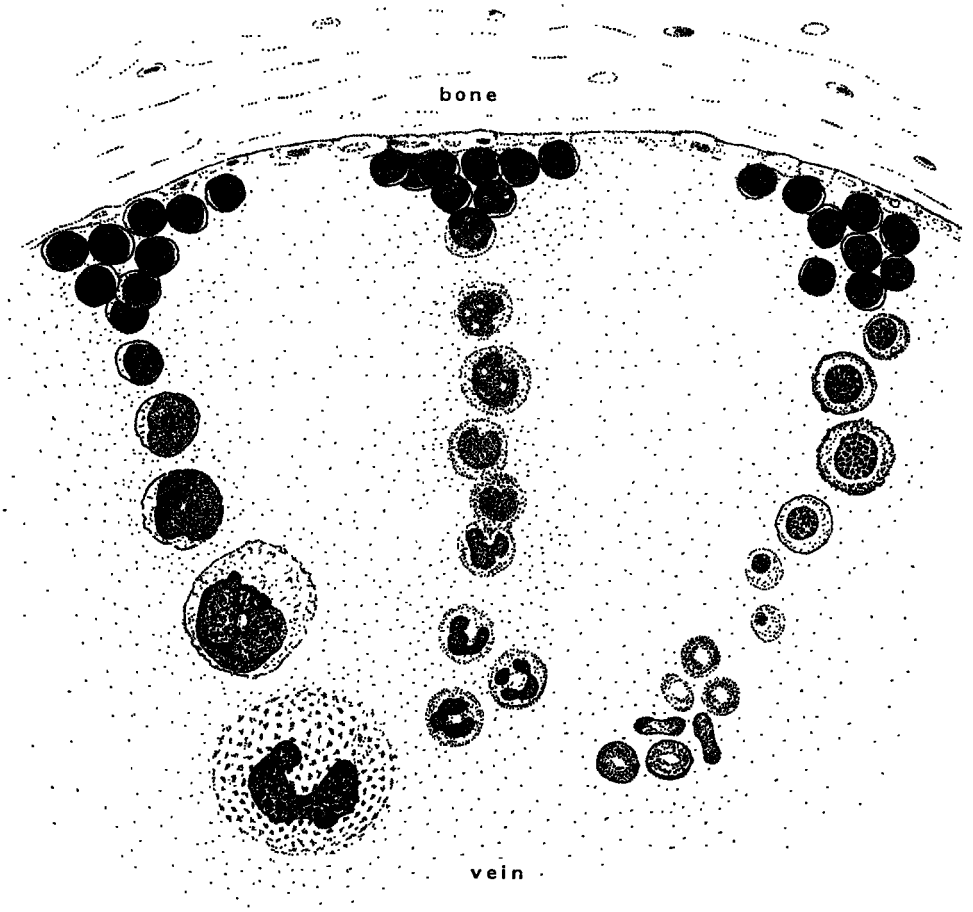

Figure 8-1:
Schematic representation of the differentiation and migration characteristic in normal hemopoiesis
Shown are the stem cells located near the endosteal region and the intermediate and mature forms of differentiating cells of three cell lineages.

By the expansion of the leukemic cell population in the marrow, more and more stem cell niches will be occupied by leukemic cells, so that relatively early during leukemia development, CFU-S numbers in bone marrow are decreasing. At the same time there is a sharp increase in the number of circulating CFU-S in

the peripheral blood [Hagenbeek et al., 1977a], accompanied by an increase of CFU-S in the spleen. Although it cannot totally be excluded that an increase in the splenic CFU-S is a response of splenic CFU-S to inadequate hemopoiesis, the alternative hypothesis is that CFU-S are leaving the marrow and are transported via the blood to the spleen (thereby explaining the sharp increase in CFU-S numbers in the circulation), where extramedullary hemopoiesis develops.

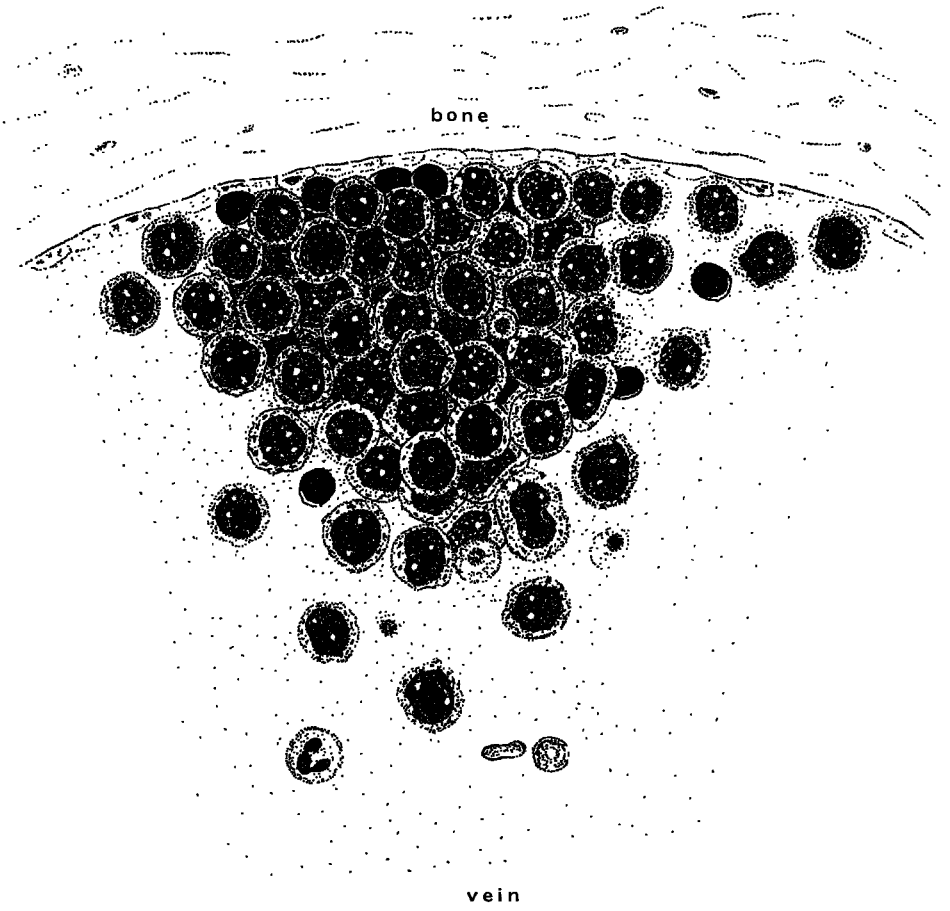

Figure 8-2:
Schematic representation of the absence of migration of the leukemic cells resulting in local development of a leukemic colony.

The kinetics of normal stem cell distribution in marrow and spleen of leukemic animals was studied in detail before, during and after application of chemotherapy [Chapter 4]. A small tumor load reduction (in the order of 50%) by low dose chemotherapy treatment was sufficient to reverse the leukemia-associated decrease in CFU-S in the marrow and reduce the number of CFU-S in the spleen. The open space in the marrow that is created by the drug treatment is apparently occupied rapidly by the normal stem cells. This normalization is, however, only temporarily, because as soon as the leukemic cells have regrown, the stem cells continue their decline.

Larger doses of chemotherapy resulted in a greater reduction of the leukemic cell load, indicated by a prolonged phase of remission during which the marrow CFU-S population recovered completely. The rapid return of CFU-S in the bone marrow to normal values, must be due to proliferation of the relatively few surviving CFU-S in the marrow, because splenectomy directly after treatment did not lead to a different pattern of CFU-S recovery. A significant contribution of CFU-S from the spleen to the regeneration process can be excluded. Again this points at the high potential for self-replication of the surviving CFU-S. This is a characteristic of the least mature stem cells.

Once the leukemia relapsed it appeared to be associated with an identical pattern of CFU-S decrease in the marrow as is observed during undisturbed leukemia growth. It is interesting to note that the increase of CFU-S in the spleen stops immediately when the bone marrow CFU-S reach control values. Thereafter the number of CFU-S in the spleen stays constant during the time that the numbers in the bone marrow are at control levels but the rise in the spleen CFU-S numbers starts again at the moment that the marrow CFU-S numbers drop .

Two explanations can be proposed for the observed changes in CFU-S numbers in marrow and spleen. The first one would be that leukemic cells are competing for space with hemopoietic stem cells (HSC). The "niches", which are supposed to be essential for the maintenance of the resting state of the HSC with selfreplicating capacity [Schofield & Lord, 1983] are being invaded by the leukemic cells. Consequently, the HSC will be located in a microenvironment (towards the central vein) which favours maturation and differentiation with a low degree of progenitor cell expansion and as a result of this, the marrow stem cell population fades out. This leads to insufficient hemopoiesis which activates a feed-back mechanism, that triggers extramedullary hemopoiesis e.g. in the spleen. However, this mechanism fails to account for the fact that blood cells levels are still normal at the time when the spleen CFU-S are already increasing. Furthermore, the proliferation of the low autochthonous number of CFU-S in the spleen, has to be extremely fast. To account for the high values obtained a few days later, this would require a population doubling within 12 hours, without any of the produced CFU-S maturing.

The alternative explanation is that CFU-S in the marrow are displaced by leukemic cells, enter the circulation and are transported to extramedullary sites, e.g. the spleen, where they start to proliferate at a high rate. Stromal structures of the bone marrow are thought to be of crucial importance for the maintenance of the pluripotent nature of the stem cells [Lord, 1986; Gordon et al., 1987]. It is likely that the microenvironment of the spleen does not provide similar conditions as found in the bone marrow; instead the spleen probably promotes proliferation and differentiation rather than selfrenewal.

At only one time point during leukemia development the ratio between day 8 and day 12 CFU-S in the leukemic spleen was investigated [data included in Chapter 3]. The day 8/day 12 CFU-S ratio was 4.5. A high proportion of the CFU-S in the leukemic spleen at this stage (measured in a day 9 SCA) appeared to be in cycle. In the leukemic spleen 60% of the CFU-S were in S-phase versus 40% in normal rat bone marrow [Hagenbeek & Martens, 1981e]. Entering the spleen might be associated with entering cell cycle which means going from a day 12 type CFU-S to a day 8 type CFU-S. This shift from day 12 to day 8 type CFU-S while entering cell cycle is also observed when day 12 CFU-S are incubated in vitro with IL3 [Mulder & Visser, 1987]. Because the self-renewal capacity of day 8 CFU-S is limited [Mulder & Visser, 1987], this may explain why during the terminal stage of leukemia the CFU-S numbers drop and why such low numbers of mature blood cells are produced.

8.3 Detection of minimal residual disease in leukemia.

The kinetics of the leukemic cell population could be studied by means of monoclonal antibody (MCA) labeling in combination with flow cytometry. The MCA-Rm124 that was raised against BNML cells did not recognize a leukemia-specific antigen, but because numerous attempts by other investigators have failed to demonstrate the existence of such antigens this was not totally unexpected. Although the antibody cross-reacts with mature granulocytes it appeared to be very well feasible to specifically recognize the leukemic cell population on the basis of differences in antigen density [Chapter 5]. This MCA could be used for specific killing of the leukemic clonogenic cells, while the hemopoietic stem cells (CFU-S) as well as the committed myeloid precursor cells (CFU-C) were spared.

On the basis of differences in fluorescence intensity, the leukemic cell population could clearly be discriminated from the cross-reacting normal cells using flow cytometry. There was one restriction, however. Due to the fact that the MCA-Rm124 belongs to the IgM-type antibodies, the formation of leukemic cell aggregates may occur in case that high numbers of heavily labeled cells are present, which indeed was observed. In flow cytometry, where cells are measured on an individual basis, this is causing serious difficulties for the exact enumeration of the leukemic cell fraction. However, when the leukemic cell frequency was below about 25 %, this problem did not occur. The MCA was

mostly used for studying the leukemic cell population during the phase of minimal residual disease (MRD) which is characterized by the presence of low leukemic cell numbers. Therefore aggregate formation was not a problem. The leukemic cells could be maximally labeled and were found as a separate cell population with the highest fluorescence intensity [Chapter 6]. The monoclonal antibody was used to study the growth kinetics of the leukemic cell population in various organs during leukemia development.

Before the flow cytometry method could be used for measuring the leukemic cell population, it had to be verified that the MCA reacted with all leukemic cells and not with only a fraction. Leukemic cell frequencies were therefore determined by using bio-assays and were compared with flow cytometry data from the same cell suspensions. Indeed, the data matched completely [Chapter 6]. This implies that flow cytometry offers a reliable method for measuring leukemic cell numbers. It was shown that the method could be used for measuring the leukemic cell production rate in organs e.g. the bone marrow, spleen and the liver.

By using the sorting option of the flow cytometer, the leukemic cell fraction could be purified and subsequently processed for DNA analysis. In this way the cell kinetic parameters of the leukemic, even in case it only represented 1% of the total cell population, could be specifically determined. The limiting factor appeared to be the fact that for reliable DNA histogram analysis at least 5000 cells are needed. This implies that in case that the leukemic cell frequency is in the order of 1 per 100,000 (which is a typical situation of MRD) 5,000 x 100,000, i.e., 5 times 10^8 cells would have to be processed. Flow sorting at a rate of 3000 cells per second implies that about 50 hours would be needed to process 1 sample. One could consider the use of certain methods e.g. density centrifugation for pre-enrichment of the leukemic cell fraction which could offer a gain of a factor 10 or 20. Results of a recently developed method based on immunomagnetic bead separation are encouraging with respect to bulk processing [Lea et al., 1985]. A "negative" selection by using a MCA coupled to the magnetic beads that reacts with a common antigen present on normal cells and absent on the leukemic cells would allow for the enrichment of the leukemic cell population that subsequent can be processed for flow cytometry using a second, "positive" selection MCA. Without an improvement of such kind it will be difficult from a practical point of view to perform the analysis of the many samples one would like to investigate.

The detection of leukemic cells at low frequencies is, however, essential for monitoring leukemic cells during therapy, during the phase of MRD and at imminent relapse. Firstly the lower limits of detection for "rare" cell populations had to be determined. Experiments with the BNML model using the MCA-Rm124 conjugated with FITC indicated that this lower limit is in the order of 1 per 10,000 to 1 per 100,000 [Chapter 7] which is in agreement with other reports using comparable conditions. Again, the large numbers of cells

required, limit the number of analyses which can be performed. For reasons of reliability 10-50 "positive" events have to be scored. Between 100,000 to 5,000,000 cells have to be analyzed which means that for each sample between 1 and 30 minutes analysis time is required.

Next, the question about the relevance of the measured leukemic cell frequency has to be answered. In general it is assumed that for instance one bone marrow aspirate or biopsy will give a reasonable indication on the amount of leukemic cells which are present in the total marrow compartment. In Chapter 6 data are presented which indicate that before anti-leukemic therapy is applied this is a reasonable assumption. However, after therapy the situation has changed completely. Leukemic cell frequencies varied considerably, not only between the animals studied, in spite of the fact that they received identical treatment, but they varied also greatly between the various bones in individual animals. The experiments indicated that the investigation of limited bone marrow samples did not always give a good impression on the leukemic state of the animal. The degree of leukemic cell infiltration at other locations in the marrow cannot simply be deduced. On the other hand, the absence of leukemic cells in a single bone marrow analysis has no predictive value either. Unfortunately it is a frequent experience for clinical hematologists that a leukemia relapse may occur totally unpredicted by previous routine bone marrow examinations. However, the differences in leukemic cell frequency between various marrow sites are not known in the clinical situation. The data in the rat model suggest that these differences may be more than 100,000 fold.

In the rat model no consistent pattern in the distribution of residual leukemia was found. It was found that inhomogeneity in leukemic cell frequency was most pronounced in the smaller bones. A detailed analysis was done in individual leukemic animals before as well as after treatment with chemotherapy [Chapter 7]. Leukemic cell frequencies in the femoral marrow cavity, representing large bones, were compared with data from a large number of ribs from the same animal, representing the small type bones. The animals were treated with cyclophosphamide, a drug which first has to be metabolized in the liver. In tumor biology it is assumed that a certain dose of drug will cause a certain fraction of cell kill [Skipper et al., 1964]. Whether this is true for all stages of leukemia development remains to be established. The results in the BNML studies indicated that following the same dose of cyclophosphamide the tumor load reducing effect was more pronounced with smaller tumor loads [Chapter 7 and Nooter et al., 1985]. Tumor-load induced differences in pharmacokinetics might be responsible for the observed phenomenon.

If one considers an individual rat, however, the assumption that a certain drug dose will result in a certain fraction of cell kill for the various compartments, seems acceptable. In the rat there are large differences in size between the various bone marrow compartments, e.g. up to 100 fold between the femur and the small ribs. Also in man, bones differ largely in size, although

some of the larger bones e.g. the femur and humerus are filled with fat, rather than with marrow. Nevertheless great differences in compartment size are present in man as well. With a constant fraction of cell kill, but with a difference in marrow compartment size, this may lead to the situation that after drug treatment leukemia may be eradicated in small bones but not in the larger bones. In that case it is important to know how soon after regrowth leukemic cells are starting to migrate to other sites. They may do so within the marrow cavity where they are located or via the bloodstream to other marrow sites or to other organs. From the fact that focal regrowth areas packed with leukemic cells were located next to areas where the leukemic cells were even below the detection level [Chapter 7] it could be concluded that the spread of leukemic cells within the marrow cavity of the femur appeared to be very limited. Furthermore, it was found that in other animals leukemia cells were present in a few rib marrow samples, while many other ribs were found to be "negative".

The study of the rib marrow compartment also demonstrated that after effective leukemia treatment, ribs could stay free of disease for periods up to 20 days. This was notably observed in those animals in which the surviving number of leukemic cells was very low (1-100 cells per animal). Assuming the survival and regrowth originating from 1 single cell it can be deduced that the primary relapse from this single cell can produce a total off-spring of 3.5×10^5 cells before the leukemia starts to disseminate. The doubling time of the leukemic cell population after treatment with cyclophosphamide was found to be 0.97 days [Schultz et al., 1985c].

The foregoing results have important implications for the strategy of marrow sampling in patients. The development of leukemia and the subsequent distribution in human patient is compared with the experimental leukemia model (BNML) and schematically illustrated in Figure 8-3. Before treatment, leukemic cells are randomly distributed and limited samples will give reliable information. After therapy the situation becomes more complex.

The rat studies indicate that at those places where leukemia cells have survived treatment they regrow locally until relatively large numbers of cells are present. In this respect leukemia relapse sites resemble the growth of metastasis from solid tumors e.g. lung or mammary carcinomas after removal of the primary tumor. For these types of tumors the current approach is to try to detect the metastasis "in situ" by NMR techniques or by radiolabeling the tumors with specific markers and subsequent "total body scanning". In this way it might also be possible to detect, localize and measure the "primary" leukemia relapse sites provided that sensitive markers e.g. MCA were available. In any case it is advisable to reconsider the strategy for detection of residual disease in the light of the findings in the BNML.

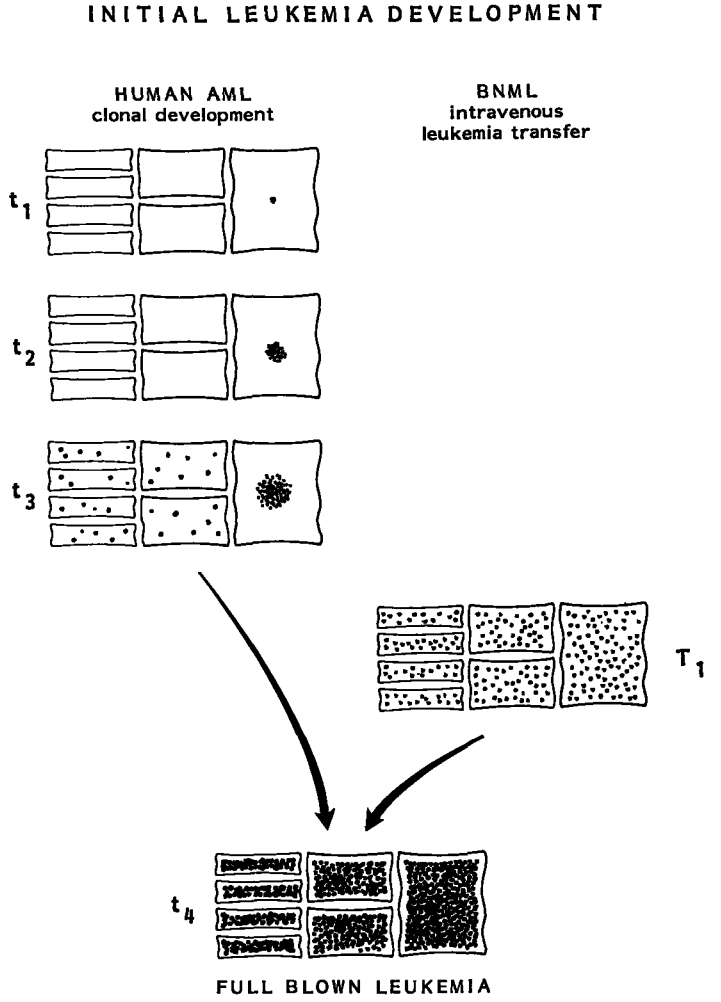

Figure 8-3:
Schematic representation of the subsequent events during development of human acute leukemia and of the BNML model.
Various stages are depicted: i.e.
t1: the appearance of the first leukemic cell. t2: localized leukemia development;
t3: metastasis formation resulting in t4: generalized leukemia growth
T1: indicates the situation after intravenous transfer of BNML cells in the rat leukemia model, which leads to homogenous spread of leukemic cells followed by full blown leukemia.
Stage t4 is comparable for the human and the rat leukemia.

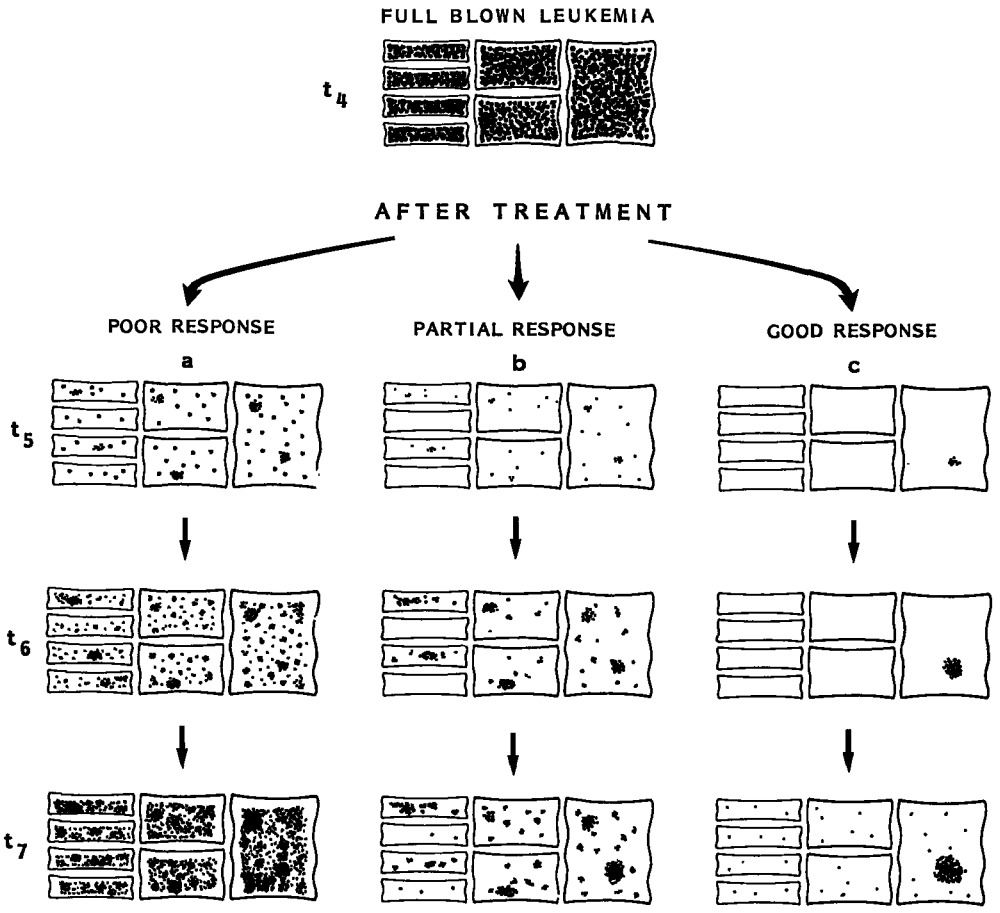

Figure 8-4:
Schematic representation of the subsequent events in the BNML model after treatment with chemotherapy, which is proposed also to occur in human leukemia.

Lane "a" shows the time course in the leukemic cell distribution after a poor response to treatment. Mainly re-growth of surviving leukemic cells, called "primary relapse growth" is observed.

Lane "b" shows the time course after a partial response. Inhomogeneity is observed because primary relapse growth as well as secondary relapse growth, after migration of leukemic cell in marrow compartments originally cleared from leukemic cells, is found simultaneously.

Lane "c" shows the time course after a good response resulting in the survival of limited numbers of leukemic cells which develop into a localized primary leukemic regrowth site. Migration occurs relatively late during the remission phase leading to equal distribution of leukemic cells in most marrow compartments.

If the conclusions from the rat studies apply to the human situation it seems to be important to sample large bones to follow the decline of the leukemia cell numbers during or after therapy. Often the crista is the bone which is preferentially investigated by means of a bone marrow aspiration. The way that the experiments were done in the rat is different. In the rat the "large" bones are flushed and the total marrow content is processed for flow cytometry. Thus, focal areas of regrowth in the rat femur were dispersed throughout the cell suspension. No matter which modern sampling technique may be proposed for the detection of residual cells, they all will face the problem of inhomogeneity in leukemic cell distribution.

As already mentioned, the data which were obtained in the rat suggest that two types of leukemia regrowth can be distinguished: local regrowth of surviving cells at a so-called "primary" relapse site and growth of cells after migration from primary sites, called "secondary" growth (i.e., a systemic relapse). This is schematically shown in Figure 8-4. Following secondary migration, the frequency of leukemic cells was found to be in the same range in the different specimens that were studied. When multiple punctures of remission marrow yield comparable leukemic cell frequencies, they reflect either one of two completely different situations. The first situation represents a poor or partial response to the treatment resulting in a certain survival of leukemic cells in all the marrow compartments. This is schematically shown in Figure 8-4 lane a. This is followed by regrowth of leukemic cells at many different sites.

The second situation is found at a certain stage during the relapse process following a remission of a long duration. Such a remission is seen after a very good response to treatment with only few leukemic cells surviving. Only after local regrowth and subsequent migration, leukemic cells become evenly distributed in the total marrow compartment (see Figure 8-4 lane c) . The latter regrowth is then predominantly of the "secondary" relapse type. The essential difference between these two conditions is the time interval between the treatment and the detection of residual disease.

As a strategy one could consider the following. For a routine analysis during the remission phase, one aspirate should be examined. If leukemic cells are found this may reflect either primary or secondary relapse growth. Additional marrow punctures, preferably from smaller marrow compartments, will indicate which of the two types is found. If the frequencies are all more or less equal to the first one, they probably all represent secondary regrowth. If, however, subsequent samples yield lower values, the first sample was apparently taken from a primary relapse site. If no leukemic cells are found in the first sample, which was taken from a large bone, additional punctures in smaller bones are not advisable, because the chances to detect leukemic cells will be equally large for both types of bones. It even might be that no leukemic cells are present at all. For the latter situation it is of utmost importance to lower the detection level.

When multiple samples are measured and highly variable frequencies are found, this will indicate that primary relapse sites as well as secondary relapse sites were punctured (schematically illustrated in Figure 8-4 lane b).

In summary, it can be concluded that the acute myelocytic leukemia model in the BN rat has contributed considerably to improved understanding of the various aspects of leukemia growth, responses to chemotherapy, application of bone marrow transplantation as therapy and the possibilities and limitations for the detection of residual disease during the remission phase. Obviously, there are restrictions with regard to the extrapolation of the rat data to the human situation. In inbred rats the leukemia is highly reproducible while in patients the leukemia presents a high degree of individual variation. However, some major characteristics are shared and the aim should be to identify the similarities as well as the dissimilarities between human and rat leukemia. In that way progress may be envisaged with respect to reaching the final goal of curing human leukemia.

CHAPTER 9

SUMMARY

Animal model studies contribute considerably to a better understanding of the nature of cancer and to the improvement of the currently used treatment protocols. This certainly is the case for leukemia. One of the animal models, which is frequently used in this respect is the acute myelocytic leukemia model in the Brown Norway rat.

In Chapter 1 an outline is given on the biology of this rat leukemia model, including the various methods which can be used to measure the fate of the two most important cell types in leukemia i.e. the normal hemopoietic stem cell and the clonogenic leukemic cell.

Chapter 1 also contains a review of most of the studies which have been performed by many different research groups in various laboratories with the BNML model. Four fields of interest were identified: the biology of leukemia growth; the interaction with normal hemopoiesis; experimental treatment of leukemia and the detection and treatment of "minimal residual disease". Chapter 1 can be considered as an update of the original description of the BNML model [Hagenbeek & van Bekkum, 1977]

Chapter 2 is a summary of the materials and methods which are relevant for the studies discussed in Chapters 3 to 7.

Chapter 3 is focused at the re-evaluation of the spleen-colony-assay which is used for the studying the pluripotent hemopoietic stem cell of the rat. It was shown that in the rat a heterogeneity within the stem cell population exists, a situation which also was observed in the mouse. Two types of stem cells were identified: one type gave rise to "early" colonies in a rat-to-mouse spleen colony assay (SCA), the second one formed "late" colonies, on day 8 and day 12, respectively. In the rat-to-rat SCA no clear indications emerged to suggest that two different stem cell types are detected in this assay. In normal rat bone marrow there is a constant ratio of 2.4 between the day 8 and the day 12 CFU-S types. This ratio was altered in the spleens of leukemic animals as well as in the bone marrow of rats which were exposed to therapeutic doses of the anti-cancer drug cyclophosphamide. The regrowth kinetics of both types was also found to be different after the cyclophosphamide treatment. In vitro incubation of rat bone marrow with maphosphamide (an in vitro active metabolite of cyclophosphamide), also resulted in a differential kill between the day 8 and the day 12 CFU-S.

In Chapter 4 the results are shown of studies which were focused at the fate of the hemopoietic stem cell in BNML rats during the progression of the disease, during the remission phase after chemotherapy and during the subsequent leukemia relapse.

During the development of leukemia a rapid decrease of the stem cell numbers in the bone marrow was observed. This reflects the invasion of

leukemic cells which home in the marrow close to the endosteal area where they start to grow at the expense of normal stem cells. A small reduction in tumor load of about 50%, induced by a low dose of cyclophosphamide was adequate to induce a rapid, although temporary recovery of stem cells. The use of therapeutic doses of cyclophosphamide resulted in a steep drop in leukemic as well as normal stem cell numbers. Splenectomy directly after treatment indicated that the very low number of surviving stem cells in the marrow (less than 1% of controls) were responsible for the rapid recovery of the stem cells to their original numbers: the stem cells that are present in the spleen after treatment do not seem to play an important role in this process. The typical rise in stem cell numbers in the leukemic spleen is not observed until the stem cell numbers in the marrow start to drop as a result of recurrent disease.

In Chapter 5 the characteristics of a monoclonal antibody (MCA) which was raised against the BNML cells are shown. As indicated by flow cytometry measurements of labeled cells, the MCA-Rm124 recognizes an antigen which is expressed on a fraction of the mature granulocytes as well as on leukemic cells but not on lymphocytes, blast cells or erythroid cells. Using a cytotoxicity test is was found that the majority of the leukemic cells could be killed, confirming the flow cytometry data, but that the hemopoietic stem cells (CFU-S) were not killed. Apparently stem cells do not express the antigen which is recognized by the MCA. Flow sorting experiments, based on fluorescence labeling intensity, indicated that the committed granulocytic precursor cell (CFU-C) also did not express the antigen.

In Chapter 6 it is illustrated how the MCA labeling in combination with flow cytometry, could be applied for studying specifically the leukemic cell population. The leukemic cell population could be discriminated from the normal cells on the basis of an increased antigen density, which is reflected in a higher fluorescence intensity compared to that of the normal cells. It was shown that the data which were derived from flow cytometry analysis corresponded with the data obtained from bio-assays. The growth kinetics of the leukemic subpopulation was followed in various organs e.g. bone marrow, spleen and liver. Doubling times for the leukemic cell population in liver and bone marrow could be determined and appeared to be clearly different, probably indicating that different growth controlling factors are operative for different organs.

It was also illustrated that by sorting the leukemic subpopulation and subsequently processing the cells for DNA analysis, cell kinetics of the leukemic cells could be determined even when they represented only 1% of the total population. It was envisaged that when much lower cell numbers (less than 1 per 10,000) are present e.g. in the MRD situation, the limiting factor would be the amount of cells which is needed to perform reliable DNA histogram analysis, which is in the order of 5,000 cells. Upto about 10^9 cells would have to be

processed which would require large bone marrow samples as well as unacceptable long sorting times, with the currently used methods.

In Chapter 7 the distribution of leukemic cells in the bone marrow of BNML rats before and after the application of chemotherapy is discussed. The studies were done using the monoclonal antibody Rm124 in combination with flow cytometry. Bone marrow from different types of bones were analyzed from each animal. The maximal ratio value (MRV, which is the ratio between the highest and the lowest measured leukemic cell frequencies) was used as a parameter for measuring the homogeneity in leukemic cell distribution. Before the animals received chemotherapy the MRV in various bones ranged from 3.7 to 11.7. During the minimal residual disease phase, however, these values were found to vary considerably i.e. from 36 upto a factor of 13,000 from one rat to another. Although in animal models it is common practice to pool data from animals which received identical treatment, it was evident that in this type of studies animals should be analyzed on an individual basis. Furthermore it was found that the variability in the larger bones e.g. femur, humerus and tibia was much less than observed for the smaller bones e.g. sternum, ribs and shoulder-blades. Therefore, it was decided to take the size of the marrow compartment into account.

The frequency of leukemic cells in ribs, representing small bones, was studied in detail in comparison to the frequencies observed in the marrow cavity of femora, representing large bones. In the first part of the study the larger bones were flushed in total. Unequal distribution of cells within the marrow cavity would therefore be unnoticed and an average value would be measured. Therefore, femoral bones were cut into many slices which were processed for flow cytometry, separately. The topographical distribution of leukemia in the marrow cavity could thus be studied. The variability was found to be extremely high, reaching differences of upto 28,000. Areas with very low values i.e. below the detection level were found next to areas with an almost 100 % leukemic cell frequency. This indicated that the reliability of diagnoses based upon analysis of single bone marrow aspirates is highly questionable.

Another interesting finding in the BNML studies was the fact that a fixed dose of cytostatic drug did not result in a constant fraction of cell kill. With increasing tumor load the treatment became less effective. This is in contradiction with a frequently proposed assumption that a constant relation does exist, and which has served as a basis for many animal model chemotherapy studies.

The leukemic cell survival in selected large and small bones from individual animals was determined during the MRD phase with flow cytometry followed by computer-aided extrapolation backwards. This yielded either the fraction of surviving cells, or the time in between the treatment and the entrance of the first

opinion, leukemia relapse was characterized by local regrowth while migration to other sites occured with a considerable time-delay. This was most pronounced when treatment was highly effective and resulted in a long lasting remission. Some similarities between leukemia growth and solid tumor growth were suggested. In this respect scanning methods for detecting the local, primary leukemia relapses, are proposed as alternatives for the bone marrow aspiration method.

In Chapter 8 the results of the studies in the BNML leukemia model are discussed in their mutual coherence. The relevance of the preclinical animal model studies for developing and exploring new methods is discussed in view of the clinical need to investigate leukemia during the state of minimal residual disease.

The final conclusion of the rat studies is that, although extrapolation to the human situation remains difficult, animal studies are important tools for the development of new strategies for the investigation and treatment of human leukemia.

Samenvatting

Studies in diermodellen leveren een aanzienlijke bijdrage aan het verbeteren van het inzicht in de verschillende aspecten van kanker. Dit is met name het geval bij leukemie. Een van de diermodellen die voor de bestudering van leukemie wordt gebruikt is het acute myeloïde leukemiemodel in the Brown Norway rat (BNML).

Hoofdstuk 1 bevat een overzicht van de voornaamste eigenschappen van de BNML, inclusief de verschillende methoden die gebruikt worden om tijdens de ontwikkeling van de leukemie, de twee belangrijkste celtypen te vervolgen. Dit betreft de normale hemopoietische stamcel en de clonogene leukemiecel. Bovendien is in dit hoofdstuk een overzicht gegeven van de studies die in de afgelopen jaren in een groot aantal onderzoeksinstituten, in Europa zowel als in Amerika, zijn uitgevoerd met de BNML als model voor humane leukemie.

Vier verschillende velden van onderzoek werden onderscheiden:

1. de biologie van leukemie groei,
2. de interactie tussen leukemie en de normale hemopoiese,
3. de experimentele behandeling van leukemie en
4. de detectie en behandeling van zogenaamde "minimal residual disease", d.w.z. de situatie waarin slechts een minimaal aantal leukemiecellen in het lichaam aanwezig is.

In hoofdstuk 2 werd een overzicht gegeven van de verschillende gebruikte methoden.

Hoofdstuk 3 bevat de resultaten van studies die gericht waren op een her-evaluatie van de "milt-kolonie-techniek" die gebruikt wordt voor de bepaling van de hemopoietische stamcel van de rat. Deze kan worden gedetecteerd d.m.v. een milt-kolonie-techniek in zowel lethaal bestraalde ratten als muizen. Twee verschillende typen werden onderscheiden. De ene stamcel vormde kolonies op een "vroeg" tijdstip in de rat-naar-muis kolonietechniek, de andere stamcel vormde kolonies op een later tijdstip, respectievelijk op dag 8 en dag 12, uitgedrukt als "colony forming unit spleen" (CFU-S). Indien de rat-naar-rat kolonietechniek werd gebruikt, waren er echter geen aanwijzingen voor het bestaan van deze twee te onderscheiden typen CFU-S.

In normaal rattebeenmerg werd een constante verhouding van 2.4 gevonden tussen het aantal dag 8 en dag 12 CFU-S. Deze verhouding is veranderd in de milt van leukemische ratten zowel als in het beenmerg van ratten na behandeling met therapeutische doses van het anti-kanker middel cyclophosphamide. De teruggroeikinetiek van beide CFU-S typen bleek verschillend te zijn na de cyclophosphamide behandeling. De in vitro incubatie van rattebeenmerg met maphosphamide (een in vitro actieve cyclophosphamide

metaboliet) resulteerde eveneens in een verschil in overleving tussen de dag 8 en de dag 12 CFU-S. De conclusie van de studie was, dat de hemopoietische stamcelpopulatie van de rat heterogeen is, zoals dat ook het geval is voor de stamcelpopulatie in de muis.

In hoofdstuk 4 werden de resultaten besproken van de studies die gericht waren op het vervolgen van de hemopoietische stamcellen tijdens de ontwikkeling van leukemie, tijdens de remissiefase na chemotherapie en tijdens het daarop volgende leukemierecidief. Tijdens de ontwikkeling van leukemie trad er een snelle daling op van het aantal stamcellen in het beenmerg. Dit werd veroorzaakt door infiltratie van leukemiecellen die zich voornamelijk bleken te nestelen op die plaatsen in het beenmerg waar de normale stamcellen zich bevinden. De leukemiegroei ging ten koste van de normale stamcellen. Wanneer een kleine reductie in de tumormassa van circa 50 % werd geïnduceerd door middel van behandeling met een kleine hoeveelheid cyclophosphamide, trad er een snel, alhoewel zeer tijdelijk, herstel op van normale stamcellen in het beenmerg. Toediening van therapeutische (dus hogere) doses cyclophosphamide resulteerde in een sterke afname van zowel leukemiecellen als normale stamcellen, in zowel het beenmerg als in de milt. Indien direct daarna de milt operatief werd verwijderd, bleek dit geen ander patroon van teruggroei van de normale stamcellen in het beenmerg te zien te geven, vergeleken met dieren waarbij de milt niet werd verwijderd. De stamcellen die na therapie overblijven in de milt, zijn dus niet van belang tijdens deze herstelfase. Het lage aantal stamcellen in het beenmerg (1 % van de controlewaarde) is dus voldoende voor een volledig herstel van de beenmerg stamcelpopulatie na de behandeling. De typische toename van het aantal stamcellen in de milt tijdens de ontwikkeling van de leukemie trad eveneens op tijdens de periode na therapie, echter niet voordat de stamcel aantallen in het beenmerg weer daalden onder invloed van de infiltratie door teruggroeiende leukemiecellen aldaar.

In hoofdstuk 5 werden de eigenschappen besproken van een monoclonaal antilichaam (MCA), dat ontwikkeld is tegen BNML cellen. Flowcytometrie experimenten leerden dat het MCA-Rm124 een antigeen herkent dat zowel op normale cellen als op leukemiecellen aanwezig is. Op erythrocyten, blasten en lymfocyten was dit antigeen niet detecteerbaar. Cytotoxiciteitsproeven gaven aan dat leukemiecellen effectief geëlimineerd konden worden m.b.v. dit MCA, waarbij de normale stamcellen gespaard bleven. Dit bevestigde dus de flowcytometrie resultaten. Stamcellen bleken het antigeen niet tot expressie te brengen. Flowcytometrie experimenten, waarbij de cellen werden gesorteerd op basis van antigeen-bindings intensiteit, gaven aan dat ook op de voorlopercellen van de myeloïde reeks (die in vitro kolonies vormt, de zogenaamde CFU-C) eveneens niet met het MCA reageerden. Leukemiecellen konden dus onderscheiden worden van normale cellen op basis van antigeen-dichtheid.

In hoofdstuk 6 werd aangetoond hoe het MCA-Rm124 in combinatie met flow cytometrie gebruikt kon worden om de leukemiecelpopulatie te bestuderen. Leukemiecellen konden onderscheiden worden van normale cellen op basis van verschillen in de mate waarin het MCA aan beide celtypen bindt. De gegevens zoals die verkregen werden met flowcytometrie bleken overeen te komen met resultaten zoals die werden verkregen met proefdierexperimenten. Op deze alternatieve wijze bleek het mogelijk om de leukemiecelgroei te vervolgen in verschillende organen, zoals beenmerg, milt en lever. De verdubbelingstijden van de leukemiecelpopulatie in lever en beenmerg werden bepaald en bleken duidelijk verschillend te zijn. Vermoedelijk duidt dit erop, dat op orgaannivau verschillende factoren het leukemisch groeiproces beinvloeden.

Eveneens werd aangetoond, dat het mogelijk is om van een leukemische subpopulatie die circa 1% uitmaakt van het totaal, een DNA analyse uit te voeren. Hiertoe worden cellen geïncubeerd met het MCA-Rm124. De cellen die het sterkst met het MCA reageren (dit zijn dus de leukemiecellen) werden m.b.v. de flowcytometer uitgesorteerd, waarna een standaard-procedure voor DNA-analyse kon worden uitgevoerd. De limiterende factor bleek het aantal cellen te zijn, d.w.z. 5000 cellen zijn nodig voor een betrouwbare analyse van het DNA-histogram. Omdat tijdens de fase van "minimal residual disease" (MRD) de leukemiecelfrequentie nog veel lager is, moet een veelvoud van cellen, in de orde van 10^9 (1 miljard), geanalyseerd worden. Hierdoor is de benodigde analyse- en sorteertijd, met de huidige typen flowcytometers, onacceptabel lang. Bovendien wordt de hoeveelheid cellen die het beenmergmonster moet bevatten een tweede beperking.

In hoofdstuk 7 werd de verdeling van leukemiecellen in het beenmerg compartiment besproken, zoals die gevonden wordt vóór en ná de toediening van therapie. De studies werden uitgevoerd m.b.v. het MCA-Rm124 in combinatie met flowcytometrie. Beenmerg van verschillende typen botten van een en dezelfde leukemische rat werd geanalyseerd. De parameter die gehanteerd werd om de resultaten van de verschillende dieren te kunnen vergelijken was de ratio tussen de hoogste en de laagste gevonden waarde voor de leukemiecelfrequentie in de geanalyseerde botten. Deze zogenaamde maximale ratiowaarde (MRW) varieerde tussen 3.7 en 11.7 voordat chemotherapie was toegediend. Gedurende de MRD fase echter varieerden deze waarden aanzienlijk meer, d.w.z. tussen de verschillende onderzochte ratten werden MRW getallen gevonden tussen 36 en 13.000. Bij dierexperimenteel onderzoek is het gebruikelijk om de gegevens van vergelijkbaar behandelde proefdieren te middelen teneinde een variatie voor de waarneming te kunnen bepalen. Het was duidelijk dat dit in dit geval uitgesloten was en dat de resultaten op individuele basis geanalyseerd moesten worden.

Bovendien bleek, dat de variatie in het beenmerg van de grotere botten, zoals het dijbeen, opperarmbeen en het scheenbeen aanzienlijk minder was dan in het beenmerg van de kleinere botten zoals het borstbeen, schouderblad, ruggewervels en de ribben. Om deze reden werd in het vervolg van de studie

rekening gehouden met de afmetingen van de te onderzoeken botten. De leukemiecelfrequentie werd nader onderzocht in de ribben, als vertegenwoordiger van de "kleine" botten, t.o.v. de waarden die gevonden werden in het beenmerg van het dijbeen, als vertegenwoordiger van de "grote" botten. In het eerste deel van de studie werd het beenmerg in zijn geheel uit het de dijbenen verzameld door deze leeg te spuiten. Echter een eventuele ongelijke verdeling binnen het bot zou op deze manier niet worden opgemerkt. Daarom werd elk dijbeenbot opgedeeld in een groot aantal plakjes elk van circa 1/20 deel van het totale bot. Op deze manier kon de verdeling van leukemiecellen binnen een botcompartiment worden bestudeerd. De variatie bleek onzettend hoog te kunnen zijn. Verschillen tot een factor 28.000 werden gevonden. Gebieden met extreem lage waarden d.w.z. zelfs beneden de detectiegrens van 1 per 10.000 tot 100.000 bevonden zich vlak naast gebieden die vrijwel uitsluitend met leukemiecellen gevuld waren. Dit betekent dus, dat de betrouwbaarheid van een diagnose die gebaseerd is op slechts een enkele beenmergpunctie, uiterst beperkt is.

Een andere opmerkelijke bevinding in de BNML studies was het feit dat met een bepaalde dosis chemotherapie (in dit geval cyclophosphamide) niet een constante fractie leukemiecellen werd gedood. Wanneer de hoeveelheid leukemiecellen toenam, bleek het effect van een bepaalde behandeling onevenredig af te nemen. Dit is in tegenspraak met een veelverkondigde stelling, die gedurende vele jaren de basis heeft gevormd voor diermodel-chemotherapiestudies.

De leukemieceloverleving in de geselecteerde botten (de ribben en de dijbeensecties) na chemotherapie kon worden bepaald door de gevonden waarden voor de leukemiecelfrequentie m.b.v. een computerprogramma voor de simulatie van leukemiegroei, te extrapoleren naar het tijdstip van behandeling. Op deze wijze kon de overlevende fractie leukemiecellen worden bepaald. Hieruit kon de effectiviteit van de behandeling berekend worden. In een aantal gevallen bleek dat na de behandeling geen enkele leukemiecel overgebleven was in het onderzochte beenmergcompartiment. Tijdens een latere fase volgde een uitzaaiing van leukemiecellen vanuit de zogenaamde "primaire teruggroei-haarden". In het algemeen wordt verondersteld dat leukemiecellen snel overgaan tot het vormen van uitzaaiingen. Dit zou dan leiden tot een snelle algemene verspreiding van de ziekte. In het BNML model bleek tijdens deze studie, dat leukemie teruggroei zich in eerste instantie voornamelijk lokaal afspeelde. De uitzaaiing naar andere locaties van het beenmerg bleek pas plaats te vinden na verloop van tijd. Dit was met name goed waarneembaar in het geval dat de behandeling zeer effectief was. Op slechts enkele plaatsen vond teruggroei plaats. Op het moment dat eenmaal uitzaaiing ging plaatsvinden, raakten de leukemiecellen zeer gelijkmatig verdeeld in het beenmergcompartiment. Dit werd "secundaire teruggroei" genoemd. Het is problematisch om onderscheid te maken tussen de "primaire" en de "secundaire" teruggroei van leukemiecellen. Het verlagen van de detectiegrens is van het uiterste belang voor het opsporen

van de secundaire teruggroei. Voor de detectie van de primaire haarden van teruggroei moet gezocht worden naar alternatieve methoden, aangezien het aantal plaatsen waar deze zich afspeelt, zeer beperkt is. In dit opzicht werden enige overeenkomsten geconstateerd met het proces van metastasering bij solide tumoren. Overwogen zou kunnen worden om voor de detectie van de primaire haarden van teruggroei bij leukemie, eveneens "scanning" methoden te ontwikkelen, zoals dit gebeurt voor de detectie van solide tumoren.

De extrapolatie van het diermodel naar de klinische situatie blijft moeilijk. De resultaten in het BNML-model tonen aan, dat diermodelstudies belangrijke instrumenten zijn voor het ontwikkelen van nieuwe strategiën voor onderzoek en behandeling van humane leukemie, met name tijdens de fase van MRD.

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Anton Martens, 27 september 1988.

Curriculum Vitae

The author of this thesis was born in Gilze-Rijen on August 21, 1952. He completed his secondary education (MULO-B) in 1968. Thereafter he was trained as a technician (clinical chemistry) at the Brabantse Medische Analisten School in Breda. This was completed in 1971. After a short period at the general laboratory of the R.K. Hospital in Dordrecht, he was appointed as a technician at the Radiobiological Institute TNO in Rijswijk. Since then he was involved in a large number of projects in the field of experimental leukemia research, which were financially supported by the "Koningin Wilhelmina Fonds" of the Dutch National Cancer League. Under the supervision of Prof. dr. D.W. van Bekkum (director of the institute) and Dr. A. Hagenbeek he developed to become a scientist in the field of experimental hematology. He frequently presented lectures during international scientific meetings and he also published a number of articles in international journals. The results of studies which were conducted at the Radiobiological Institute TNO during recent years are presented in this thesis.