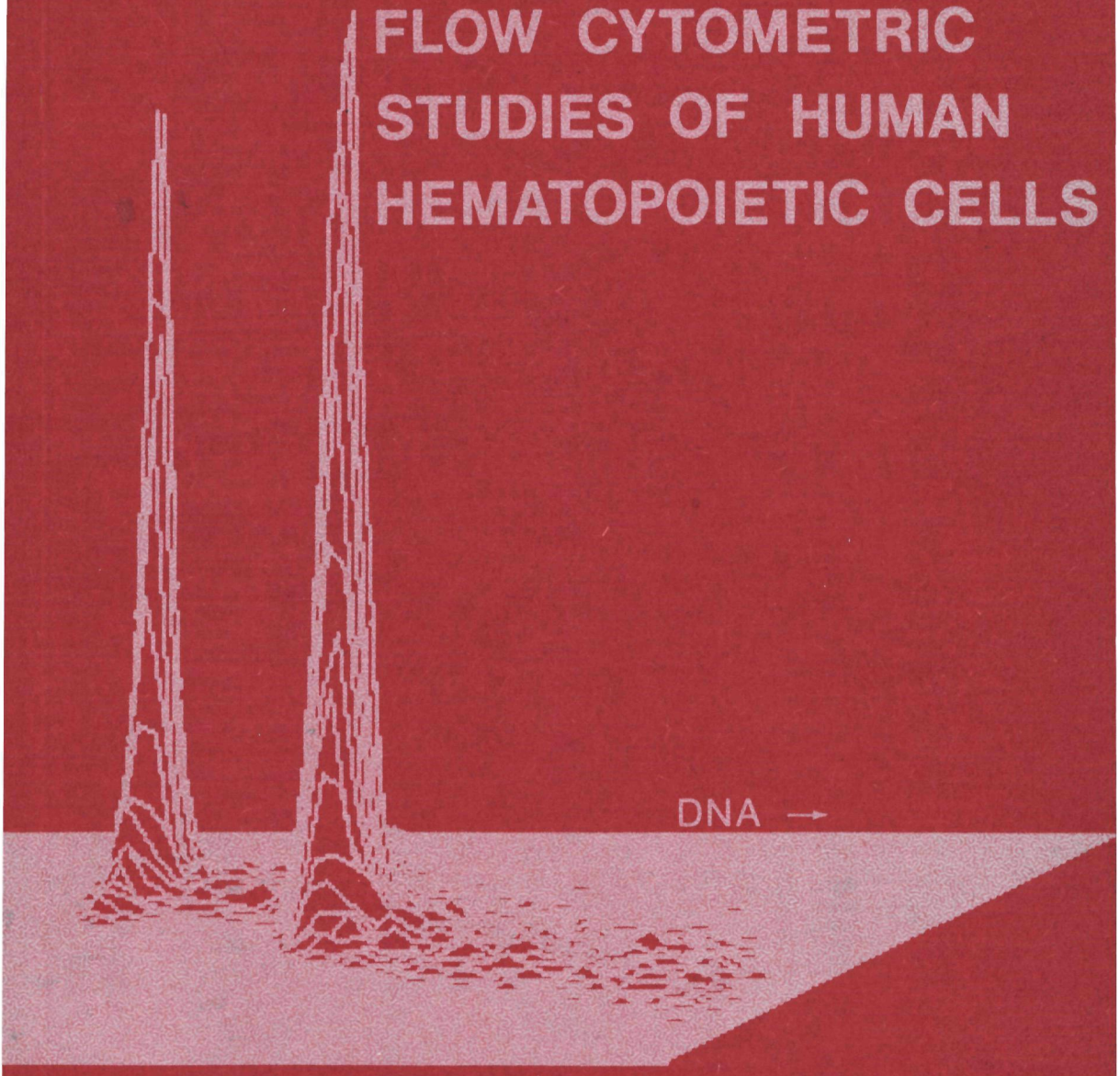
FLOW CYTOMETRIC STUDIES OF HUMAN HEMATOPOIETIC CELLS



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PROEFSCHRIFT

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Prof. Dr. P. G. A. B. Wijdeveld
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door

ROBERT SIMON GEMMA HOLDRINET
geboren te Oosterhout (NB)

KRIPS REPRO MEPPEL

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To Patricia,

Kai, Olaf, Muriel and Flora

To my parents

CONTENTS

CHAPTER I

INTRODUCTION AND OBJECTIVES OF THE STUDY

Flow cytometry is a new technology which allows rapid quantitative evaluation of cell numbers, cell properties or cell constituents by electrical or optical signals, which are generated and sensed while cells in an aqueous suspension pass a sensing region.

Flow cytometry started in 1956 with the development of a cell counting instrument by Coulter (1). In 1956 Kamentsky et al (1) introduced an improved flow system which enabled the simultaneous analysis of electrical and optical signals. van Dilla et al (3) and Göhde et al (4) independently developed flow systems using various flow chambers and improved optical and electronical devices. Later on Fulwyler adapted a flow system with droplet generation and a deflection method as introduced by Sweet (5,6) to permit cell sorting. Bonner et al (7) introduced a sensor which enabled cell sorting based on fluorescence. In the last ten years interest in flow cytometry has gradually increased,which is reflected in further instrumentational development and a growing number of biomedical applications. The developments and achievements in flow cytometry are collected in the proceedings of the four international symposia on flow cytometry (8,9,10,11) and recently reviewed extensively by Melamed and Mullaney (12).

Flow cytometry was introduced in clinical research by Göhde, Schumann, Büchner, Andreeff and Barlogie in Germany, by Haanen, Wessels and Hillen in the Netherlands and by Darzynkiewicz and Kamentsky in the U.S.A. (13). Initially, flow cytometry was limited to the measurement of the relative DNA-content of individual cells (DNA-flow cytometry); a preference which is understandable by the fundamental importance of DNA and by the availability of reproducible DNA-staining methods. DNA-flow cytometry was mainly employed to cytokinetic studies in various tumors, and its potential for widespread application was supposed to exist especially in the field of cancer research and medical oncology (13,14).

The clinical application of DNA-flow cytometry in Nijmegen started in 1972 and was applied to the study of proliferation kinetics in patients with acute leukemia. The effort to apply DNA-flow cytometry to leukemia was inspired by new knowledge about cell kinetics in leukemia obtained by ^{3}H-thymidine autoradiography (15). With this technique it was found that leukemic blast cells have a varying, but generally prolonged generation time (16,17,18,19,20). The disappointing results in the chemotherapeutic treatment of acute leukemia were attributed to the presence of a large number of slowly proliferating or resting cells,which are difficult to eradicate with cytostatic drugs. However,

it was expected that, with properly timed drug scheduling causing cellrecruitment or cellsynchronization, the cytotoxic effects were to be enhanced (21,22). For this reason cellkinetic monitoring became essential, for which ^{3}H-thymidine autoradiography was unpractical because this method is too laborious and time consuming. Consequently, DNA-flow cytometry as a rapid and reliable method for cell cycle analysis was considered to be the obvious way.

The results of cell kinetic studies in acute leukemia performed with DNA-flow cytometry by Hillen et al (23) were consistent with previous studies, accomplished with ^{3}H-thymidine autoradiography. Moreover Hillen et al were the first to demonstrate with DNA-flow cytometry the cytokinetic effects on leukemic cells of prednisone, cytosine arabinoside, adriamycine and vincristine (24). They demonstrated also the prognostic potential of the pretreatment S-phase compartment in patients with acute myeloblastic leukemia to attain complete remission (25). However, dispite these encouraging results, clinical application of DNA-flow cytometry was hampered by the inadequacy of one-parameter measurements,which will make no distinction between various cell types or maturation levels in heterogeneous bone marrow samples. Moreover, one became aware of a source of error caused by contamination of bone marrow aspirates with nucleated cells from the circulating blood.

The objective of this thesis was to extend the applicability of flow cytometry in clinical hematology by

1) determination of the clinical significance of DNA-content analysis in leukemic bone marrow,
2) by addressing the problems connected with the heterogeneity of cells in bone marrow and blood samples.

Basicly, this problem was approached in three ways:

a) by the development of a procedure to quantify the amount of peripheral nucleated cells in bone marrow aspirates,
b) by the application of counterflow centrifugation (elutriation) to attain enrichment of cell classes, prior to flow cytometric analysis,
c) by the application of multiparameter flow cytometry to analyze cells simultaneously on different biological properties to enable discrimination between various cell classes.

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CHAPTER II

THE CELL CYCLE AND KINETICS OF HEMATOPOIESIS

During the interval between two successive mitoses (called the interphase), a series of cell metabolic states can be distinguished which represent various phases of the cell cycle (1,2,3).

After mitosis it takes some time before DNA-replication starts. This gap-period is called the *G_1-phase*. The period during which the genetic material deoxyribonucleic acid (DNA) is replicated is called the *S-phase*. This period is followed by a premitotic resting or gap period, the *G_2-phase*. The G_2-phase ends at mitosis, the *M-phase* (figure 1). It has been recognized that during the G_1-phase, RNA-synthesis increases before DNA replication starts. Based on differences in RNA content therefore, early G_1-cells can be distinguished from late G_1 cells (4,5).

It appears that the S-phase in most mammalian cells takes about 6-8 hours. The duration of the G_2-phase is about 1-2 hours, whereas mitosis takes place in 30-70 minutes. The G_1-phase is the most variable period of the cell cycle

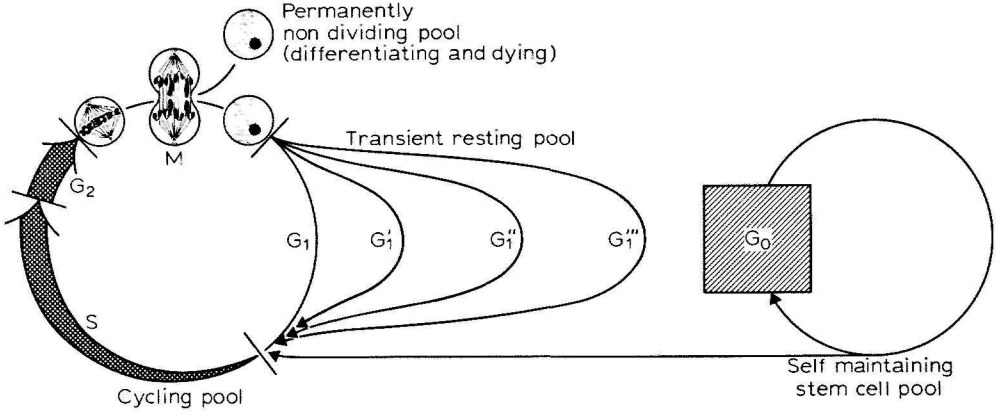

Figure 1. Cell populations and the phases of the cell cycle.

and may vary from a few hours to several months depending on the type of animal or tissue from which the cell originates.

KINETICS OF HEMATOPOIESIS

With regard to its kinetics, bone marrow consists of a mixture of three different cell populations (1,2,3). The first cell population consists of cycling cells; i.e. cells that are continuously dividing and traversing the cell cycle (progenitor cells). The second cell population contains cells that leave the cell cycle after a certain number of divisions, in order to differentiate and to die (myeloid, erythroid and lymphoid cells). The third cell population contains cells that leave the cell cycle temporarily and remain in a dormant state until environmental conditions stimulate their reentry into the cell cycle (hematopoietic stem cells). The dormant state of these cells is commonly designated as the G_0-phase of the cell cycle (figure 1).

Hematopoietic tissue is one of the most rapidly proliferating cell compartments in the body and consists mainly of proliferating cells that are differentiating to permanent non-dividing cells. The selfmaintaining capacity of the hematopoietic system is confined to a morphological unrecognizable population of stem cells and progenitor cells (less than 0.1 % of bone marrow cells).

Like all tissues, the generative activity of bone marrow depends on the cell cycle time (the time interval between two successive mitoses), the fraction of cycling cells (the growth fraction) and the rate of cell loss (the fraction of cells that die or migrate to other tissues). The number of cells produced per unit of time depends on the cell cycle time and the growthfraction, whereas the rate of cell loss determines the number of cells that leaves or die per unit of time. In normal steady state conditions the number of cells produced per unit of time is equal to the number that is lost.

CELL CYCLE ANALYSIS

The fraction of cells in S-phase can be determined with ^{3}H-thymidine autoradiography, ^{3}H-thymidine uptake or with flow cytometry (1,6).

^{3}H-thymidine is incorporated into DNA during the S-phase. The percentage of cells taking up the label after a given exposure can be determined by autoradiography and expressed as the *labeling index* (LI) which corresponds with

with the percentage of cells in S-phase.

After flash labeling with ^{3}H-thymidine the cohort of cells in S-phase which is labeled, may be followed as it enters mitosis and the time course of labeled mitosis can subsequently be recorded. Theoretically such *percent labeled mitosis* curves (PLM-curves) permit measurement of the time durations of the various cell-cycle phases. However, in actual practice, biological variation damp these PLM-curves and render such measurements less precise.

The continuous labeling method permits determination of the growth-fraction, whereas duration of the cell-cycle and the S-phase can also be determined by this method.
The number of cell divisions as a function of time after application of ^{3}H-thymidine can be derived from the decreases of the mean grain grount, whereas under certain conditions, the *mean grain number* per nucleus gives information of the DNA-synthesis rate per cell.

Double labeling provides another method to estimate the duration of the S-phase. In this method two isotopic labels, e.g. ^{3}H-thymidine and ^{14}C-thymidine are injected with a known short interval of about 1-2 hours. Cells labeled with ^{14}C alone can be distinguished from those labeled with ^{3}H, and allow the computation of S-phase and cell-cycle duration.

^{3}H-thymidine autoradiography has been applied succesfully in numerous cellkinetic studies and has added greatly to the knowledge of cell growth. However, autoradiographic methods are time-consuming and their applicability to studies in vivo is limited.

Automated methods for studying cell kinetics, which are less time-consuming, have become available. The uptake of ^{3}H-thymidine may be assessed rapidly by *scintillation counting*, measuring the desintegrations per minute of incorporated labeled thymidine.
Unfortunately thymidine availability and uptake show marked variations in individual cells so that comparison of total scintillator counts may not reflect the proliferative rate.

A new technology concerns *DNA-flow cytometry* of which the application in clinical hematology forms the subject of this thesis. As indicated in chapter I, DNA-flow cytometry is an objective, reliable and rapid method to determine the relative number of cells in the various cell-cycle phases. However, up to now estimation of absolute phase durations or growth fraction was not possible with this method, whereas the heterogeneity of the cell-suspensions has limited its clinical application thus far.

CELL-KINETIC DATA OF NORMAL BONE MARROW IN MAN

Estimates of the labeling index (LI) for granulocyte precursors have ranged from 30-75 % (mean 43 %) for myeloblasts, and about 20 % for myelocytes, implying a rapid rate of myeloid cell proliferation (7,8). Cell cycle time and phase duration for granulocyte precursors are poorly defined. An estimate of S-phase duration by double labeling gave a value of 5-6 hours (9), but this is probably an underestimate, due to artifacts in the method. Percent labeled mitosis (PLM-) curves have been derived for granulocyte precursors in only two patients with normal bone marrow (10). Computed estimates of median values give a S-phase duration of about 12-13 hours and a cell-cycle time of 16-25 hours. However, the shape of the PLM-curves did not allow accurate measurements of the S-phase duration.

Erythroid precursors also have a rapid rate of proliferation. Estimates of the LI range from 30-75 % with higher values for earlier cells in the maturation sequence (11,12,13). The S-phase duration is about 13 hours while the median values for the cell-cycle time are in the range of 16-26 hours (10).

Little is known of the kinetics of other precursor cells in human bone marrow such as these from monocytes, lymphocytes, plasma cells and megakaryocytes.

CELL-KINETIC DATA OF BONE MARROW IN PATIENTS WITH ACUTE LEUKEMIA

The mean LI of myeloblasts in patients with acute myelogenous leukemia ranges in the bone marrow from 5 to 11 %, and in the blood from 3 to 9 % (8,14,15,16). Similar values are obtained for lymphoblasts in bone marrow and blood in patients with acute lymphoblastic leukemia (17). Mean values of S-phase duration and cell-cycle durations for leukemic blast cells are about 20 to 60 hours respectively and are longer than the estimates for granulocyte and erythroid precursors in normal bone marrow.

Accumulation of cells in leukemia is supposed not to be caused by increased proliferation, but by a maturation block leading to a decreased rate of cell death and a delayed removal from the hematopoietic compartment.

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CHAPTER III

PRINCIPLES OF FLOW CYTOMETRY

DNA-flow cytometry was performed with the Impulscytophotometer, ICP-11 (Phywé, Göttingen, West-Germany). Two parameter flow cytometry was accomplished with an ICP-11, modified for two color analysis.

The ICP-11 consists of a detector unit and electronic components (figure 1). The detector unit accomodates an illuminating system, including a high-pressure mercury lamp, a measuring chamber, a photomultiplier tube with pre-amplifier, and a pump to drive the cell suspension and the rinsing fluid. The electronic components consist of power supplies, a multichannel analyzer with display facilities, an X-Y recorder and an oscilloscope adapted with a data storage paper-tape punch unit.

The actual measuring procedure is as follows: the stained cell suspension is sucked through a (100 µm wide) capillary. The exit of this capillary, constituting the measuring field of the instrument, is adjusted to the focal

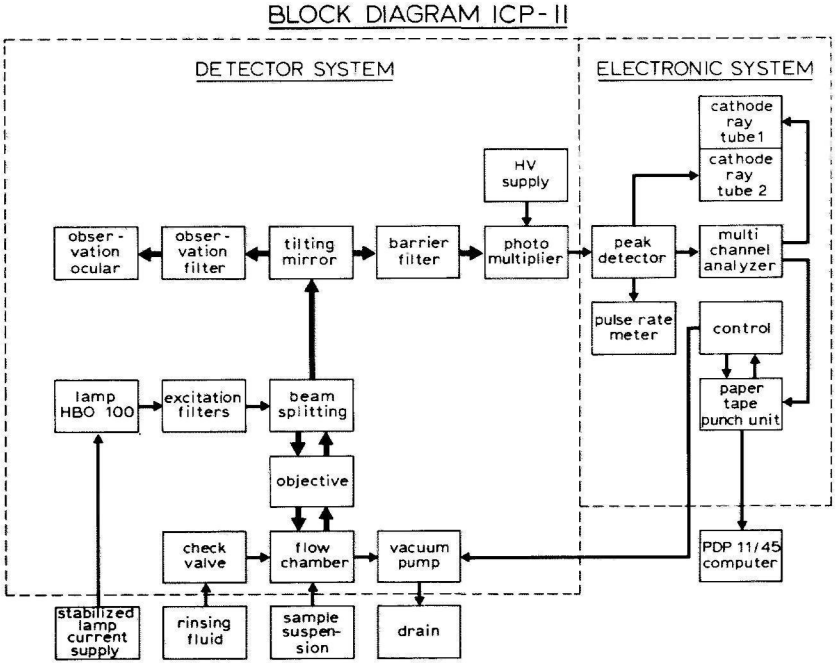

Figure 1. Block diagram of ICP-11

plane of an objective. The measuring field is homogeneously illuminated (Köhlers' principle) by incident excitation light. The wave length of the excitation light can be adapted to particular fluorochromes by different excitation filters (figure 2). Every stained cell passing the measuring field, emits a fluorescent light-pulse. The height of each pulse is proportional to the amount of the dye present, which reflects the quantity of the substance to be measured. The emission light passes a dichroic mirror and is reflected to a photomultiplier or to an observation device. The fluorescent light is converted by the photomultiplier to an electrical signal, which is proportional to the intensity of the optical pulse. Interference filters in front of the photomultiplier are adaptable to desired optimal wave length. The electric signals are analyzed for amplitude and stored in a multichannel pulse-height analyzer. The analyzer delivers a frequency-distribution of the fluorescent light-emission per cell in the measured cell suspension. The results of a measurement are automatically recorded in a histogram and are typed out by a paper-tape punch unit. The histogram is read out and analyzed by a PDP 11/45 computer. Hard copies are provided by a Gould 5005 unit.

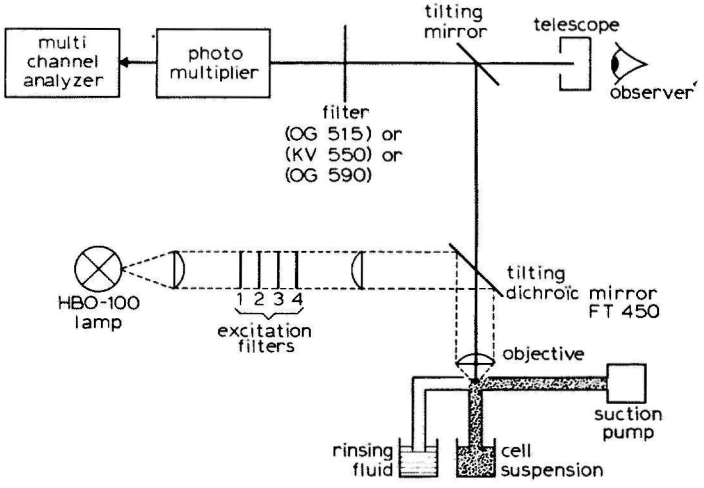

Figure 2. Schematic drawing of the optical arrangement of the ICP-11

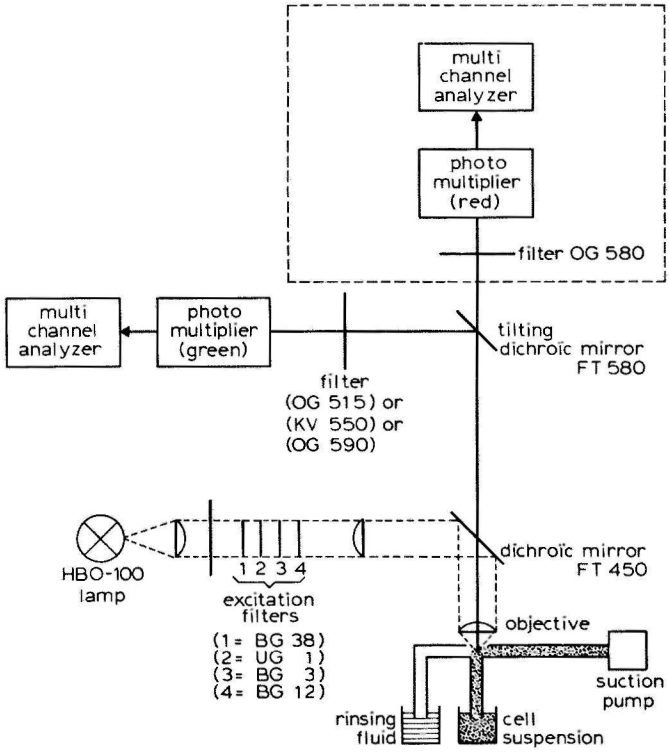

Figure 3. Schematic drawing of the optical arrangement of the ICP-11 modified for two color analysis.

In the ICP-11 modified for two parameter analysis a second photomultiplier is installed (figure 3). By combination of specific dichroic mirrors and interference filters one of the photomultipliers converts for example green light, and the additional photomultiplier red light. After digital conversion, signals are stored in a multichannel analyzer (Nuclear Data-100) according to their relative magnitude. The digitalized signals are stored on a magnetic tape and processed through a PDP 11/45 computer. Hard copies of the histograms are provided by a Gould 5005-unit.

THE DNA-HISTOGRAM

A DNA-histogram of an asynchronously growing cellpopulation is shown in

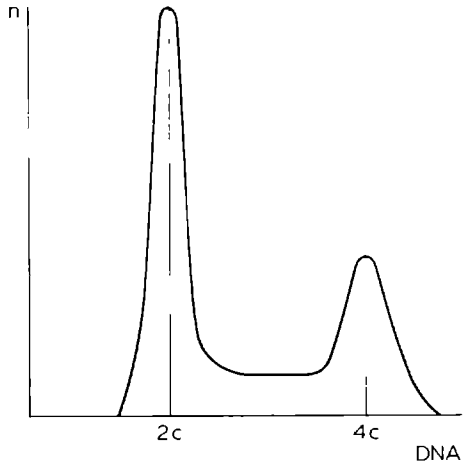

Figure 4. A DNA-histogram of an asynchronously growing cellpopulation. The cell number is shown on the ordinate, and the relative DNA-content on the abscissa.

figure 4. The large peak represents the G_1-phase cells, the smaller peak at twice this DNA-content the G_2 + M-phase cells and the continuum between these peaks reflects the S-phase cells. Although the DNA-content of normal diploid cells are found to be very constant the G_1 peak actually shows a variation of measured values around a mean. The extent of this variation is expressed as coefficient of variation (CV) and is mainly affected by the cytochemical and instrumental variability.
DNA-histograms of bone marrow or blood samples show CVs' of the G_1 peak ranging from 2.5 - 5.0 % (median: 3.2 %). Factors that affect measured DNA-distributions in homogeneous cell populations are the growth fraction and the relative durations of the cell-cycle phases. Additional variations are introduced by the heterogeneity of the cell populations.

The percentages of cells in the various phases of the cell cycle can be calculated from DNA-histograms by different mathematical techniques. In this thesis the planimetric method described by Baisch and Göhde was used (1). With this method the percentage of S-phase cells is calculated from the distance on the abscissa between the middle of the G_1-peak and the G_2 + M-peak, multiplied by the height of the curve at half distance between the G_1

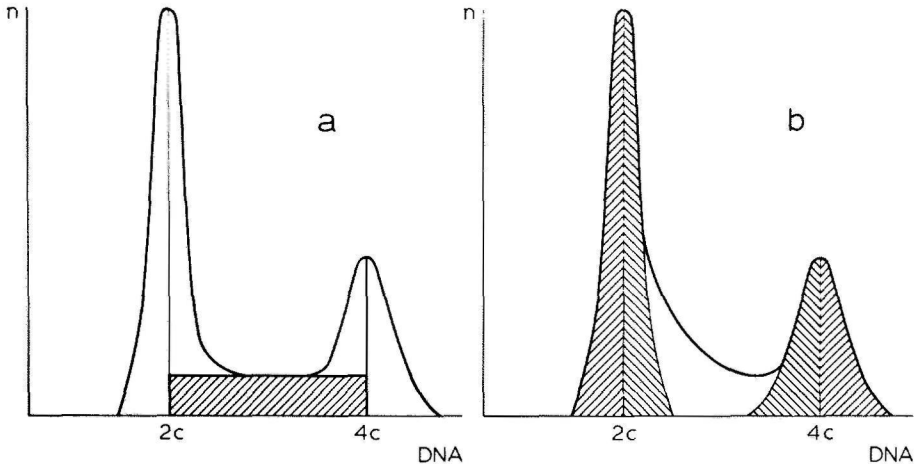

Figure 5. Two techniques for the analysis of DNA-distributions.

a. A planimetrical method in which a rectangular box is used to approximate the S-phase population.

b. A graphical method in which the left half of the G_1 peak and the right half of the G_2 + M peak are used to estimate the G_1 and G_2 + M-phase fractions.

and the G_2 + M-peak (Figure 5a). This sample estimation of the S-phase compartment, in general arrives at the same results as obtained by sophisticated computerized analysis of histograms. However, this planimetric method is not applicable to histograms of synchronous growing cell populations. Histograms from synchronous growing cell populations were analyzed according to a method described by Barlogie et al (2). This method is based on the assumption that the left half of the G_1-peak and right half of the G_2 + M-peak represent only G_1- or G_2 + M-phase cells respectively, with no contribution of S-phase cells. The peak halves are folded around their respective modes, forming two symmetric peaks whose areas yield the fraction of cells in each of these two cell-cycle phases. The S-fraction is then obtained by subtraction (Figure 5b).

DISPLAY AND ANALYSIS OF TWO-PARAMETER MEASUREMENTS

The simpliest way of displaying two-variable data, both electronically

and computationally is in a *scatter-plot* (3). Each cell in such a plot is represented by a dot whose X and Y coordinates are proportional to the two cell characteristics. One disadvantage of scatter plots, however, is their limited dynamic range. Regions, where the frequency of cells is high may become completely black before a sufficient number of data-points are plotted to the low-frequency areas.

A more quantitative method for the presentation of two-variable data is *the perspective plot*. The values of the two variables are presented as coordinates on a horizontal plane; the frequency of objects appears as distance above this plane, so that the distribution appears as a solid figure e.g. as peaks and valleys. Figure 6 shows the perspective display (with hidden lines removed) of the propidium iodide (red) and FITC (green) fluorescence of a bone marrow sample. Although perspective displays present two variable data in an easily visualized form, they provide little quantitative topographic information. For example it is almost impossible to determine bivariate

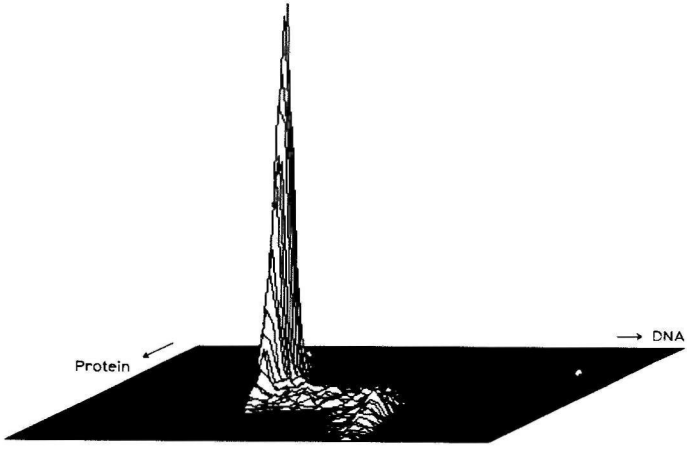

Figure 6. A perspective plot of two variable data (e.g. DNA and protein content of individual cells).

modes or standard deviations from a perspective display. *Density interval maps*, which were used in this thesis, overcome this limitation. Figure 7 shows an example of such a plot, representing a two-dimensional array of grey levels, which correspond to equal steps (or intervals) in the logarithm of the cell numbers.

The quantitative results of two parameter measurements are usually based on the information contained in subsets of cells selected by electronic gating. This information includes the distribution statistics of the cells within a certain window of which the position, the size and shape are defined (4).

CELL-STAINING

DNA-staining. For DNA-flow cytometry cells were stained with ethidium-bromide (EB). EB binds specifically to nucleic acids,mainly by intercalation in double-stranded DNA and RNA. This binding results in about 20-fold increase of the fluorescence quantum-efficiency of the dye. In consequence the

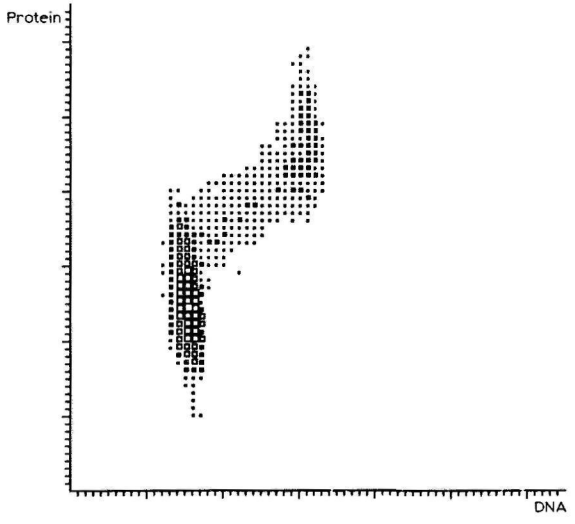

Figure 7. Density interval map of two variable data (e.g. DNA and protein content of individual cells). The two dimensional array of grey levels correspond to equal steps in the logarithm of the cell numbers.

intensity of fluorescence emission increases by a factor between 50-100 (5). The maximum excitation of EB is about 500 nm, and the maximum emission about 600 nm (orange-red).

In the studies of this thesis,cell staining for DNA was performed according to the method of Krishan (6). In this method about 10^6 cells of blood or bone marrow sample are added to 5 ml hypotonic EB-solution containing 0.1 % trisodium citrate and 25 mg/l EB (pH 7 6). Cells are lysed in this solution and nuclear DNA is immediately stained with EB at room temperature, allowing flow cytometric analysis within few minutes Staining of nuclear RNA can be prevented by adding RNA'se (1 mg/ml) to the cell suspension.

Simultaneous staining for DNA and R.A. Acridine Orange (AO) was used as metachromatic fluorochrome for simultaneous staining of DNA and RNA in unfixed bone marrow or cultured cells,according to the method described by Traganos and Darzynkiewicz (9). Upon excitation with blue light, AO intercalated into double strands nucleic acids (DNA, some RNA) fluoresces green (530 nm), while stacked on single stranded nucleic acids (RNA) it fluoresces red (640 nm). Since a large amount of RNA in situ is double stranded it must be selectively denaturated to the single stranded form prior to staining. This is accomplished by using EDTA as chelating agent.
The unfixed cells are made permeable to AO and chelating agents by short pretreatment (30 sec) with Triton X-100 at pH 1.0. The staining procedure actually is as follows: aliquots of 0 2 ml of cells suspended in PBS, containing 2 mM $MgCl_2$ and 10 % fetal calf-serum are mixed with 0.4 ml of a solution containing 0.1 % (v/v) Triton-X-100, 0.08 N HCl and 0.15 M NaCl. After 30 sec 1.2 ml AO (20 µg/ml) in 10^{-3}M EDTA, 0.15 M NaCl, 0.1 M phosphate-citrate buffer (pH 6.0) is added, resulting in a final dye concentration of 3 0 x 10^{-8}M AO. Measurements are performed within 10 min

Simultaneous staining for DNA and protein. Double staining of cells for both DNA and protein can be accomplished by using the fluorescent dyes propidium iodide (PI) and fluorescein isothiocyanate (FITC), which have about similar excitation spectra but differing and separable emission spectra. PI intercalates into DNA and RNA analogous to EB. Since PI bound to DNA has an emission maximum near 615 nm, compared to 600 nm for EB, and FITC near 530 µm, spectral separation is obtained using the PI/FITC combination (8). Although

some overlapping of the two colors occurs, adequate resolution is obtained by using appropriate color filters (figure 3).

The staining method as recently adjusted to hematopoietic cells by Crissman et al is described in chapter VIII (9).

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CHAPTER IV

A METHOD FOR QUANTIFICATION OF PERIPHERAL BLOOD ADMIXTURE IN BONE MARROW ASPIRATES

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Experimental Hematology, *8*, 103, 1980

ABSTRACT

Peripheral blood admixture in bone marrow aspirates of twenty patients with hematological diseases were studied either with ^{51}Cr-labeled autologous erythrocytes or ^{125}I-labeled albumin.

In these bone marrow aspirates 97.0 $\pm$ 4.2 % (mean $\pm$ SD) of the hemoglobin content appeared to be derived from peripheral blood. Assuming the presence of a proportional number of peripheral nucleated cells, one may calculate therefore the fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb}) using the ratios of Hb-level and nucleated cell count in respectively blood- and bone marrow samples. The F_{pb} in the twenty patients studied, showed a considerable variation (6 - 93 %). In a group of 25 healthy controls the mean F_{pb} $\pm$ SD was 14 $\pm$ 8 %.

From the present study it is evident that reliable results in quantitative bone marrow studies can only be obtained, if the fraction of peripheral nucleated cells in bone marrow aspirates is considered.

INTRODUCTION

Bone marrow aspirates are to a variable extent contaminated with peripheral blood. Usually, bone marrow is aspirated to determine the presence or absence of disease and for this purpose peripheral blood contamination is generally not supposed to be a major problem. However, if bone marrow aspirates are used for quantitative measurements e.g. as in determining its proliferative activity or its ability to form colonies, peripheral blood contamination may have a considerable influence on the results. In studying bone marrow proliferative activity in leukemias, we were also confronted with this problem. For this reason we determined the extent of peripheral blood admixture in bone marrow aspirates of 25 healthy controls and of 20 patients with various hematological diseases.

Based on results of the present study, a method is introduced for the calculation of the fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb}).

PATIENTS AND METHODS

Twenty patients with hematological diseases (age 17-64 years) and twenty-five healthy controls (age 22-34 years) were studied. Ten patients were injected intravenously with 20 µCi ^{51}Cr-labeled autologous erythrocytes whereas in ten additional patients 2 µCi ^{125}I-labeled albumin was administered. Three hours after the injection of ^{51}Cr-autologous red cells and ten minutes after the ^{125}I-albumin injection, a sternal and a venapuncture were performed almost simultaneously.

Both bone marrow aspirates (0.5 - 1.0 ml) and venous blood samples were anticoagulated with 1.0 ml acid citrate dextrose (ACD, formula A). The weights (volumes) of the samples were measured on a microbalance and of all specimens the nucleated cell count and the hemoglobin level were determined. The radioactivity of the specimens was measured with a gamma-scintillation counter (Baird Atomic). The total radioactivity in the bone marrow aspirate and the peripheral blood sample was related to the hemoglobin content of the samples (cpm/mg Hb). The values obtained in the bone marrow aspirate and the peripheral blood sample, were compared to each other.

The fraction of peripheral nucleated cells present in the bone marrow aspirate (F_{pb}) was calculated according to the formula as described in RESULTS. The F_{pb} was determined also in twenty-five additional healthy controls.

Patient	Bone marrow CPM∞	Bone marrow Hemoglobin content (mg)†	Peripheral blood CPM∞	Peripheral blood Hemoglobin content (mg)	CPM/mg Hb in BM‡ / CPM/mg Hb in Pb x 100 ∞
1	132 5	96 9	128 5	91.9	98
2	138 9	168 1	240 4	293 5	101
3	180 3	107 2	96 1	50 9	89
4	146 3	142,3	110 4	102 2	95
5	103 4	70 0	159 4	100 1	93
6	114 3	91.9	90 0	75 7	102
7	129 1	132 2	95 9	92 9	95
8	108 8	58 1	137 5	71 3	97
9	158 7	89 0	149 0	82 7	99
10	39 1	39 6	123 9	127 0	101
				Mean ± SD	= 97.0 ± 4.1 %

Table 1. Peripheral blood in bone marrow aspirates measured with ^{51}Cr-labeled autologous erythrocytes.

Patient	Bone marrow CPM∞	Bone marrow hemoglobin content (mg)-	Peripheral blood CPM∞	Peripheral blood Hemoglobin content (mg)	CPM/mg Hb in BM‡ / CPM/mg Hb in Pb x 100 ,
11	112 7	63 1	104 7	52 0	89
12	150 8	146 3	128 3	122 1	98
13	116 1	67 9	118 0	64 9	94
14	100 1	63 7	111 8	69 2	97
15	84 4	68 6	90 7	72 4	98
16	87 5	38 2	113 7	49 0	99
17	66 1	72 0	75 4	77 6	94
18	111 5	44.2	124 4	47 2	96
19	93.8	48 4	110 9	57 6	101
20	52 7	50 7	53 8	54 3	105
				Mean ± SD	= 97 1 ± 4 3 %

∞ CPM = counts per minute in total bone marrow aspirate or blood sample
† Hemoglobin content = hemoglobin (mg/dl) x volume (ml)
‡ BM = bone marrow, Pb = peripheral blood

Table 2. Peripheral blood in bone marrow aspirates measured with ^{125}I-labeled albumin.

Measurement of peripheral blood in bone marrow aspirates.

If a bone marrow sample contains non-circulating hemoglobin belonging to the marrow compartment, one would expect the radio-activity per mg Hb in the bone marrow aspirate to be less than in the peripheral blood.

Table I summarizes the results from patients injected with ^{51}Cr-labeled autologous erythrocytes. The radioactivity per mg Hb in the aspirates was 97.0 % ± 4.1 % (mean ± SD) of the radioactivity per mg Hb in the peripheral blood. In patients injected with ^{125}I-labeled albumin similar findings were obtained: 97.1 % ± 4.3 % (mean ± SD) of the radioactivity per mg Hb in the peripheral blood was present in the aspirates (Table 2).

We may conclude therefore that almost all hemoglobin in bone marrow aspirates is in due fact derived from peripheral blood.

Calculation of the peripheral nucleated cell fraction in bone marrow aspirates.

In a bone marrow aspirate the number of nucleated cells derived from peripheral blood apparently is proportional to the amount of peripheral hemoglobin present in the sample. As shown, nearly all hemoglobin in a bone marrow aspirate is derived from circulating blood. Consequently, neglecting a small error, the fraction of peripheral nucleated cells in a bone marrow sample (F_{pb}) may be calculated from the nucleated cell count and the Hb level in both blood sample and bone marrow aspirate.

In formula:

$$F_{pb} = \frac{HB_{bm}/Hb_{pb} \times NC_{pb}}{NC_{bm}} \times 100\ \%$$

where pb = peripheral blood, bm = bone marrow and NC = nucleated cell count/ml.

The F_{pb} in the twenty patients is shown in Table 3. In these cases the F_{pb} ranges from 6 % to 93 %. In some cases findings were rather surprising as almost all nucleated cells actually proved to be derived from peripheral blood, although the aspirates were considered cell rich (case 8, 18 and 20). On the other hand in a cell poor bone marrow aspirate (case 14) 69 % of the cells were derived from the bone marrow compartment.

The F_{pb} in the twenty-five healthy controls was less divergent (Table 4). The mean F_{pb} was 14 % ± 8 % (range 3 % - 37 %).

Patient	Diagnosis	Nucleated cell count x 10^6/g Hb		F_{pb} (%)
		Peripheral blood	Bone marrow	
1	Lymphoma	71	237	30
2	AML	472	888	53
3	Myeloma	106	801	13
4	Vaquez	104	497	21
5	Hodgkin	53	430	12
6	Myeloma	80	142	56
7	Anamia	43	237	18
8	CLL	1019	1497	68
9	ALL	150	1460	10
10	Lymphoma	35	142	25
11	Myeloma	109	401	27
12	Anemia	61	1092	6
13	Myeloma	55	191	29
14	AEL	12	39	31
15	ALL (Remission)	43	91	47
16	AML	53	335	16
17	Hodgkin	51	309	17
18	CLL	361	460	78
19	ALL (Remission)	71	115	62
20	CML.	4020	4304	93

AML = Acute Myeloblastic Leukemia
ALL = Acute Lymphoblastic Leukemia
AEL = Acute Erythroblastic Leukemia
CLL = Chronic Lymphocytic Leukemia
CML = Chronic Myeloid Leukemia

Table 3. The calculated fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb}) of 20 hematological patients.

No	Nucleated cell count x 10^6/g Hb		F_{pb} (%)
	Peripheral blood	Bone marrow	
1	30	584	5
2	43	360	12
3	31	509	6
4	38	180	21
5	44	118	37
6	45	373	12
7	30	994	3
8	37	261	14
9	76	317	24
10	32	248	13
11	18	304	6
12	68	310	22
13	32	211	15
14	36	522	7
15	38	348	11
16	44	261	17
17	29	721	4
18	36	242	15
19	44	199	22
20	36	360	10
21	50	497	10
22	32	186	17
23	31	118	26
24	42	379	11
25	31	242	13
		Mean + SD	14 ± 8 %

Table 4. The calculated fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb}) of 25 healthy controls.

It is known that red blood cells leave the bone marrow compartment as reticulocytes (1). The hemoglobin present in the bone marrow compartment therefore is derived mainly from erythroblasts and young reticulocytes. The reticulocyte number of the bone marrow compartment is estimated to be of the same order of magnitude as the total amount of erythroblasts (2,3). Consequently, one may expect the amount of hemoglobin derived from the bone marrow compartment to be small compared to the total hemoglobin content in an aspirate.

It has already been established by radioactive labeling studies in animals that mature erythrocytes in bone marrow samples represent circulating blood (4). Also in human beings it was already shown that bone marrow aspirates may be contaminated with peripheral blood to a large extent (5), while the influence on quantitative bone marrow studies of this artifact has been demonstrated as well (6,7). However, hitherto no practical method has been proposed to quantify the fraction of peripheral nucleated cells in bone marrow aspirates.

As demonstrated in the present study the amount of peripheral blood in a bone marrow aspirate is indicated by its hemoglobin content. Hence, one may calculate the peripheral nucleated cell fraction from the ratios of the Hb-level and nucleated cell count in both bone marrow aspirate and a simultaneously obtained peripheral blood sample. As shown, the fraction of peripheral nucleated cells in bone marrow aspirates may be considerable especially in hematological diseases. Consequently, the relevance of considering this possible artifact in quantitative bone marrow studies is evident. For example, in measuring the proliferative activity in bone marrow aspirates of patients with leukemia (using autoradiography or DNA-flow cytometry), peripheral blood contamination may cause a fall in the proliferative cell fraction, as circulating blasts usually are less proliferative cells. The significance of this consideration is apparent as the proliferative activity in bone marrow aspirates is used as a kinetic rational in the chemotherapeutic treatment of leukemia (8). Moreover, several authors have stressed its prognostic significance in predicting the likelihood of attaining remission, remission duration or survival time (9,10). The same reasoning can be applied for example to studies on the colony forming capacity of human bone marrow, as peripheral blood usually contains only a few colony forming cells.

Application of the proposed correction method to studies in which bone marrow aspirates are used to quantify some bone marrow cell characteristics,

will contribute to an enhanced reliability of the obtained data.

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CHAPTER V

FLOW CYTOMETRIC DETERMINATION OF THE S-PHASE COMPARTMENT IN BONE MARROW OF ADULT PATIENTS WITH ACUTE LEUKEMIA

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ABSTRACT

Flow cytometric analysis of bone marrow aspirates and blood samples were performed in 106 adult patients with acute leukemia in order to assess the size and the prognostic significance of the percentage of S-phase cells in the bone marrow (% S_{bm}). A correction procedure was applied for the fraction of contaminating peripheral nucleated cells in bone marrow aspirates (% F_{pb}). In 82 out of the 106 patients studied the % S_{bm} could be reliably determined and was compared to the % S_{bm} in 25 healthy controls. The % S_{bm} in these healthy controls ranged from 8.4 - 14.6 %.

The median % S_{bm} in 31 patients with acute non-lymphocytic leukemia (ANLL) at diagnosis (11.3 %) and in 14 patients with ANLL at relapse (11.8 %) did not differ significantly from the median % S_{bm} in normal bone marrow (11.7 %). On the other hand in 12 out of 23 patients with acute lymphocytic leukemia (ALL) at diagnosis and in 6 out of 11 patients with ALL at relapse the % S_{bm}, ranging from 17.8 - 44.0 %, was much increased compared to normal.

The prognosis,with regard to remission duration and survival of patients with ALL having a high % S_{bm} (> 15 %) was significantly worse than those with normal or low % S_{bm} (≤ 15 %).

Aneuploidy was noticed in 7.7 % of the patients with acute leukemia at diagnosis, the incidence being significantly higher especially in patients with ALL at relapse (42.1 %).

INTRODUCTION

The size of the S-phase compartment in bone marrow,measured with either flow cytometry (FCM) or tritiated thymidine autoradiography in normals and patients with acute leukemia,shows a considerable individual variation (1,2). Recently it was shown that bone marrow aspirates in patients with leukemia may be expected to be contaminated to a large amount with peripheral nucleated cells (3). Since the percentage of cells in S-phase in blood may differ from that in bone marrow, it is reasonable to assume that variations in the S-phase compartment in patients with leukemia, as determined in bone marrow aspirates, may partly be explained by the large variation of peripheral blood contamination in bone marrow aspirates.

In the present study we determined the S-phase compartment in blood and bone marrow of patients with acute leukemia, taking into account, and correcting for the amount of peripheral nucleated cells in bone marrow aspirates. The S-phase compartments of bone marrow and blood of patients with acute leukemia were compared with those from healthy controls, and the prognostic significance evaluated with regard to remission rate, length of remission and survival.

MATERIALS AND METHODS

Patients

Flow cytometric determination of DNA-content (DNA-FCM) was performed of bone marrow aspirates and blood samples from 106 patients with acute leukemia. Studied were 40 patients with acute non-lymphocytic leukemia (ANLL), 3 patients with acute erythroblastic leukemia (AEL) and 25 patients with acute lymphoblastic leukemia (ALL) at the onset of disease, whereas 19 patients with ANLL and 19 patients with ALL were evaluated at first relapse. The diagnoses were made according to the currently accepted FAB-criteria (3), and confirmed at the W.H.O. Reference Centre for Leukemias, Villejuif, France and by the Netherlands' Bone Marrow Committee, Rotterdam.
Only patients with more than 70 % leukemic blast cells in bone marrow were evaluated.The ANLL patients were treated according to the LAM-5 protocol of the EORTC, consisting of vincristine, adriamycin and cytosine arabinoside as remission induction therapy. The ALL-patients were treated with prednisone, vincristine, adriamycin and asparaginase supplemented with intrathecal methotrexate.

Studied were also 25 healthy controls of whom the bone marrow aspirates were subjected to both DNA-FCM and tritiated thymidine autoradiography.

Sample processing

From sternum or posterior iliac crest 0.5 - 1.0 ml bone marrow material was aspirated and anticoagulated with 2 ml acid citrate dextrose (ACD formula A). A venous blood sample was taken from an antecubital vein. Of both bone marrow and blood samples the Hb content and the cellnumber were determined. Staining was performed by adding 10^6 nucleated cells of blood or bone marrow sample to 5 ml hypotonic ethidium bromide solution (0.1 % trisodium citrate, 25 mg/l ethidium bromide) according to the method of Krishan (4).
The relative fluorescence of about 10^5 cells was measured in a ICP-11 pulse cytophotometer (Phywé Company, Germany). Distributions of DNA-content were analyzed utilizing the method described by Baisch et al (5). The coefficient of variation for the G_1 compartment ranged from 2.2 - 5.3 % (median 3.2 %).

Correction for peripheral blood contamination of bone marrow aspirates

The fraction of peripheral nucleated cells present in bone marrow aspirates (F_{pb}) was calculated as recently described (6,7). Briefly, this method is based on the observation that almost all hemoglobin in bone marrow aspirates is derived from peripheral blood. Therefore determination of hemoglobin content and the number of nucleated cells in blood and bone marrow samples enables the calculation of the fraction of nucleated cells in the aspirate originating from the peripheral blood, according to the formula:

$$F_{pb} = \frac{NC_{pb}}{NC_{asp}} \times \frac{Hb_{asp}}{Hb_{pb}}$$

where NC_{pb} and NC_{asp} stand for the nucleated cell count respectively in peripheral blood and bone marrow aspirate and Hb_{pb} respectively Hb_{asp} for hemoglobin content in peripheral blood and aspirate.
Consequently, the percentage of S-phase cells in the bone marrow compartment can be calculated from the findings in the blood sample and the bone marrow aspirate according to the formula:

$$\% S_{bm} = \frac{\% S_{asp} - (F_{pb} \times \% S_{pb})}{1 - F_{pb}}$$

where % S_{bm}, % S_{asp} and % S_{pb} stand for the percentage of S-phase cells in respectively bone marrow compartment, bone marrow aspirate and peripheral blood. However, with increasing F_{pb}, calculation of % S_{bm} from % S_{asp} and % S_{pb} progressively becomes less reliable. Consequently, according to an error analysis (given in the *Addendum*) bone marrow aspirates with a F_{pb} of more than 40 % were rejected for calculation of the S-phase compartment.

Tritiated Thymidine Autoradiography

In addition to DNA-FCM,the bone marrow aspirates of 25 healthy controls were studied with ^{3}H-thymidine autoradiography. To 100 µl of the bone marrow cell suspension, 0.5 µCi ^{3}H-thymidine was added and incubated for 60 min at 37^{o}C. Smears were prepared, air dried, fixed in methanol and stained with May-Grünwald Giemsa.

The slides were covered with polyvynilidenechloride to prevent chemography of the overlayered Illford L4 photographic emulsion (8). Autoradiographs were examined after exposure during 14 days at 4^{o}C. The percentage of labeled cells was determined by counting 2000 nucleated cells. Cells with more than 4 grains were considered to be labeled. The Labeling Index (LI) of erythroid and myeloid cells were determined separately and corrected for peripheral nucleated cell admixture, according to the formula

$$LI\text{-}M_{bm} = \frac{LI\text{-}M_{asp}}{1-(F_{pb} \times \% M_{pb})}$$

where $LI\text{-}M_{bm}$ and $LI\text{-}M_{asp}$ stand for the LI of myeloid cells respectively in bone marrow compartment and aspirate, and % M_{pb} stands for the percentage of granulocytes in blood as determined with the Hemalog-D (Technicon).

The determination of the LI of the various subclasses within the myeloid cell series was omitted since an accurate distinction between myeloblasts, promyelocyte and myelocyte was not quite possible on the autoradiographs.

Statistical methods

Analysis for differences in S-phase compartments between various groups of patients was performed utilizing the Student's-t-test. Comparisons of duration of response and survival were made utilizing the Gehan modification of the Wilcoxon analysis (9). Survival curves were calculated by the life table technique according to Kaplan and Meier (10).

The contamination of bone marrow aspirates with peripheral nucleated cells

The fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb}) of healthy controls and patients with acute leukemia show a considerable variation (Figure 1). In normals the F_{pb} ranged from 3-36 % (median 15 %), in patients with ANLL and AEL from 1-95 % (median 12 %) and in patients with ALL from 1-76 % (median 7 %). In 7 patients with ANLL and in 1 patient with ALL the F_{pb} of bone marrow aspirates repeatedly exceeded 40 % at several attempts, as indicated, these cases were excluded from evaluation.

The S-phase compartment in healthy controls

Table 1 summarizes the data obtained in the study of S-phase compartments (% S_{bm}) in 25 normals. The mean labeling index (LI) of all bone marrow cells was 11.0 $\pm$ 1.9 % (SD). The mean LI of the myeloid cells was 9.1 $\pm$ 2.1 % and the mean LI of the erythroid cells 27.1 $\pm$ 4.8 %. The mean % S_{bm} determined with DNA/FCM was 11.3 $\pm$ 1.5 % (range 8.4 - 14.6 %).

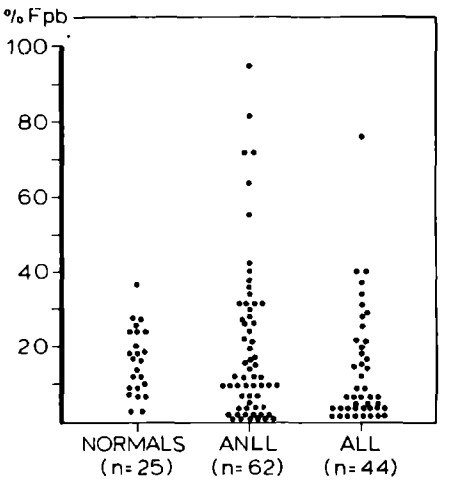

Figure 1. The fraction of peripheral nucleated cells in bone marrow aspirates (% F_{pb}) in healthy controls and patients with acute leukemia.

	^{3}H-thymidine Autoradiography			DNA Flow Cytometry	
	LI(%) Myeloid Cells	LI(%) Erythroid Cells	LI(%) Total BM	% S Total BM	% S Periph Blood
Median	9 2	27 0	10 8	11 2	2 0
Mean	9 1	27 1	11 0	11 3	2 0
SD	2 1	4 8	1 9	1 5	0 5
Range	8 7-14 5	17 0-34 7	6 5-13 9	8 4-14 6	1 6-3 1

Table 1. The S-phase compartment in 25 healthy controls

DNA-FCM of normal blood reveals the precense of a median percentage of "S-phase" cells of 2.0 % (mean 2.0 $\pm$ 0.5 %). However, this percentage must be considered artefactual as it is partly caused by interference of the relative large amounts of erythrocytes in blood samples. Consequently S-phase cells were supposed to be present in peripheral blood only, when the % S_{pb} exceeded a minimum threshold of 3.0 %.

The S-phase compartment in bone marrow (% S_{bm}) in patients with acute leukemia

Reliable calculation of the percentage of S-phase cells in bone marrow (% S_{bm}) was possible in 31 ANLL patients, 3 AEL patients and 23 ALL patients at diagnosis and in 14 ANLL patients and 11 ALL patients at relapse.

The % S_{bm} in normals and patients with acute leukemia is shown in Figure 2. In ANLL patients the median % S_{bm} at diagnosis was 11.3 % (mean 11.2 $\pm$ 3.2 % SD) which does not differ from the median % S_{bm} in the heterogeneous normal bone marrow (11.2 %), and which is slightly higher than the LI of normal myeloid cells (9.1 %). In ANLL patients at relapse the median % S_{bm} (11.8 %) was not significantly different from the median % S_{bm} at diagnosis.
In three patients with AEL the % S_{bm} was considerably higher (i.e. 24.5 %, 28,5 % and 34,5 %) which corresponds with the high LI of erythroid cells in normal bone marrow (27.1 %).

In patients with ALL at diagnosis and at relapse the % S_{bm} ranged from 5.2 - 37.5 %, respectively from 7.1 - 44.5 %. In 12 out of 23 ALL patients at diagnosis and in 6 out of 11 patients at relapse the % S_{bm} was higher than normal (> 15.0 %). In 4 patients at diagnosis and in 1 patient at relapse the % S_{bm} was low (< 8.0 %). The median % S_{bm} at diagnosis was 17.6 % and at relapse 23.8 %, which is not significantly different.

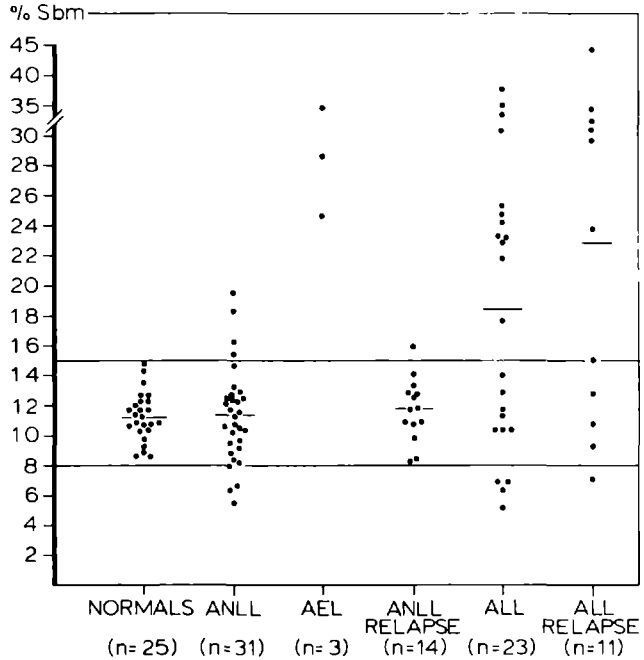

Figure 2. The percentage of S-phase cells in the bone marrow in normals and patients with acute leukemia at diagnosis and at relapse.

Aneuploidy occurred in 3 patients with ANLL and 2 patients with ALL at diagnosis, and in 3 patients with ANLL and 8 patients with ALL at relapse. All patients with aneuploidy at the onset of disease showed the same DNA-content abnormality at relapse. In 5 patients with ALL aneuploidy occurred only at relapse.

The percentage of S-phase cells in peripheral blood in patients with acute leukemia (% S_{pb})

In almost all patients the percentage of S-phase cells in peripheral blood (% S_{pb}) was lower than in the bone marrow (% S_{bm}), as demonstrated in Figure 3.

In ANLL patients at diagnosis a high % S_{pb} (> 3 %) was observed in 14 out of 35 patients (range 3.1 - 7.5 %) and in 2 out of 19 patients at relapse

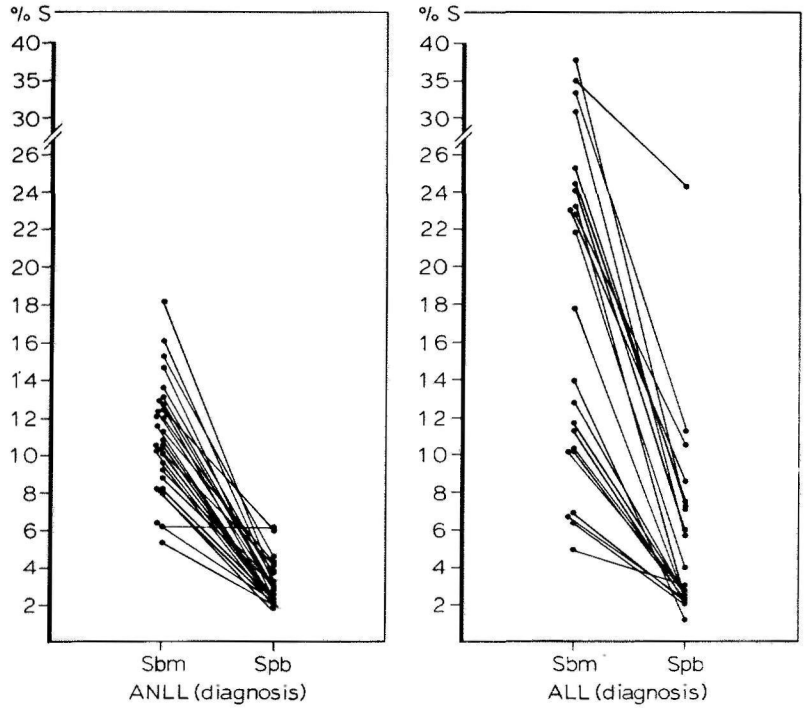

Figure 3. The percentage of S-phase cells in peripheral blood (% S_{pb}) compared to the percentage of S-phase cells in bone marrow (% S_{bm}) in patients with acute leukemia.

(3.4 % respectively 3.9 %). In 3 AEL patients the % S_{pb} was pronounced (14.2 %, 17,2% respectively 22.6 %) corresponding with the presence of erythroid cells in peripheral blood.

The % S_{pb} was more than 3 % in 12 out of 23 patients with ALL at diagnosis (range 3.1 - 24.3 %) and in 8 out of 11 patients at relapse (range 4.6 - 20.4 %).

In peripheral blood aneuploidy was noticed in all patients in which this abnormality could be demonstrated in the bone marrow aspirate.

Relationship of % S_{bm} to age, initial white blood cell count, remission-rate and prognosis

No correlation existed between either the % S_{bm} and the initial white blood cell count in ANLL patients ($r = 0.17$) and ALL patients ($r = 0.42$) nor between age and % S_{bm} ($r = 0.23$ respectively 0.31).

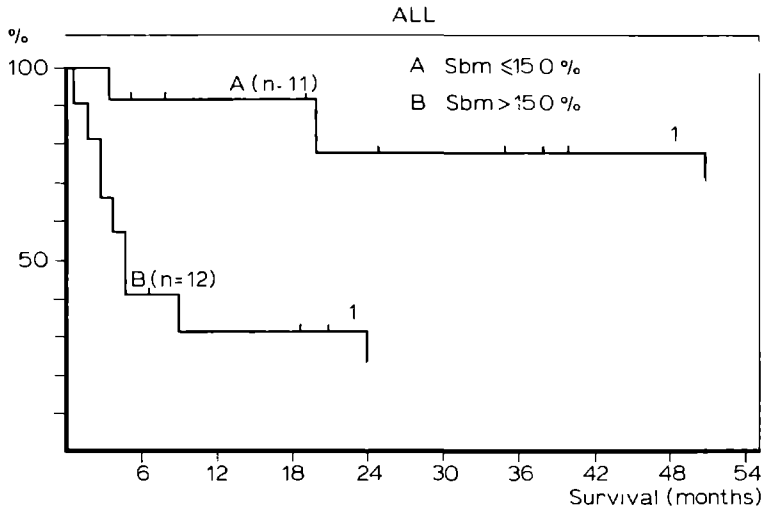

Figure 4. Survival curves (according to Kaplan and Meier) of ALL patients with high % S_{bm} (> 15 %) and normal or low % S_{bm} (≤ 15 %).

	% S_{bm} ≤ 15 %	% S_{bm} > 15 %	
Number of patients	11	12	
Age, median (range)	18 year (13-28)	20 year (14-69)	NS*
WBC, median (range)	10 5 x 10^9/l (0 6-71 0)	12 6 x 10^9/l (0 6-477)	NS
Complete remission	10	7	NS
Estimated median remission duration (range)	33 months (2 - 37+)	12 months (1 - 22)	P=0 016
Estimated median survival (range)	51 months (7-51)	5 months (2-24)	P= 0 006

* NS = non significant
+ = still alive

Table 2. Characteristics of 23 adult patients with ALL at diagnosis subclassified according to the % S_{bm}.

The % S_{bm} in ANLL was not related to the likelihood of achieving a complete remission, i e complete remission was obtained in 9 out of 15 patients with initial % S_{bm} above the median value and in 11 out of 16 patients with a % S_{bm} below the median.
The median survival of 31 patients with ANLL of which the % S_{bm} could be determined was 13 months The % S_{bm} at diagnosis in ANLL was not related to remission length or survival.
On the other hand, in 12 out of 23 ALL patients a high % S_{bm} (> 15 %) corresponded evidently with an unfavourable prognosis In ALL-patients with a % S_{bm} > 15 % the median length of remission was 12 months, while in patients with a % S_{bm} < 15 % the median remission duration was 33 months ($p = 0.016$). The median survival in both groups was also significantly different, respectively 5 and 51 months ($p = 0.006$)(Figure 4). Table 2 summarizes the differences between the two groups of ALL-patients with regard to remission rate, length of remission, survival, white blood cell count and age. Remission duration and survival were significantly related to the percentage of S-phase cells in the bone marrow at diagnosis.

DISCUSSION

Bone marrow aspirates may contain considerable amounts of nucleated cells, derived from peripheral blood (1,2,6). This contamination has negatively affected results of bone marrow studies with regard to proliferation kinetics and may partly account for the discrepancy and the great variation of the pretherapeutic S-phase compartment in leukemias as reported in literature (11,12, 13,14,15). The influence of peripheral nucleated cells in bone marrow aspirates upon the results of cytokinetic studies was recently discussed by Hiddemann et al (2) and Dosik et al (16), who advocated the use of bone marrow biopsies instead of aspirates to circumvent this problem. As shown in the present study, cytokinetic data of the bone marrow could be obtained, in 82 out of 106 patients with acute leukemia and in all 25 healthy controls from bone marrow aspirates, applying a correction procedure for peripheral blood admixture. A high F_{pb} hampered a reliable determination of the % S_{bm} in only 8 patients.

The mean % S_{bm} in 25 healthy controls was 11 3 % and 11.0 % as determined with respectively DNA-FCM and ^{3}H-thymidine autoradiography. The mean LI of the myeloid cell series and erythroid cells were respectively 9.1 % and 27.1 %. In the majority of patients with ANLL the % S_{bm} was of the same order of mag-

nitude as the LI of normal myeloid cells and in 3 patients with AEL the % S_{bm} was as high as the LI of the normal erythroid precursors.
These values suggest a normal cell proliferation rate in a majority of patients with AML and AEL.
On the other hand in 12 out of 23 patients with ALL at diagnosis and in 6 out of 11 patients at relapse the % S_{bm} was much higher compared to normal, which corresponds with an unfavourable prognosis in regard to remission duration and survival. In ANLL patients these findings could not be demonstrated, whereas in both ANLL and ALL patients no relation was found to exist between % S_{bm} and age, or % S_{bm} and initial white blood cell count. In addition, there was no significant difference between the % S_{bm} at diagnosis and the % S_{bm} at relapse in both groups of patients.

The unfavourable prognosis of a high % S_{bm} with respect to remission duration and survival in patients with ALL is consistent with findings reported by some other investigators (17,18).
An initially rapid cell turnover, causing a rapid increase in cell number at relapse and the quick development of resistance to chemotherapeutic agents by rapidly proliferating cells, are postulated as probable explanations for this observation. Anyhow, one would anticipate that patients with a high % S_{bm} would require quite different treatment from those with a normal or low % S_{bm}.

The frequency of aneuploidy observed with DNA-FCM in the present study (7.5 % of ANLL, and 8.0 % of ALL patients at diagnosis) is lower than the 16.5 % incidence reported by Barlogie et al (19). On the other hand, in ALL patients at relapse a 42.1 % of aneuploidy was noticed suggesting a growth advantage of cells with high degree chromosomal aberrations in ALL.

Acknowledgement
The authors are indebted to Miss G.Blankenborg for assistance in this study.

Correction of data obtained in bone marrow aspirates for deviations due to peripheral blood admixture is subjected to the "propagation of errors" inherent to the figures used for the calculation (20).

Generally speaking if a result R depends on measurements M according to the equation (1).

$$R = f(M_1, M_2, M_3 \ldots\ldots M_n) \qquad (1)$$

the standard error ΔR can be calculated according to the function (2)

$$(\Delta R)^2 = \sum_{i=1}^{n} \left(\Delta M_i \frac{\delta R}{\delta M_i}\right)^2 + \sum_{i}^{n} \sum_{j+i}^{n} \Delta M_{ij}^2 \frac{\delta R}{\delta M_i} \cdot \frac{\delta R}{\delta M_j} \qquad (2)$$

where ΔM is the standard error of measurement M_1.
The second term of covariates is discarded in the present study, because these parameters can be expected to be uncorrelated.
The standard error of % S_{bm} was calculated with use of experimentally determined standard deviations of the individual variables such as the nucleated cell count (NC), the hemoglobin content (Hb) and the percentage of S-phase cells (S). The functions which were found to exist for the various parameters are expressed in the formulas (3), (4) and (5):

$$\Delta NC = 0.107\ NC^{(.52)} + 0.01\ NC \qquad (3)$$

$$\Delta Hb = 0.007\ Hb^{(1.5)} + 0.05 \qquad (4)$$

$$\Delta S = 0.2 + 0.03\ S \qquad (5)$$

The standard error of % S_{bm} as function of F_{pb} was calculated.
In figure 5 a plot is given of the expected error in % S_{bm} as a function of F_{pb}, keeping all parameters of the peripheral blood constant. The calculated Standard Error (SE) at various F_{pb}-values (line) correlates closely to an actual set of measurements (dots).
If the F_{pb} is 40 % or higher the SE increases progressively beyond 10 percent

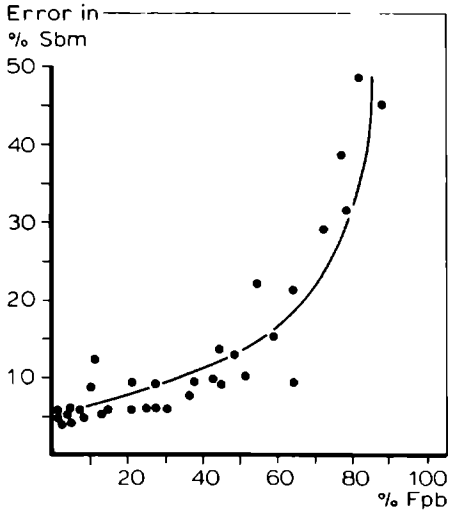

Figure 5. The relation between the F_{pb} and the expected error in the % S_{bm}. The line indicates expected values, the dots indicate the results of an actual set of measurements.

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CHAPTER VI

DNA-FLOW CYTOMETRY OF BLOOD AND BONE MARROW IN CHRONIC MYELOGENOUS LEUKEMIA (CML)

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ABSTRACT

Flow cytometric analysis of bone marrow aspirates and blood samples in 42 patients with chronic myelogenous leukemia (CML) at various stages of their disease was performed to determine the size of the S-phase compartment of bone marrow and blood. Twenty-five healthy controls were studied for comparative information with both DNA-flow cytometry (DNA-FCM) and ^{3}H-thymidine autoradiography. A correction procedure was applied for peripheral nucleated cell admixture in bone marrow aspirates.

The fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb}) in individual patients was considerable, especially in those with a very high white blood cell count (> 100 x 10^{9}/l).

The size of the S-phase compartments of bone marrow (% S_{bm}) in patients with CML at diagnosis and in patients at apparent hematological remission is of the same order of magnitude as in normal bone marrow. However, in 3 out of 4 patients at malignant metamorphosis in which the % S_{bm} could be reliably determined, this percentage was signiciantly higher than normal ($p = 0.013$). In 4 out of 11 patients at malignant metamorphosis aneuploidy was noticed.

From these findings it is concluded that bone marrow cell proliferation in CML patients at diagnosis and during apparent remission is not essentially different from normal. However, at malignant metamoprhosis changes occur in ploidy level or proliferative activity.
DNA-FCM provides means for early detection of malignant metamorphosis in CML.

INTRODUCTION

Cytokinetic studies in patients with chronic myelogenous leukemia (CML) at various stages of their disease are commonly performed with tritiated thymidine autoradiography of blood samples and bone marrow aspirates (1). Recently it was demonstrated that bone marrow aspirates in patients with leukemia may be contaminated with peripheral nucleated cells (2), constituting a major error factor in cytokinetic studies of bone marrow (3,4).

In the present study the amount of peripheral nucleated cells in bone marrow aspirates of patients with CML at various stages of the disease was determined, and the S-phase compartment of bone marrow and blood investigated with DNA-flow cytometry (DNA-FCM). The results are compared with data obtained in healthy controls who where studied with both DNA-flow cytometry and tritiated thymidine autoradiography.

MATERIALS AND METHODS

Patients

DNA-FCM was performed on bone marrow aspirates and blood samples in 42 patients with CML and 25 healthy controls. 18 Patients were studied at diagnosis, 13 patients in apparent hematological remission and 11 patients at malignant metamorphosis of the disease. All patients received busulfan as remission-induction and maintenance therapy.
Malignant metamorphosis was defined by a rising white blood cell count with increasing numbers of immature cells, associated with a fall in Hb-level, occurring in spite of intensified treatment.

Sample processing

From sternum or posterior iliac crest 0.5 - 1.0 ml bone marrow was aspirated and anticoagulated with 1.0 ml acid citrate dextrose (ACD formula A). A venous blood sample was taken from an antecubital vein. The Hb-content and cellnumber were measured in both bone marrow and blood-samples.

Staining was performed by adding 10^6 nucleated cells to 5 ml hypotonic ethidium-bromide solution (0.1 % trisodium citrate, 25 mg/l ethidium bromide), according to Krishan (5). The relative fluorescence of about 10^5 cells was measured in a ICP-11 pulsecytophotometer (Phywé Company, FRG). Distributions of DNA-content were analyzed utilizing the method described by Baisch et al (6).

The median coefficient of variation for the G_1-compartment was 3.2 % (range 2.2 % - 5.3 %).

Correction for peripheral blood admixture in bone marrow aspirates

The fraction of contaminating peripheral nucleated cells present in bone marrow aspirates was calculated as recently described (2,1). Briefly, this method is based on the observation that almost all hemoglobin (or erythrocytes) present in a bone marrow aspirate, is derived from peripheral blood. Consequently, determination of hemoglobin content and the number of nucleated cells in the blood and bone marrow samples permits the calculation of the fraction of nucleated cells in the aspirate originating from peripheral blood, according to the formula:

$$F_{pb} = \frac{NC_{pb}}{NC_{asp}} \times \frac{HB_{asp}}{Hb_{pb}}$$

where NC_{pb} and NC_{asp} stand for the nucleated cell count respectively in peripheral blood and bone marrow aspirate and HB_{pb} respectively Hb_{asp} for hemoglobin content in peripheral blood and aspirate.
Consequently, the percentage of S-phase cells in the bone marrow compartment can be deduced from the percentages of S-phase cells in blood and bone marrow aspirate, according to the formula:

$$\% S_{bm} = \frac{\% S_{asp} - (F_{pb} \times \% S_{pb})}{1 - F_{pb}}$$

where $\% S_{bm}$, $\% S_{asp}$, and $\% S_{pb}$ stand for the percentages of S-phase cells in respectively bone marrow compartment, bone marrow aspirate and peripheral blood.

Calculation of the $\% S_{bm}$ in bone marrow aspirates of which the F_{pb} amounts to more than 40 % is not reliable, as appears from an error-analysis (see Addendum). Therefore these bone marrow aspirates were rejected for calculation of the $\% S_{bm}$.

Tritiated Thymidine Autoradiography

In addition to DNA-FCM, bone marrow aspirates of 25 healthy controls were studied with ^{3}H-thymidine autoradiography. To 100 µl of the anticoagulated

bone marrow aspirate, 0.5 μCi ^{3}H-thymidine (Radiochemical Centre Amersham, specific activity 5 Ci/mmol) was added and incubated for 60 min at 37°C. Smears were prepared, air dried, fixed in absolute methanol and stained with May-Grünwald Giemsa. The slides were covered with polyvynilidenechloride to prevent chemography of the overlayered Illford L4 photographic emulsion (7). Autoradiographs were examined after an exposure of 14 days at 4°C. The percentage of labeled cells was determined by counting 2000 nucleated cells. Cells with more than 4 grains were considered to be labeled.
The Labeling Index (LI) of myeloid cells (from blast to polymorphs) and erythroid cells were determined separately and corrected for contributing peripheral myeloid cells, according to the formula:

$$LI\text{-}M_{bm} = \frac{LI\text{-}M_{asp}}{1-(F_{pb} \times \% M_{pb})}$$

where $LI\text{-}M_{bm}$ and $LI\text{-}M_{asp}$ stand for labeling index of myeloid cells in bone marrow compartment and bone marrow aspirate respectively, and $\% M_{pb}$ stands for the percentage of granulocytes in blood as determined with the Hemalog-D (Technicon). The determination of the LI of the various subclasses of the myeloid cells was omitted since an accurate distinction of these subclasses is not quite possible on autoradiographs.

Statistical methods

Analysis for differences in size of S-phase compartments between the various groups of patients was performed utilizing Students'-t-test.

RESULTS

Patient characteristics and the fraction of peripheral nucleated cells in bone marrow aspirates (F_{pb})

Table 1 summarizes the clinical characteristics of the CML-patients studied. The median white blood cell count (WBC) at diagnosis, at hematological remission and at malignant metamorphosis were respectively 132.1, 15.7 and 23.0 x 10^{9}/l. In patients with malignant metamorphosis the percentage of blast cells ranged from 15 to 75 % (median 29 %).

As may be evident from the formula, one may expect a high F_{pb} especially in patients with high WBC counts. In 11 out of 13 patients having WBC counts of more than 100 x 10^{9}/l the F_{pb} exceeded 40 % indeed, while this was only the

	n	Sex M/F	Median Age years (range)	Median WBC° 10^9/l (range)	Median BM+-blast % (range)	Median Fpb× % (range)
At Diagnosis	18	12/6	36 (24-79)	132.0 (15 9-500)	2 (1-7)	51 (25-100)
At "Remission"	13	11/2	39 (28-56)	15 7 (59.0-61.7)	1 (1-5)	11 (2-100)
At Malignant Metamorphosis	11	9/2	53 (21-64)	23.0 (10 3-137.6)	29 (15-75)	31 (8-100)

° WBC = White Blood Cell Count
+ BM = Bone marrow
× Fpb = Fraction of peripheral nucleated cells in bone marrow aspirates

Table 1. Characteristics of the patients with CML at different disease stages

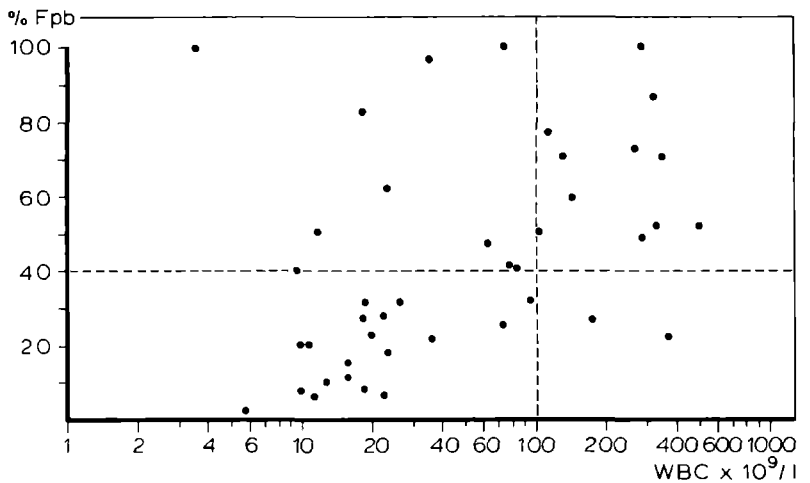

Figure 1. The relation between the F_{pb} and WBC count in patients with CML.

	^{3}H-thymidine Autoradiography			DNA Flow Cytometry	
	LI(%) Myeloid Cells	LI(%) Erythroid Cells	LI(%) Total BM	% S Total BM	% S Periph Blood
Median	9 2	27 0	10 8	11 2	2 0
Mean	9 1	27 1	11 0	11 3	2 0
SD	2 1	4 8	1 9	1 5	0 5
Range	8 7-14 5	17 0-34 7	6 5-13 9	8 4-14 6	1 6-3 1

Table 2. The S-phase compartment in 4 healthy controls.

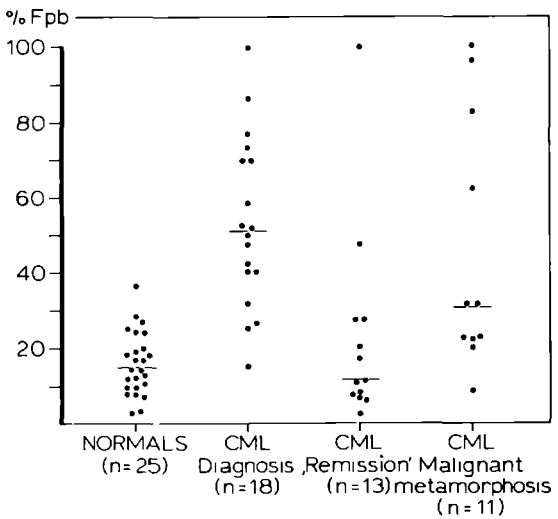

Figure 2. The F_{pb} in healthy controls and patients with CML.

case in 8 out of 29 patients with WBC counts below 100 x 10^9/l (Figure 1). Consequently, in the majority of patients with CML at diagnosis (12 out of 18 patients) bone marrow aspiration yielded cellular material which did not represent the cellular composition of the bone marrow compartment. In these patients the median F_{pb} was 51 % (range 25 - almost 100 %) whereas during hematological "remission" or at malignant metamorphosis the median F_{bp} was 11 % respectively 31 % (Figure 2). In healthy controls the median F_{pb} was 15 % (range 2 - 36 %).

The S-phase compartment in normals

The cytokinetic data obtained with DNA-FCM and ^{3}H-thymidine autoradiography in 25 controls are summarized in Table 2.

The median ^{3}H-thymidine LI of the total bone marrow (corrected for peripheral blood admixture) was 10.8 % (mean 11.0 $\pm$ 1.9 % SD). the median LI of all myeloid cells in the bone marrow compartment was 9.2 % (mean 9.1 $\pm$ 2.1 %), and of the erythroid cells 27.0 (mean 27.1 $\pm$ 4.8 %).
The median percentage of S-phase cells in the total bone marrow determined with DNA-flow cytometry (% S_{bm}) was 11.2 % (mean 11.3 $\pm$ 1.5 %).

DNA-FCM of normal blood reveals the presence of a median percentage of "S-phase" cells of 2.0 % (mean 2.0 $\pm$ 0.5 %). However, this percentage must be considered as partly artefactual, because it is caused by interference of the relative large amount of erythrocytes in blood samples. Consequently S-phase cells in paripheral blood were supposed to be present only, when the % S_{pb} exceeded a minimum threshold of 3.0 %.

The S-phase compartment in blood and bone marrow in patients with CML

The results of marrow and blood kinetic studies are shown in Figure 3, with comparative information obtained from normal bone marrow.
Since the F_{pb} exceeded 40 % in 12 out of 18 patients with CML at diagnosis, the % S_{bm} could be evaluated reliably in 6 patients only.
The median % S_{bm} in these 6 patients was 12.8 % (mean 13.2 $\pm$ 2.2 %), whereas the median % S_{pb} of all 18 patients at diagnosis was 9.2 % (mean 9.1 $\pm$ 2.0 %).
In 11 patients with CML at apparent remission the median % S_{bm} was 11.6 % (mean 12.4 $\pm$ 1.7 %). The median % S_{pb} in these patients was 4.1 % (mean 4.4 $\pm$ 1.5 %). The % S_{bm} in CML patients at diagnosis and at apparent remission are not significantly different from normal.

In 4 out of 11 patients at malignant metamorphosis, DNA-FCM revealed the

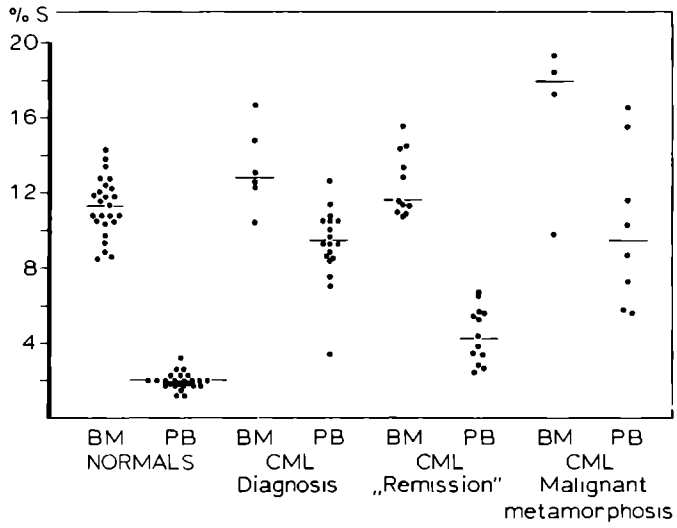

Figure 3. The percentages of cells in S-phase in bone marrow (BM) and blood (PB) in CML patients at different disease stages.

presence of cells with an abnormal DNA-content (aneuploidy). In only 4 out of 7 remaining patients the % S_{bm} could be reliably determined. In 3 out of these 4 patients the % S_{bm} was significantly higher than normal ($p = 0.013$) i.e. 19.3, 18.4 and 17.1 % (median 17.7 %).
The median % S_{pb} in 8 patients at malignant metamorphosis was 10.2 % and varied from 5.6 % - 16.5 %. In one patient with aneuploid cells in the bone marrow, this abnormality could not be demonstrated in the blood.

DISCUSSION

The present study was undertaken to determine the S-phase compartment of blood and bone marrow in patients with CML, taking into account the amount of peripheral nucleated cells in the aspirate. The results of this analysis show that the fraction of peripheral nucleated cells in bone marrow aspirates may be considerable in CML-patients, especially in those with a high WBC ($> 100 \times 10^9/l$) as is usual in about 80 % of the patients at diagnosis (8). Studies in CML-patients, in which bone marrow cell characteristics are quantified therefore are more or less hampered by this artefact. This conclusion is consistent with the recent findings of Dosik et el (4), who showed

that cytokinetic data derived from bone marrow aspirates are less reliable than those obtained from bone marrow biopsies. These authors supposed peripheral blood admixture in bone marrow aspirates to be the explanation for all discrepancies and advocated the use of cell suspensions derived from bone marrow biopsies for cytokinetic studies of bone marrow. Nevertheless, it could be shown in the present study that corrected cytokinetic data can be calculated reliably from bone marrow aspirates, in all 25 healthy controls and in 21 out of 28 CML-patients with a WBC count lower than 100 x 10^9/l. Recently, we obtained similar results in patients with acute leukemia (9,10).

The variation in the F_{pb} in bone marrow aspirates from patients with CML at diagnosis did not result however in a large underestimation of the S-phase compartment in bone marrow, because at diagnosis the % S_{bm} and the % S_{pb} in CML approximate each other rather closely.

The similarity of the % S_{bm} in normals and patients with CML at diagnosis and at remission, is accordant to presumed normal cell proliferation in CML (11,12,13). On the other hand in patients studied at malignant metamorphosis the % S_{bm} was higher than normal in 3 out of 4 patients, whereas in 4 out of 11 patients aneuploidy was noticed at this disease stage. The changes in both proliferation pattern and ploidy level of hematopoietic cells in CML patients at malignant metamorphosis, supports the suggestion that this disease-phase results from the evolution of new clones of neoplastic cells with an apparent growth advantage over normal cells and chronic phase CML clones (14,15,16). Moreover it indicates that flow cytometry may be applied as a useful tool for early diagnosis of malignant metamorphosis in CML.

ADDENDUM

Correction of data obtained in bone marrow aspirates for deviations due to peripheral blood admixture is subjected to the "propagation of errors" inherent to the figures used for the calculation (17).

Generally speaking if a result R depends on measurements M according to the equation (1)

$$R = f(M_1, M_2, M_3 \ldots . M_n) \qquad (1)$$

the standard error R can be calculated according to the function (2).

$$(\Delta R)^2 = \sum_{i=1}^{n} (\Delta M_i \frac{\delta R}{\delta M_i})^2 + \sum_{i}^{n} \cdot \sum_{j+i}^{n} \cdot \Delta M_{ij}^2 \cdot \frac{\delta R}{\delta Mi} \cdot \frac{\delta R}{\delta Mj} \qquad (2)$$

where ΔM is the standard error of measurement M_1.
The second term of covariates is skipped in the present study, because these parameters can be expected to be incorrelated.
The standard error of % S_{bm} was calculated with use of experimentally determined standard deviations of the individual variables such as the nucleated cell count (NC), the hemoglobin content (Hb) and the percentage of S-phase cells (S). The functions which were found to exist for the various parameters are expressed in the formulas (3), (4) and (5):

$$\Delta \ NC = 0.107 \ NC^{(.52)} + 0.01 \ NC \qquad (3)$$

$$\Delta \ Hb = 0.007 \ Hb^{(1.5)} + 0.05 \qquad (4)$$

$$\Delta \ S = 0.2 + 0.03 \ S \qquad (5)$$

The standard error of % S_{bm} as function of F_{pb} was calculated.
Figure 4 shows the expected error in % S_{bm} as a function of F_{pb}, keeping all parameters of the peripheral blood constant.
The calculated Standard Error (SE) at various F_{pb}-values (line) correlates closely to an actual set of measurements (dots). If the F_{pb} is 40 % or higher the SE increases progressively beyond 10 percent.

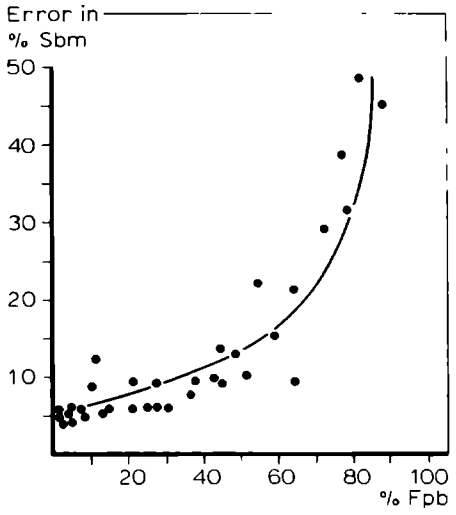

Figure 4. The relation between the F_{pb} and the expected error in the % S_{bm}. The line indicates expected values, the dots, the results of an actual set of measurements.

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CHAPTER VII

CYTOKINETIC CHANGES AFTER CYTOSINE ARABINOSIDE IN ACUTE NON-LYMPHOCYTIC LEUKEMIA

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ABSTRACT

Changes in the S-phase compartment of blasts were measured after intravenous push injections of cytosine-arabinoside (Ara-C, 100 mg/m^2, 380 mg/m^2 or 1000 mg/m^2 body surface) in 11 patients with acute non-lymphocytic leukemia At several intervals after Ara-C injection, blood and bone marrow samples were taken for autoradiography, DNA-flow cytometry and ^{3}H-thymidine incorporation

The data obtained from the bone marrow aspirates were corrected for peripheral blood admixture In untreated patients the three methods correlated very well. The perturbation of the cell-cycle after Ara-C administration shows for each method a specific pattern *labeling indices* reached, after an initial decrease, pretreatment levels at about 24 h after injection. Recruitment of cells into the cycling compartment could not be demonstrated. *DNA-flow cytometry* reveals a significant increase in the percentage of cells with > $2n$ < $4n$ DNA, with a maximum after about 30 h The discrepancy between autoradiography and DNA-flow cytometry after Ara-C injection may be due to the presence of cells arrested in S-phase. *^{3}H-thymidine incorporation* increased after an initial inhibition to about twice pretreatment values at 36 to 40 h after injection This observation suggests partial synchronization. Incorporation of ^{3}H-thymidine in peripheral nucleated cells correlated well with the corrected values of ^{3}H-thymidine incorporation in the bone marrow The perturbation of the cell-cycle appeared identical in the three different dosages used. The observations suggest that 24 h scheduling might be optimal for Ara-C given by push-injections

INTRODUCTION

In the treatment of acute non-lymphocytic leukemia (ANLL) cytosine arabinoside (1-β-D-arabinofuranosylcytosine, Ara-C) is one of the major chemotherapeutic agents (1,2). Its cytostatic activity is based on inhibition of DNA-synthesis by competitive interference of the intracellular metabolite cytosine arabinoside triphosphate with deoxycytidine triphosphate for the enzyme DNA-polymerase (3,4,5,6).

The depression of cellular DNA synthesis caused by intravenous administration of Ara-C varies considerably between leukemic patients (7,8,9) and may be related to individual differences in half-life time of Ara-C in the blood (7,8,10) and to differences in the phosphorylating capacity of the leukemic cells (3,11). The anti-leukemic effect of a treatment-course depends on the dose of Ara-C, the number of injections and the percentage of cells in S-phase at the moment of injection (12,13).

In animal models high dosages of Ara-C cause accumulation of cells in S-phase and treatment results are substantially improved when subsequent injections are given at the moment of maximal S-phase accumulation (14,15,16). Schedules used in human ANLL are almost completely empirical. Adjustment of administration intervals to the moment of maximal S-phase accumulation may improve treatment results in human ANLL as well.

This paper reports the results of a study in which cytokinetic changes were investigated in bone marrow and peripheral blood of patients with ANLL after intravenous bolus injections of different dosages of Ara-C.

MATERIALS AND METHODS

Patients

Cell-kinetic studies were performed in 11 patients with ANLL before and at different time intervals after an intravenous bolus injection of Ara-C. Diagnoses were confirmed at thw W.H.O. Reference Rentre for Leukemias, Villejuif, France and by the Bone Marrow Committee at Rotterdam, The Netherlands. Clinical data at the time of investigation are summarized in Table 1. Only one patient received previous cytostatic treatment. All patients received one intravenous push-injection of Ara-C, respectively 1000 mg/m^2 (3 patients), 380 mg/m^2 (4 patients)or 100 mg/m^2 (4 patients).

Patient	Age (years)	Sex	Morphological diagnosis*	Initial WBC ($\times 10^9$/l)	% Blasts (pb)	% Blasts (bm)
1	53	M	M 1	75	70	73
2	60	F	M 4	6	1	50†
3	31	F	M 4	50	69	88
4	68	M	M 2	66	32	90
5	53	M	M 1	71	89	74
6	41	M	M 1	127	69	93
7	40	F	M 2	16	17	45‡
8	40	M	M 1	96	83	95
9	34	M	M 1	8	25	90
10	24	F	M 1	16	60	71
11	44	M	M 2	26	30	70

* Classification according to the French-American-British (FAB) Co-operative Group Classification (29)
† Remainder are pathological (pro)myelocytes
‡ Bone marrow contained 17 % erythropoiesis

Table 1. Patient characteristics

Sampling

Bone marrow aspirates and peripheral blood samples were collected in buffered acid citrate dextrose solution (ACD formula A, pH 7.4). In one patient (no. 11), for technical reasons, only peripheral blood was monitored. Cells were washed in order to prevent fibrin formation during incubation. Since the presence of erythrocytes did not interfere with the measurements, no procedures were used to remove erythrocytes from the samples.

Measurement of the labeling index

To 100 µl of blood or bone marrow sample, 0.5 µCi ^{3}H-thymidine (Radiochemical Centre Amersham, specific activity 5 Ci/mmol) was added and incubated for 60 min at 37°C. Smears were prepared, air-dried, fixed in methanol and stained with May Grünwald-Giemsa. The slides were covered with polyvynilidenechloride to prevent chemography of the overlayered Ilford L4 photographic emulsion (7). Autoradiographs were exmined after an exposure of 14 days at 4°C. The percentage of labeled cells was determined by counting at least 2000 nucleated cells. Cells with more than 4 grains were considered to be labeled.

Measurement of DNA-content with flow cytometry.

The nuclear DNA of about 10^6 cells of each blood or bone marrow sample was stained by adding a small volume of the cell-suspension to 10 ml hypotonic ethidium-bromide solution (0.1 % tri-sodium citrate, 25 mg/l ethidium-bromide) according to Krishan (18). The relative fluorescence of about 10^5 individual cells was measured in an impulse cytophotometer (ICP-11, Phywé). To determine the percentage of cells containing $> 2n < 4n$ DNA, the histograms were analysed according to Göhde (19) with a computer program as described by van Egmond and Hillen (20).

Measurement of ^{3}H-thymidine incorporation

Blood and bone marrow samples were diluted in medium 199 (Flow Laboratories) buffered with 10 mM Tris (pH 7.4) and supplemented with 10 % heat inactivated fetal calf serum (Flow Laboratories, Lot. 2919107) to a final cell concentration of 10^6 nucleated cells per ml. Four aliquots of 1 ml were incubated with 2 µCi ^{3}H-thymidine (specific activity 5 Ci/mmol) for 60 min at 37°C.

The incubation was stopped by adding 2 ml ice-cold medium with an excess of unlabeled thymidine. The cells were collected on glass fiber filters (Schleicher and Schüll No. 9), washed with water and precipitated with 1 M perchloric acid. The total amount of radioactivity in the perchloric acid precipitable fraction was counted in a liquid scintillation counter (LKB 81000) with a counting efficiency of about 30 %.

Correction for peripheral admixture and error analysis

The fraction of contaminating peripheral nucleated cells, present in the bone marrow aspirates, were calculated according to Holdrinet et al (21). This method is based on the observation that almost all red cells, present in a bone marrow sample, are derived from peripheral blood admixture. Determination of hemoglobin content and the number of nucleated cells in the blood and bone marrow samples allows the calculation of the fraction of nucleated cells in the aspirate originating from the bone marrow compartment (F_{bm}), according to the formula:

$$F_{bm} = \frac{NC_{pb}}{NC_a} \times \frac{Hb_a}{Hb_{pb}}$$

where NC_{pb} and NC_a stand for nucleated cell count in peripheral blood and

aspirate respectively and Hb_{pb} and Hb_a respectively stand for hemoglobin content in peripheral blood and aspirate.

The cell-kinetic data (D) of the bone marrow compartment are calculated from the measurements on both the aspirate and the peripheral blood, according to

$$D_{bm} = \frac{D_a - (1-F_{bm})D_{pb}}{F_{bm}}$$

where D stands for labeling index, percentage cells with $> 2n < 4n$ DNA or ^{3}H-thymidine incorporation respectively.
The error in F_{bm} is a function of the individual errors of cell counts and hemoglobin measurements, determined in bone marrow aspirate and peripheral blood and can be calculated with the formula (22):

$$\Delta D = \sqrt{\Sigma_i \left(\frac{\partial D}{\partial x_i} \cdot \Delta x_i \right)^2}$$

where $\partial D/\partial x_i$ = first derivative to the i th variable; Δx_i = standard error of the i th variable; D = any parameter to be calculated from the variables.

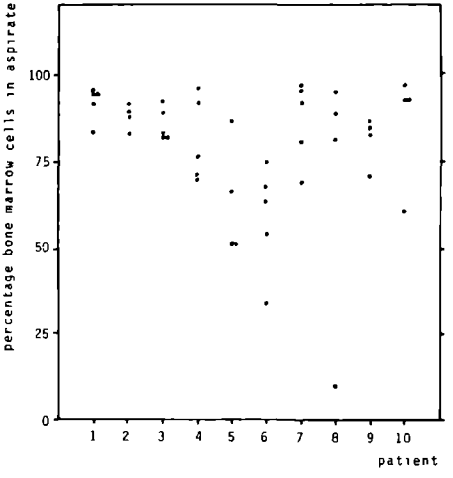

Figure 1. Fraction of nucleated cells in the bone marrow aspirates originating from the bone marrow compartment (F_{bm}).

Peripheral blood admixture in bone marrow aspirates

The fraction of peripheral nucleated cells in the bone marrow aspirates varies considerably, even within the same patient (figure 1). Four aspirates contained more than 50 % peripheral nucleated cells and the relative error on the F_{bm} exceeded 10 % in these cases. These samples were therefore excluded from further evaluation.

Accuracy of data

Errors on all data were calculated as described under methods. For labeling indices over 6 % the relative error is about 15 %. Flow cytometric determination of the percentage S-phase cells is more accurate, for values over 4 %, the relative error is less than 10 %. The relative error is also smaller than 10 % for values of thymidine incorporation over 1000 counts/min/10^6 cells. For data below these limits the relative errors showed a rapid increase.

Bone marrow S-phase compartment before treatment

The relative number of proliferating cells before Ara-C injection differs between patients. The percentage S-phase cells determined by labeling index varied from 3.4 to 20.5 %. The S-phase compartment as determined by DNA-flow cytometry ranged from 4.7 to 21.6 %. Correlation between these methods was good (correlation coefficient 0.93). In one patient (No. 9) the number of S-phase cells could not be calculated from the DNA-histogram because of polyploidy. Incorporation of ^{3}H-thymidine ranged from 4400 to 59.700 counts/min/10^6 cells. For all patients, except one (No. 2) ^{3}H-thymidine incorporation correlated with the labeling index (correlation coefficient 0.87, patient 2 omitted) and with flow cytometry (correlation coefficient 0.93, patient 2 omitted).

Perturbation in S-phase compartment of bone marrow cells after Ara-C injection

Changes in labeling index. Labeling indices determined 7 h after Ara-C administration were significantly below pretreatment values in all evaluable patients. The data, found 23 h after injection, were comparable to pretreatment values. Labeling indiced obtained after Ara-C administration did not exceed significantly pretreatment values, except in patient No. 3 (Table 2).

Patient	Doses Ara-C (mg/m^2)	Hours after Ara-C administration 0	1	7	23	31	47	70
1	1000	12 0		0 9	8 1	11 7	14 2	
2	1000	20 5		4 0		13 4	12 0	
3	1000	6 8		1 6		16 4	13 9	6 4
4	380	3 4		0 0	3 0	3 4	3 3	
5	380	18 1		2 5	12 2	∞	13 7	
6	380	13 4		5 2	∞	13 2	16 2	
7	380	8 2		1 3	8 7	6 0	6 7	
8	100	19 0	1 0	∞	16 9	17 2		
9	100	3 9	0 0	0 0	6 1	∞		
10	100	12 4		3 9	12 2	9 5		
11	100							

∞ Aspirate contained more than 50 % peripheral nucleated cells

Table 2. Changes in labeling index in bone marrow cells after Ara-C administration.

Patient	Doses Ara-C (mg/m^2)	Hours after Ara-C administration 0	1	7	23	31	47	70
1	1000	11 0		7 3	10 0	11 1	17 9	
2	1000	21 6		19 2		27 2	18 2	
3	1000	9 8		4 5		14 0	12 3	7 1
4	380	4 7		4 0	6 7	8 3	6 8	
5	380	14 3		10 3	17 7	∞	16 4	
6	380	14 7		6 1	∞	19 3	19 1	
7	380	9 2		9 4	13 3	13 6	10 5	
8	100	17 4	16 3	∞	18 0	23 8		
9	100	polyploidy						
10	100	11 4		7 8	14 7	16 0		
11	100							

∞ Aspirate contained more than 50 % peripheral nucleated cells

Table 3. Changes in percentage bone marrow cells with > 2n < 4n DNA after Ara-C administration.

Changes in flow cytometric measurements. A slight decline in the percentage S-phase cells was observed 7 h after Ara-C injection. After 23 h in 5 out of 7 evaluable patients, these values returned to pretreatment levels and showed a further increase thereafter. In all 8 patients, evaluable 31 h after injection the percentage S-phase cells exceeded significantly pretreatment values, and tended to decline thereafter (Table 3).

Changes in ^{3}H-thymidine incorporation. ^{3}H-thymidine incorporation was significantly decreased 1 and 7 h after Ara-C administration. The incorporation measured 23 h after injection was comparable with the incorporation before Ara-C administration. A marked increase was observed thereafter in most patients. In 7 our of 10 patients maximal values obtained ranked higher than pretreatment values (1.5 to 2.5 times higher). In patient 4, the elevation may have been missed as the 31 h sample could not be analysed.

In patient No. 2, thymidine incorporation did not return to the pretreatment level. In this case, thymidine incorporation before Ara-C was extremely high and not proportional to the percentage of S-phase cells. In patient No. 10, only a slight increase was observed. The results are given in Table 4.

Patient	Doses Ara-C (mg/m^2)	0	1	7	23	31	47	70
1	1000	16,867		7405	25,776	41,116	42,428	
2	1000	59,745		8275		20,759	17,775	
3	1000	11,027		2033		17,337	14,467	8818
4	380	4436		1195	3917	‡	3985	
5	380	14,343		2057	15,329	*	26,338	
6	380	19,735		4746	*	35,512	31,769	
7	380	10,772		4495	14,764	19,315	12,325	
8	100	24,957	534	*	17,667	44,019		
9	100	6230	1503	1038	20,358	7543		
10	100	18,300		7613	19,125	8119		
11	100							

∞ Counts/min/10^6 cells

* Aspirate contained more than 50 % peripheral nucleated cells

‡ Sample contaminated with bacteria.

Table 4. Changes in ^{3}H-thymidine incorporation$^{\infty}$ in bone marrow cells after Ara-C administration.

Patient	Doses Ara-C (mg/m^2)	Hours after Ara-C administration 0	1	7	12	23	27	31	47	70	r†
1	1000	565		281		848		953	1054		0.97
2	1000	6496		909				1996	1626		0 99
3	1000	1490		878				2185	1836	1118	0 96
4	380	676		473		1456		‡	1078		0 81
5	380	554		340		837		1397	1283		0 96
6	380	8708		4057		11,272		10,922	6509		0 89
7	380	3741		1472		5125		7125	5535		0.98
8	100	5378	259	569	657	3596	4823	6403			0.96
9	100	1982	279	910		3936	1604	1640	2294		0 98
10	100	370									
11	100	2699	955	2045	3083	2935					

Δ Counts/min/10^6 cells

† Correlation coefficient of ^{3}H-thymidine incorporation in bone marrow and peripheral blood

‡ Sample contaminated with bacteria

Table 5. Changes in ^{3}H-thymidine incorporation in peripheral blood cells after Ara-C administration.

Cytokinetic measurements in peripheral blood

As the percentage of proliferating cells in peripheral blood is mostly very low, relative errors in labeling indices and flow cytometric data are too high for accurate measurements. ^{3}H-thymidine incorporation is a more sensitive parameter and in 10 out of 11 patients it was high enough to produce evaluable results. Only in one patient (No. 10) ^{3}H-thymidine incorporation was too low for evaluation (less than 500 counts/min/10^6 cells). In all patients thymidine incorporation in peripheral blood showed the same pattern as in bone marrow, although at a lower level. Correlation between thymidine incorporation in bone marrow and peripheral blood in individual patients is expressed in their correlation coefficients, ranging from 0.81 to 0.99 (Table 5).

Effect of the dosage of Ara-C

The perturbation of the cell-cycle by the three different dosages Ara-C (100 mg/m^2, 380 mg/m^2, 1000 mg/m^2) shows no essential differences.

As is shown in this study, the percentage of nucleated cells derived from the peripheral blood in a bone marrow aspirate may vary considerably To our knowledge, this contamination was never taken into account in studies about cell-kinetic changes after cytostatic therapy which may explain fluctuations in previously published results (23,24,25,26). After elimination of the influence of peripheral blood admixture, the cytokinetic data of the bone marrow show good similarity in all patients Furthermore, correction unmasked a good correlation between ^{3}H-thymidine incorporation in the bone marrow cells and in the peripheral nucleated cells

No studies are available which compare the results of autoradiography, DNA-flow cytometry and ^{3}H-thymidine incorporation of leukemic cells before and during 48 h after Ara-C administration. Before Ara-C injection a good correlation exists between the three methods. After Ara-C administration this correlation appeared to be disturbed The differences between the patterns obtained with each method provides additional information about the cytokinetic effects of Ara-C.

After an initial decrease, *labeling indices* returned to pretreatment values. A pronounced increase in the labeling index as described in animal models (16) was not observed. Our data confirm the results of Ernst et al (23) and Lange Wantzin et al (26) Lampkin et al (25) found a significant increase of the labeling index in half of the patients 18 to 24 h after Ara-C administration. This observation may be caused by different amounts of peripheral blood admixture in the bone marrow sample before Ara-C injection and the sample obtained 18 to 24 h after injection

The decrease in the percentage S-phase cells, measured with *DNA-flow cytometry*, was less pronounced than that estimated from labeling indices. Maximal values estimated with flow cytometry were found to be about 1.5 times pretreatment values The discrepancy between labeling indices and flow cytometric data may be explained by the presence of cells arrested in S-phase. The decrease in the *^{3}H-thymidine incorporation* after Ara-C injection was proportional to the decrease in the labeling index. Pretreatment levels were reached after about 24 h. Thereafter thymidine incorporation showed a further rise to maxima of about twice pretreatment values. Since the labeling indices did not increase significantly, the increased thymidine incorporation may be due, for instance, to partial synchronization of cells in mid-S-phase (27,28).

In conclusion, changes suggesting recruitment of leukemic cells by Ara-C were not observed; the data are suggestive for the occurrence of partial synchronization; evidence is found for the presence of cells arrested in S-phase after Ara-C injection.

Thymidine incorporation in peripheral blood cells showed a close correlation with data obtained in the corresponding bone marrow (Table 5). Therefore, incorporation studies in peripheral blood may be a suitable method for monitoring the cell-kinetic response in individual patients after Ara-C injection.

No essential differences in the perturbation of the cell-cycle were observed with respect to the three different dosages, albeit that a dose of 100 mg/m^2 Ara-C may not always be high enough to produce a sufficient inhibition of the DNA-synthesis (24) and to damage S-phase cells irreversibly. The early increase of the thymidine incorporation in patient No. 11 may be due to such a phenomenon.

In almost all patients the relative size of the S-phase compartment returned to pretreatment levels about 24 h after Ara-C administration. Therefore treatment schedules with intervals of 24 h will probably result in more effective cell kill, compared to schedules with shorter intervals.

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CHAPTER VIII

SIMPLIFIED METHOD FOR DNA AND PROTEIN STAINING OF HUMAN HEMATOPOIETIC CELL SAMPLES

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ABSTRACT

A rapid reproducible method yielding high resolution analysis of DNA and protein in human hematopoietic cell samples has been developed by modification of the propidium iodide (PI) and fluorescein isothiocyanate (FITC) procedure. Cell staining involves sequential addition of each reagent (RNase, FITC and PI) to ethanol-fixed cells and requires no centrifugation steps.

Stained cells are analysed in the reagent solutions. Analysis of bone marrow samples from multiple myeloma patients showed mixed normal and aneuploid populations with the aneuploid cells having a significantly higher protein content.

This approach permitted differential cell cycle kinetic analysis of normal and the aneuploid populations.

INTRODUCTION

Analysis of cellular DNA content by flow cytometry (FCM) provides a rapid and convenient method for studying cell cycle kinetics (1,2) and population ploidy levels (3,4). However, in heterogeneous cell populations such as found in human bone marrow, the kinetic patterns are a composite of all hematopoietic cell types and cell cycle progression profiles, for subpopulations of interest,are not easily derived. Furthermore, when malignant aneuploid cells become prominent in the FCM-distributions (5), cell cycle kinetic analysis by single parameter DNA content measurements is not possible.

Protein content of cells reflects in a general way, the metabolic capacity of cells and, in most instances, is also a good descriptor for protoplasmic mass. Simultaneous FCM analysis of cells stained for both DNA and protein can reveal subpopulations of different protein content located throughout the cell cycle or within aneuploid cell populations. Additional descriptive information such as the nuclear to cytoplasmic relationship (6) and the DNA to protein ratios for subpopulations can be determined by FCM (7) and this approach can further enhance the analysis of heterogeneous cell populations.

This report describes a rapid and simplified method for staining hematopoietic cells with propidium iodide (PI) and fluorescein isothiocyanate (FITC). In contrast to our previous protocol (7), all reagents are added sequentially to ethanol-fixed cells and no centrifugation steps are required. Staining time is reduced to 10 minutes as compared to 3 h by our previous method (7).

MATERIALS AND METHODS

Fresh aspirated bone marrow obtained by sternal puncture was diluted in ACD-solution (acid citrate dextrose). Red blood cells were removed by centrifugation over Percoll (density 1.085 g/ml) (Pharmacia Fine Chemicals). The supernatant cell suspension was diluted for fold with phosphate buffered saline solution containing 13 mM disodium EDTA (PBS-EDTA). White blood cells were recovered by centrifugation, and fixed in 70 % ethanol as a total bone marrow sample. To avoid cell clumping, cells were first thoroughly resuspended in 1 ml of cold PBS-EDTA solution and then 3 ml of cold 95 % ethanol was added to the cell suspension.
Alternatively, unfixed bone marrow samples were separated by cell elutriation into subpopulations on the basis of cell size. A small proportion of

unfixed cells from each subpopulation was stained for cellular DNA directly, by a method similar to that described by Krishan (8) except that ethidium bromide (25 µg/ml in 0.1 % sodium citrate) was used in place of propidium iodide (PI). The remaining portion was fixed in 70 % ethanol as above.

For two color DNA-protein staining cell reagents were added sequentially to ethanol-fixed cells and no centrifugation steps were required. Four ml of PBS-EDTA solution were added to 0.2 ml of ethanol fixative containing 5 x 10^6 cells. Fluorescein isothiocyanate (1 mg/ml) was initially dissolved in absolute ethanol *immediately prior to use*, and 0.1 ml of this stock solution was added to 10 ml of PBS-EDTA solution. Two-tenths ml of the FITC in PBS-EDTA solution was added to the cell suspension and after approximately 5 min, one ml of propidium iodide (46 µg/ml) was also added to the cell suspension. After 2-3 min, 0.2 RNase (1 mg/ml in PBS-EDTA) at room temperature was added for at least 1-2 min prior to analysis. Final concentrations of PI, FITC and RNase were 8.5, 0.37, and 37 µg/ml respectively. Stained cells in the reagent solutions were analyzed in a Phywé ICP 11 pulse cytophotometer modified for two-color analysis.

RESULTS

Figure 1 (A-D) and Figure 2 (A-D) show the DNA-protein distributions respectively obtained by analysis of total bone marrow samples from a normal donor and a multiple myeloma patient following staining with PI and FITC. The DNA-profiles (Fig. 1A and 2A) each show a G_0/G_1 peak (major peak) representing a 2c DNA content and a corresponding 4c DNA content (G_2 + M) population based on analysis of the DNA content of nucleated peripheral blood cells from a healthy person. However, the arrow in Figure 2A indicate a small but significant population of aneuploid cells with an elevated DNA-content. Comparison of Figure 1D and Figure 2D also shows that this population has a higher average protein content than the remaining cells in the 2c-4c DNA-content range.

Verification of these observations was made by DNA/protein analysis of a subpopulation of cells from the same myeloma patient obtained following cell elutriation. Figure 3 and Figure 4 respectively show composites of the DNA profiles of subpopulations of cells from a normal donor (Figure 3) and from the multiple myeloma patient (Figure 4). The DNA profiles are arranged in order of increasing cell size (front to rear) as indicated by the arrow in Figure 3. In general, Figure 4 shows an enrichment of the aneuploid

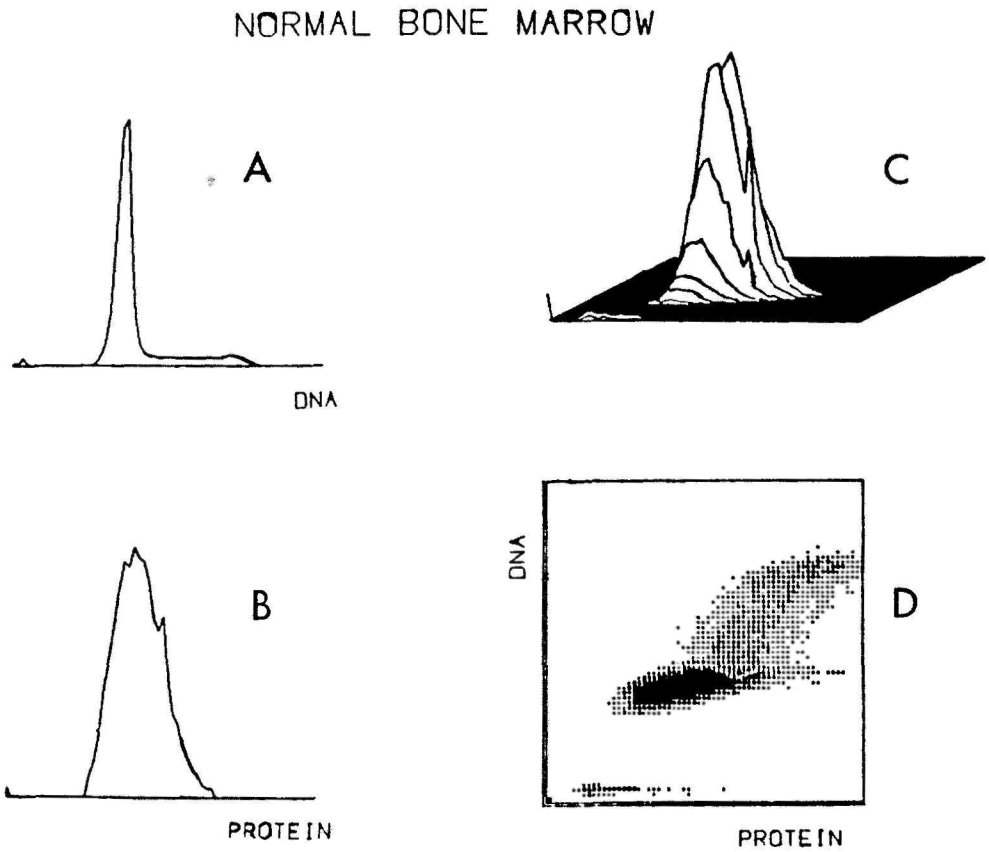

Figure 1. Single parameter DNA (A), protein (B) and two parameter DNA-protein (C, isometric and D, contour) profiles of bone marrow cells from a normal donor.

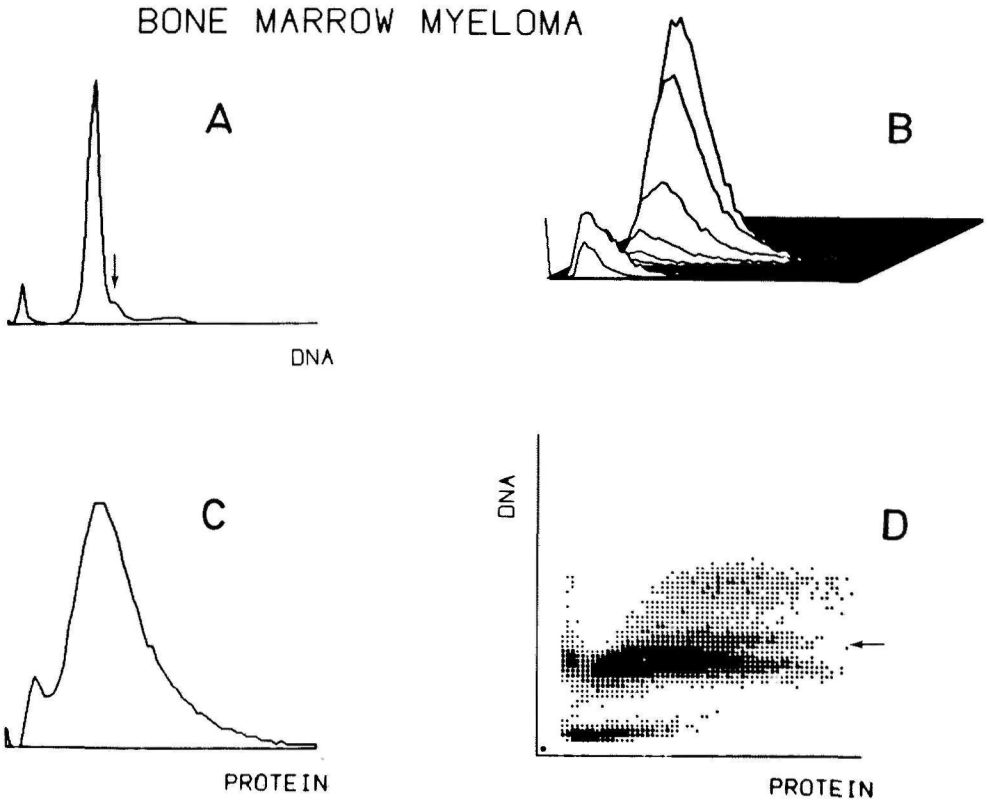

Figure 2. Single parameter DNA (A), protein (B) and two parameter DNA-protein (C, isometric and D, contour) profiles of cells of a total bone marrow sample obtained from a multiple myeloma patient. Arrows in A and D indicate position of G_0/G_1 cells of the aneuploid population.

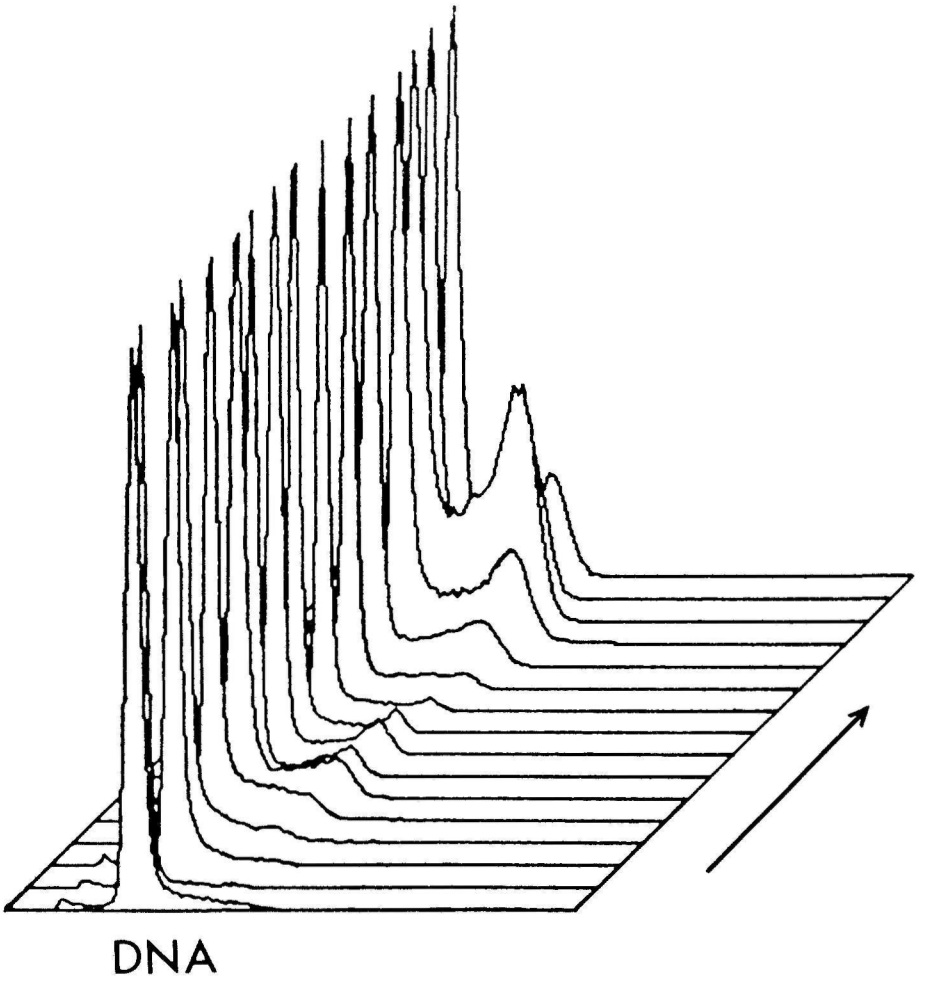

Figure 3. Composite of ethidium bromide-DNA profiles for subpopulations of cells obtained by cell elutriation of a normal bone marrow sample. Arrow indicates increasing cell size.

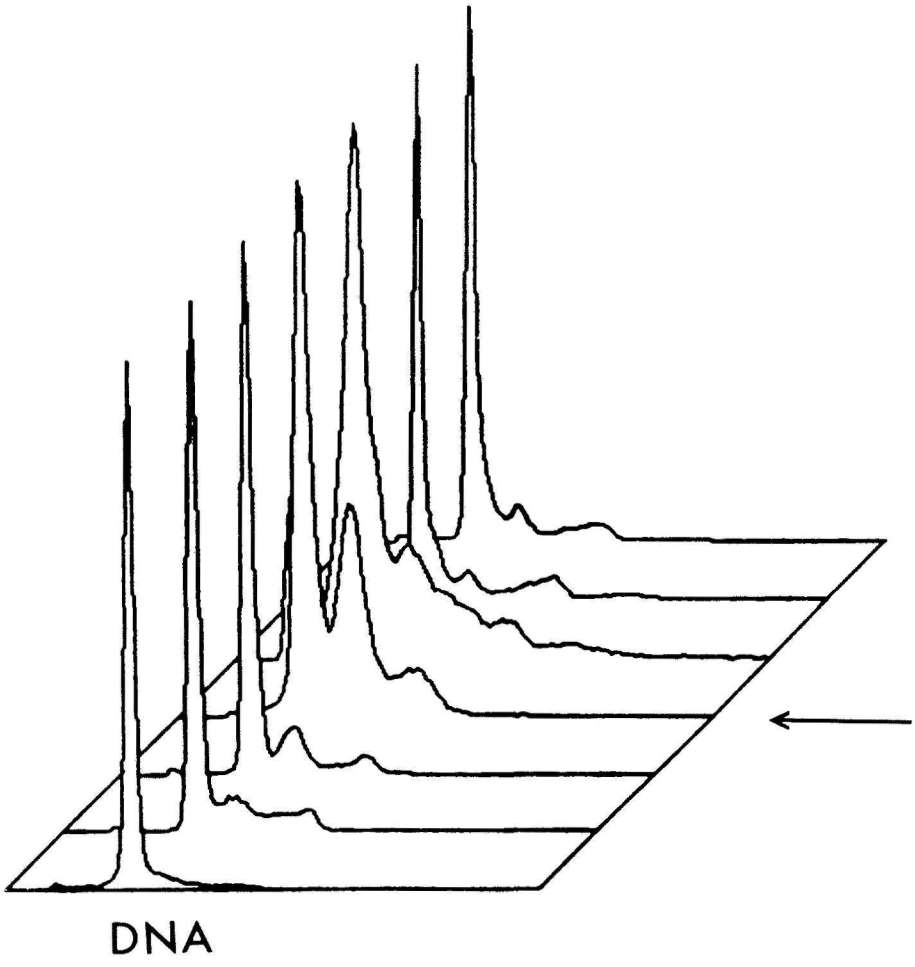

Figure 4. Composite of ethidium bromide-DNA profiles for subpopulations of cells obtained by cell elutriation of a bone marrow sample from a multiple myeloma patient. Arrow indicates subpopulation described in Figures 5 and 6.

cells in some subpopulations of larger cells, while Figure 3 shows primarily an enrichment of the G_2 + M population with increasing cell size.

Figure 5 shows the DNA-protein distributions for the subpopulation of cells indicated by the arrow in Figure 4. The contour DNA-protein profile (Figure 5D) clearly demonstrates the elevated protein content in the aneuploid cells. In fact the protein content of the G_0/G_1 aneuploid cells exceeds the protein content levels of the normal S-phase cells of this subpopulation having the same DNA content as the aneuploid cells.

Figure 6 shows the complete DNA-histogram (Figure 6A) and the contour DNA-protein distribution (Figure 6B) as shown for the myeloma sample in Figure 5. The DNA profile shown in Figure 6C was obtained by computer extraction of the aneuploid population in Figure 6B based on the elevated DNA and protein contents of the aneuploid cells.

DISCUSSION

Protocols for cell staining, particularly in routine clinical studies, should be simple, rapid, highly reproducible and still provide for quality analytical resolution. In this study we have described a useful method for DNA and protein staining of human hematopoietic cell samples using PI and FITC, respectively. Cells are treated sequentially with the reagent solutions, and rinsing or centrifugation steps are not required.
The approach not only increases the rapidity of the method compared to our original procedure (7), (i.e. 10 min vs 3 h, respectively), but it also minimizes cell clumping and cell loss which can be a particular problem in hematopoietic cell samples.

The two parameter profile obtained by analysis of the PI-FITC stained bone marrow sample demonstrates the high quality color resolution that is obtainable with this technique. By maintaining the stain concentration at a low level, particularly the highly fluorescent FITC, background fluorescence remains minimal. However, the dye concentrations are still adequate to provide good resolution for detecting abnormalities such as the aneuploidy population in the DNA-profile.

From a clinical standpoint, the procedure appears particularly attractive for separating the cell cycle distribution profile of the aneuploid population from the normal 2c-4c DNA-content population. In a recent autoradiographic study by Durie et al (9), analysis of the percentage of cells in

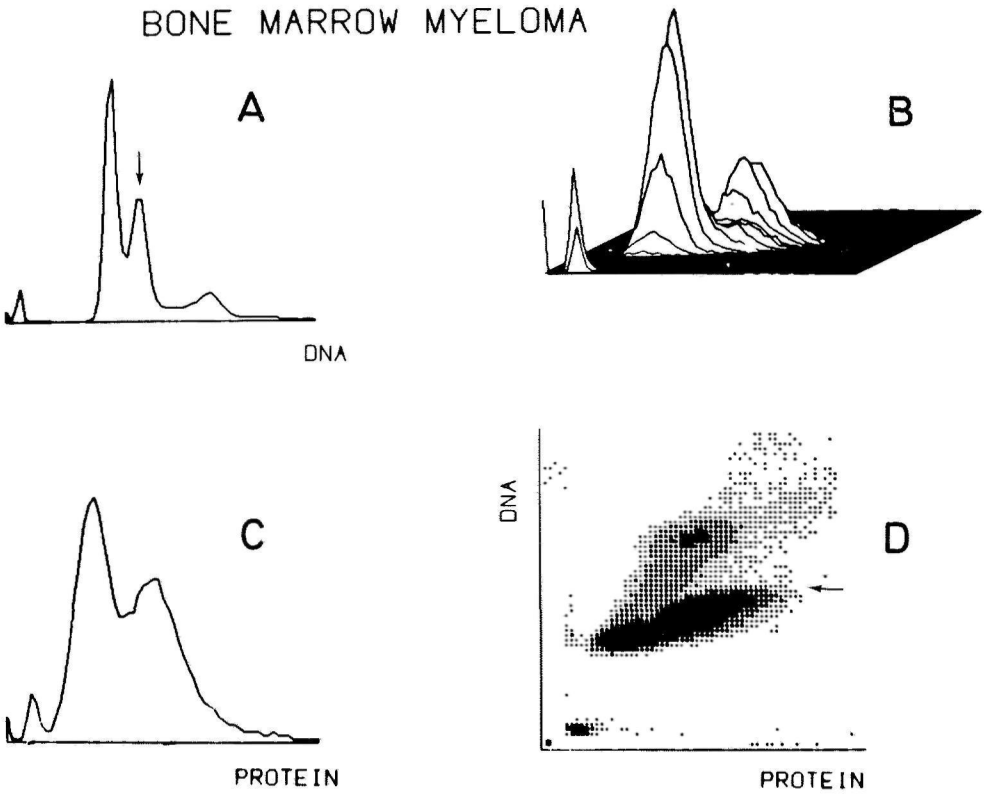

Figure 5. Single parameter DNA (A), protein (B) and two parameter DNA-protein (C, isometric and D, contour) profiles of a subpopulation of cells following cell elutriation of a bone marrow sample obtained from a multiple myeloma patient.
Arrows in A and D indicate position of G_0/G_1 cells of the aneuploid population.

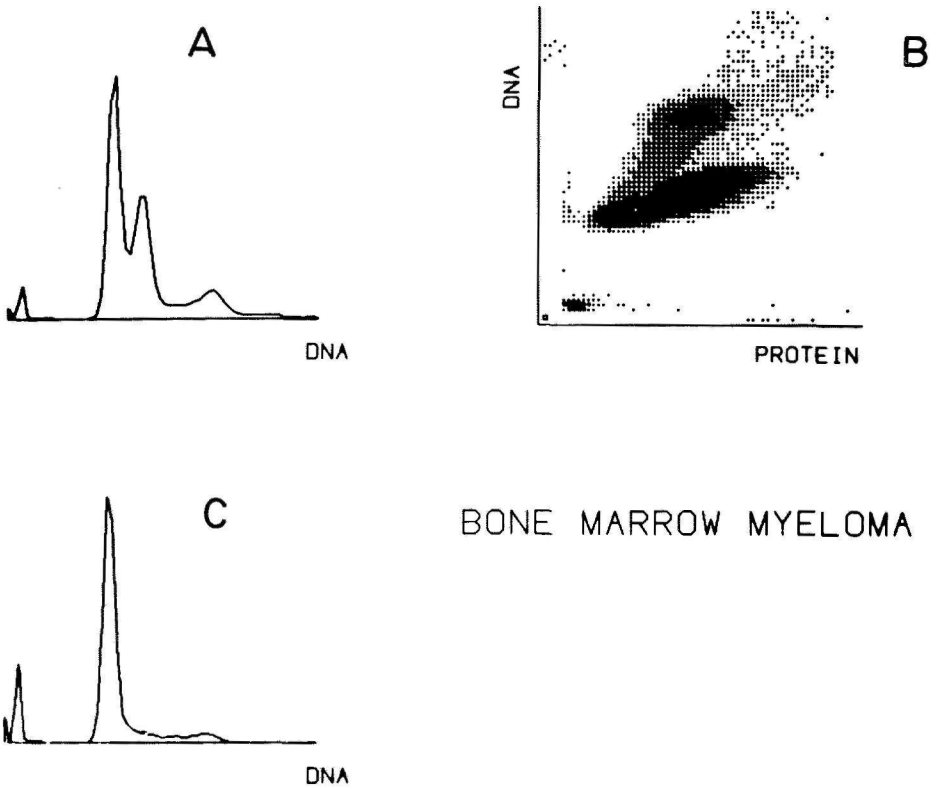

Figure 6. Single parameter DNA (A) and two parameter DNA-protein contour (B) profiles for cells described in Figure 4. The DNA profile in C was obtained by computer extraction (gated analysis) of the aneuploid cells.

S-phase (tritiated thymidine labeling index, LI) in bone marrow cells of untreated multiple myeloma patients provided helpful prognostic information. In general that study showed that pretreatment LI > 3 % in high cell mass patients conferred a poor prognosis compared to patients with LI < 1 %. A recent report by Latreille et al (5) revealed a 65 % incidence of aneuploidy in myeloma patients and demonstrated that the frequency of myeloma plasma cells correlated with the percentage of cells with abnormal DNA-content.
Based on these studies it appears that simultaneous FCM DNA-protein analysis can further increase the sensitivity of the diagnostic procedure of Durie et al (9). By analyzing the kinetic profile of the aneuploid population separate from the other cell populations it is possible to determine, in each instance, which population contributes most significantly to the elevated percentage of cells in S-phase.

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CHAPTER IX

FLOW CYTOMETRIC ANALYSIS OF BONE MARROW SUBPOPULATIONS OBTAINED WITH COUNTER-FLOW CENTRIFUGATION IN PATIENTS WITH MULTIPLE MYELOMA AND BENIGN MONOCLONAL GAMMOPATHY

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ABSTRACT

To augment the potential of flow cytometric analysis (FCM) of plasma cells, bone marrow aspirates of 12 patients with multiple myeloma (MM) and 3 patients with benign monoclonal gammopathy (BMG) were subjected to counterflow centrifugation (elutriation) to obtain enriched plasma cell fractions.

DNA-FCM analysis of these cell fractions in particular patients revealed the occurrence of ploidy abnormalities, proliferating plasma cells and multinucleated plasma cells, which was not apparent from DNA-FCM analysis of whole bone marrow aspirates.

Two parameter DNA/protein-FCM of plasma cell enriched subpopulations additionally allows discrimination between plasma cells and other bone marrow cells by the high protein content of plasma cells, and enables the separate analysis of plasma cell proliferative activity.

INTRODUCTION

Introduction of DNA-flow cytometry (FCM) has provided means to obtain information about proliferation patterns and ploidy abnormalities in hematologic malignancies (1). However, application of FCM to the study of plasma cells in patients with gammopathies is limited by the heterogeneity of bone marrow cells and the low percentages of plasma cells in some cases.

In the present study, bone marrow aspirates of patients with multiple myeloma (MM) and benign monoclonal gammopathy (BMG) were subjected to counterflow centrifugation (elutriation) to obtain enriched plasma cell fractions for subsequent FCM-analysis.

The results in a limited group of patients indicate, that plasma cell enrichment with elutriation considerably augments the discriminatory potential of FCM with regard to various plasma cell characteristics.

MATERIALS AND METHODS

Sample processing

Bone marrow aspirates from 12 patients with MM and 3 patients with BMG, were anticoagulated with acid citrate dextrose (ACD formula A) and wahsed once with phosphate buffered saline (PBS). Red blood cells were removed by centrifugation over Percoll (density 1.085 g/ml, Pharmacia Fine Chemicals) the supernatant cells were washed and resuspended in PBS and subsequently counted.

Counterflow centrifugation (elutriation

A Beckmann elutriator system (J2-21C + JE-6 rotor), adapted with a fine scaled speed selector and a 47 PF condensor for improved stability of the rotorspeed was utilized in this study. The collecting tubes were filled with PBS containing 1 % fetal calf serum, whereupon the cell suspension was slowly introduced into the elutriation rotor at a rate of 0.3 ml/min. Elutriation was initiated at a rotorspeed of 2500 rpm and a flow rate of 20 ml/min and was accomplished by a 5-steps decrease of the rotorspeed to 1000 rpm. A T-tube mounted up the effluent line enabled a small sample stream to pass an electro-optical scatter unit, which permitted a continuous control of cellnumber and cellsize in the elutriator effluent during the whole procedure (2). When the output became less than 100 cells/sec, the rotorspeed of the centrifuge was decreased stepwise as indicated.

The 6 cell fractions were collected, centrifuged and resuspended in 3 ml PBS. Part of the specimens were used for morphological analysis of May Grünwald-Giemsa stained cytocentrifuge slides. The bulk of the cellsuspensions were used for FCM-analysis.

Cell staining and flow cytometry (FCM)

Staining for DNA occurred according to Krishan by adding 0.5 x 10^6 cells to 10 ml hypotonic ethidium bromide (0.1 % trisodium citrate, 25 mg/l ethidium bromide) (3). The relative fluorescence of about 10^5 cells was measured with an ICP-11 pulse cytophotometer (Phywé, FRG) at a flow rate of 400 cells/sec.

Simultaneous staining for DNA and protein was performed as described recently by Crissman et al (4). Briefly, 1 ml of the cell suspension was fixed in 75 % ethanol. After 24 hours 0.2 ml of the fixed cell suspension was added to 4 ml PBS containing 13 mM disodium EDTA (PBS-EDTA) and stained with FITC and Propidium Iodide (PI). For this purpose FITC was dissolved in absolute ethanol (1 mg/ml) of which 0.1 ml was added to 10 ml PBS-EDTA. Of this solution 0.2 ml was immediately added to the cellsuspension. After approximately 5 minutes 1 ml of PI (46 mg/ml) was also added to the cell suspension. After 2-3 minutes 0.2 ml RNA-se (1 mg/ml PBS-EDTA) was added for at least 1-2 minutes prior to FCM-analysis. Stained cells in the reagent solution were analyzed in a Phywé ICP-11 pulse cytophotometer, modified for two color analysis. The green (< 550 nm) and red (> 590 nm) fluorescence emissions were separated by dichromatic filters and measured by separate photomultipliers. The signals were integrated in a multichannel analyzer with display facilities on a magnetic tape and further processed by a PDP 11/45 computer. Routinely 50.000 cells were measured for DNA-content analysis, and 15.000 cells in 2 parameter experiments.

RESULTS

Plasma cell enrichment with counterflow centrifugation (elutriation)

Table I shows total cell numbers and plasma cell percentages present in bone marrow aspirates and fractions obtained with elutriation. In most cases a substantial increase in the relative plasma cell concentration was obtained.

The mean maximal increment in plasma cell percentages was 3.7 x the plasma cell percentage of the whole bone marrow aspirate (range 1.4 - 14.1 x). The

No.	Diagnosis	Bone marrow aspirate		Fraction 1 (2500 rpm)		Fraction 2 (2250 rpm)		Fraction 3 (2000 rpm)		Fraction 4 (1750 rpm)		Fraction 5 (1500 rpm)		Fraction 6 (1000 rpm)		Cell recovery	
		PC %	$n(10^6)$	PC %	$n(10^6)$	PC %	$n(10^6)$	PC %	$n(10^6)$	PC %	$n(10^6)$	PC %	$n(10^6)$	PC %	$n(10^6)$	PC %	Total %
1	BMG	2	98.5	0	11.7	1	16.7	2	23.7	2	7.2	2	2.2	3	1.7	45	64
2	BMG	4	42.1	0	12.1	1	4.6	2	7.2	6	5.1	14	2.1	13	0.3	49	74
3	MM	6	129.0	0	30.8	0	25.2	0	24.2	1	10.5	63	4.2	85	2.8	66	76
4	MM	8	108.0	0	22.2	0	23.2	2	18.9	17	15.7	24	7.5	63	4.6	90	85
5	BMG	11	22.0	0	3.4	1	3.6	8	2.7	25	5.6	27	1.4	38	0.3	89	77
6	MM	12	100.0	0	26.6	8	16.5	11	28.6	34	12.9	34	1.4	37	1.1	81	87
7	MM	20	61.1	0	19.0	8	6.9	22	11.9	58	4.0	62	6.7	40	0.6	81	80
8	MM	21	51.4	0	16.2	12	15.9	58	7.3	68	2.2	66	0.7	72	0.8	80	84
9	MM	21	73.1	0	15.5	0	4.7	20	20.0	57	12.7	46	1.8	55	1.4	84	77
10	MM	22	101.0	0	15.0	3	7.0	8	11.2	12	20.2	71	18.0	76	2.2	81	73
11	MM	24	42.7	0	12.8	6	3.4	48	8.3	51	4.8	19	1.4	10	0.5	68	73
12	MM	28	91.4	0	28.4	2	14.3	66	12.1	67	8.6	70	4.3	75	2.4	74	76
13	MM	43	35.1	0	21.4	10	11.3	32	18.4	72	23.1	81	22.1	68	4.6	77	75
14	MM	45	40.0	0	8.8	45	1.4	19	4.4	48	8.2	82	7.6	85	5.2	88	89
15	MM	67	158.0	0	15.0	90	18.0	79	47.2	45	17.2	67	3.8	94	1,7	63	65

BMG = Benign Monoclonal Gammopathy
MM = Multiple Myeloma.
PC% = Plasma Cell Percentage.

Table 1. Plasma cell percentages and absolute cell numbers in different bone marrow fractions obtained with counterflow centrifugation

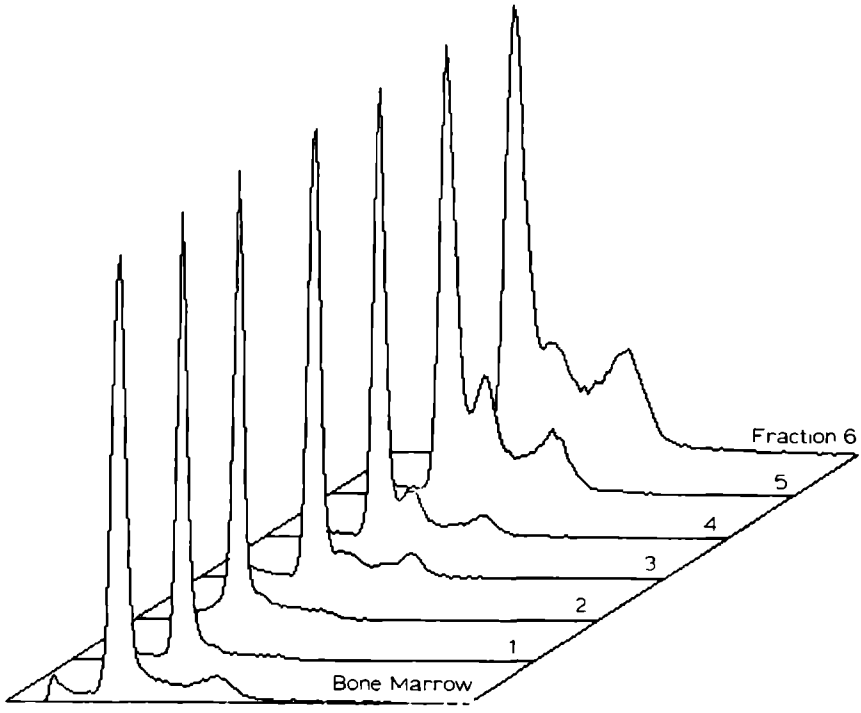

Figure 1. Composite of DNA-profiles of unfractionated bone marrow and bone marrow subpopulations, obtained with elutriation.

total cell recovery was 77 ± 7.1 % (mean ± SD) and the plasma cell recovery 74.4 ± 13.7 %.

FCM-analysis of enriched plasma cell fractions

One parameter DNA-FCM revealed the presence of aneuploidy in the whole bone marrow aspirate in 9 out of 12 patients with MM and in none of those with BMG. After elutriation, the presence of aneuploidy could be demonstrated in two additional patients with MM (patient No. 3 and 4) and in one patient with BMG (Patient No. 5) in whom the DNA-profiles of the unfractionated bone marrow samples were entirely normal (Figure 1).

Figure 2 shows the DNA-profiles of the bone marrow (2a) and one of the bone marrow

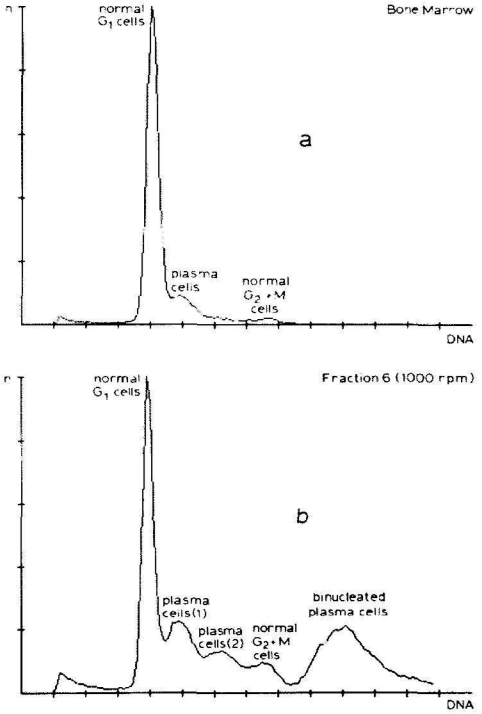

Figure 2. DNA-profiles of unfractionated bone marrow (a) and of a bone marrow fraction (b).

fractions (2b) in a myeloma patient (No. 10). In this patient elutriation unmasked the presence of a second aneuploid plasma cell population and a large number of binucleated cells. In another myeloma patient (No. 15) binucleated cells and multinucleated plasma cells were identified (Figure 3), which was confirmed by morphological examination of the cytocentrifuge slides. The presence of multinucleated cells was disclosed with elutriation in 6 out of 12 myeloma patients.

In one myeloma patient (No. 14) both aneuploidy and tetraploidy could be demonstrated in bone marrow. The tetraploid cells in this patient were actively proliferating as was disclosed with elutriation by the demonstration of S- and G_2 + M-phase cells of this particular cell population (Figure 4).

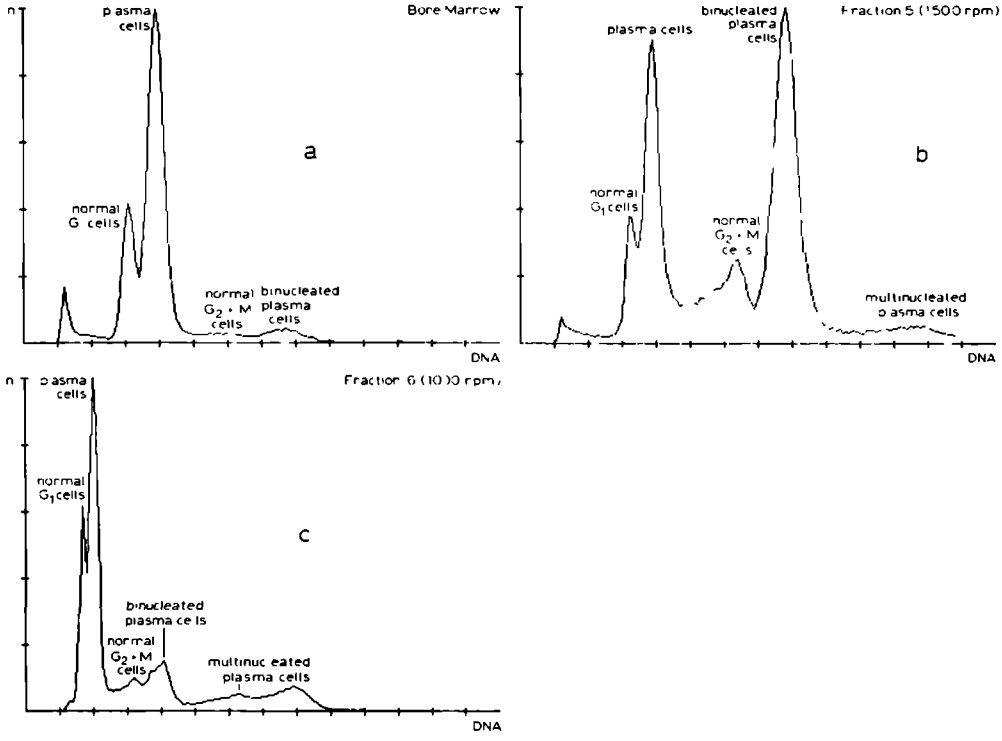

Figure 3. DNA-profiles of unfractionated bone marrow (a) and two successive bone marrow fractions (b and c).
To demonstrate the multinucleated cells in fraction 6 the gain setting of the instrument was changed.

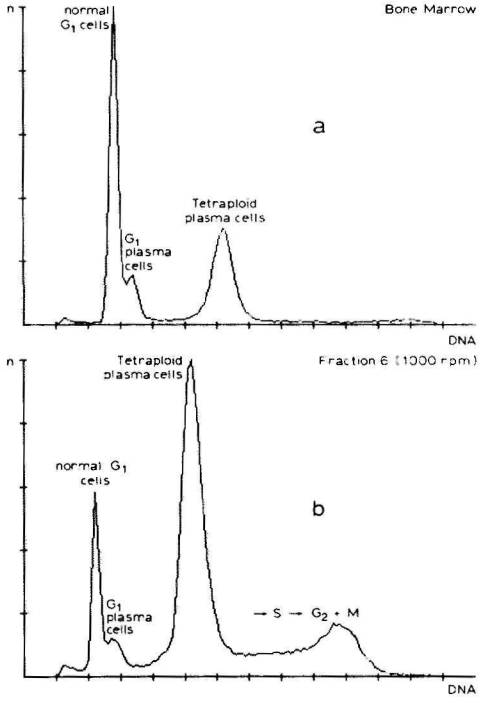

Figure 4. DNA-profiles of unfractionated bone marrow (a) and one of the bone marrow fractions (b).

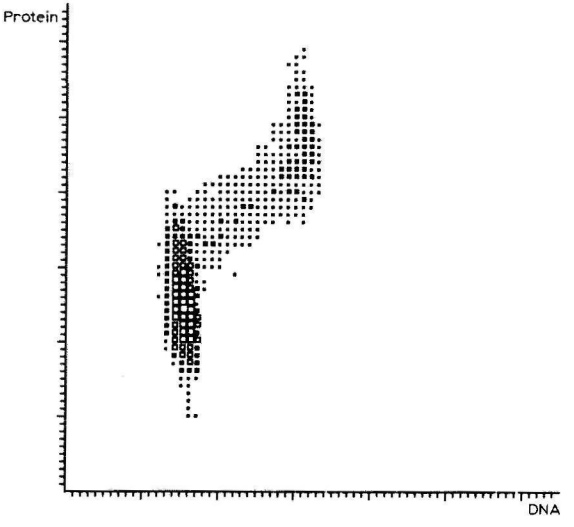

Figure 5. A DNA/protein histogram of normal bone marrow.

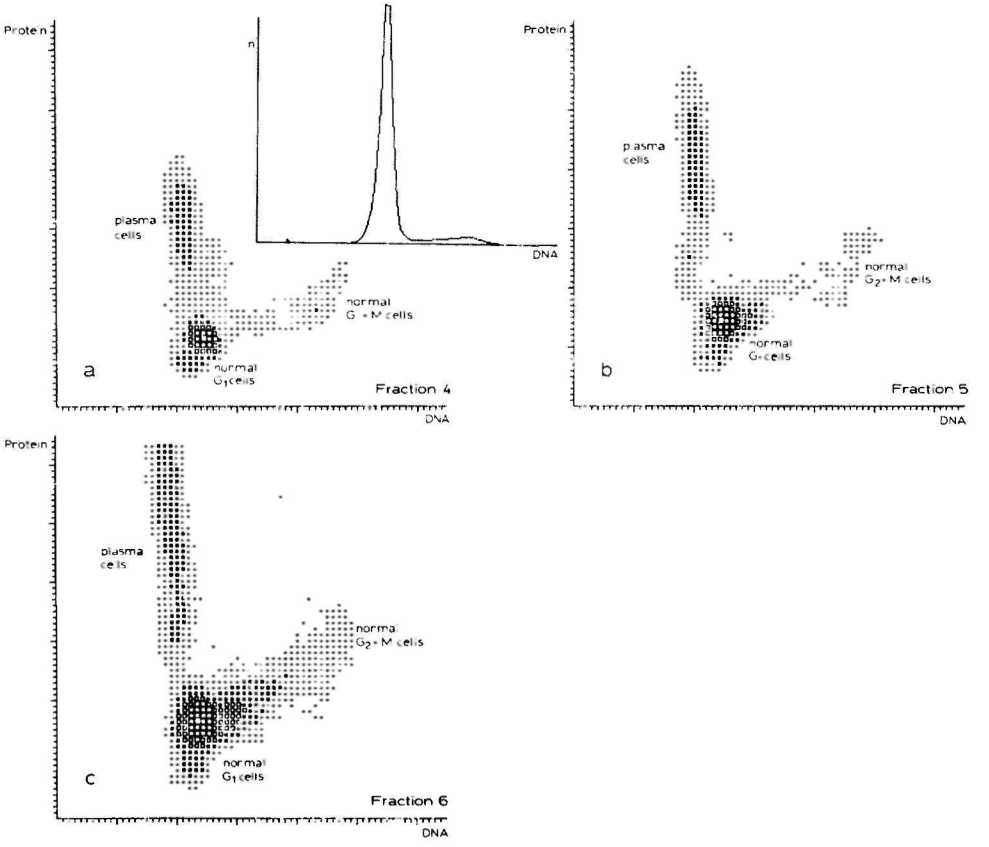

Figure 6. DNA/protein histograms of three successive bone marrow fractions of a myeloma patient with hypodiploid plasma cells. (the one parameter DNA-histogram is shown in figure 6-a, as well).

Two parameter DNA/protein-FCM of normal bone marrow cells (Figure 5) revealed a progressive increase in protein content of cells during the cell cycle, with an average protein content of cells in G_2 + M-phase nearly twice as much as the average of G_1-cells In case of bone marrow plasmocytosis, the relative high protein content of plasma cells makes them easily to be discerned on a two parameter DNA/protein plot.

Figure 6 (a-c) shows the DNA/protein histograms of three successive cell fractions, obtained with counterflow centrifugation of a bone marrow sample from a myeloma patient (No. 13). Evidently the average protein content of the plasma cells increased at each successive elutriation step, which corresponds to the increase in average cell size. Moreover, these plots demonstrate, that two parameter FCM analysis disclosed two additional important plasma cell characteristics in this particular patient. First, the DNA-content of the plasma cells seems less than the DNA-content of the normal G_1-cells (hypodiploid), which was not apparent from the one parameter DNA-profiles (see Figure 6a box). Secondly, even after plasma cell enrichment, which usually is associated with the enrichment of S- and G_2 + M-phase cells, no transition of plasma cells into S-phase could be demonstrated, indicating a low proliferation activity of these cells in this particular patient. In another myeloma patient however(No 12) DNA/protein-FCM of one of the fractions disclosed transitions of both diploid (normal) and hypodiploid cells (plasma cells with high average protein content) to the S- and G_2 + M-phase Moreover, the tetraploid plasma cells even show a further increase in protein and DNA content suggesting ongoing proliferation of tetraploid plasma cells as well (Figure 7).

DISCUSSION

FCM analysis of elutriated bone marrow cell fractions enabled the detection of aneuploid cells in two patients with MM and one patient with BMG, in whom the DNA-profiles of the unfractionated bone marrow aspirates were entirely normal. A second aneuploid peak was disclosed, and a proliferating tetraploid plasma cell clone could be demonstrated after elutriation in two additional myeloma patients, whereas in other patients multinucleated cells could be shown.

Two parameter DNA/protein-FCM disclosed the existence of small degree hypodiploid plasma cells and provides means to analyze the cell kinetic profiles of plasma cells in heterogeneous cellfractions.

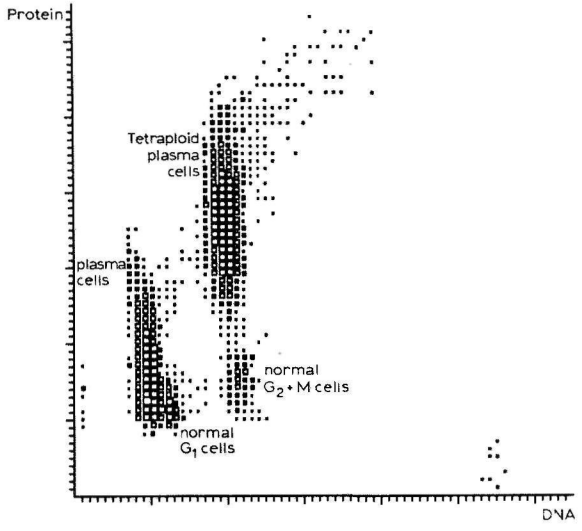

Figure 7. DNA/protein histogram of one of the bone marrow fractions of a myeloma patient.

Elutriation has the advantage over other cell-fractionating methods, to result in a high cell-recovery with intact cell-viability, whereas the simultaneous separation of multiple cell-types makes this method especially suitable for human bone marrow (4,5). In the present study, elutriation was accomplished by a 6-steps decrease of the centrifuge rotor speed, which resulted in a substantial enrichment of plasma cells in the majority of patients.

Latreille et al have reported a 65 % incidence of aneuploidy in myeloma patients (7). The results of the present study indicate, that the incidence of aneuploidy in MM might in fact be higher when plasma cell enriched fractions are studied. Of particular interest in this regard is the finding of aneuploid cells in a patient with BMG. As the presence of aneuploid cells is supposed to be indicative for malignant disease (8), the demonstration of these cells might indicate in fact the existence of a malignant gammopathy in this particular patient. In general, this finding suggest the potential of this method to differentiate between benign and malignant monoclonal gammopathies.

As shown, elutriation combined with FCM enables the study of plasma cell proliferation kinetics. Especially when cells are stained simultaneously

for DNA and protein, plasma cells can be distinguished from other bone marrow elements, which allows the simultaneous measurement of proliferation characteristics of plasma cells and other hematopoietic cells. The clinical relevance of such measurements is indicated by recent studies in which a correlation was demonstrated between disease progression and the proliferative activity of plasma cells (9,10). Moreover, such measurements may be valuable in monitoring the effects of treatment.

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CHAPTER X

CELL CYCLE ANALYSIS WITH USE OF TWO PARAMETER FLOW CYTOMETRY OF CYCLE SPECIFIC CELL-COHORTS, OBTAINED WITH COUNTERFLOW CENTRIFUGATION

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ABSTRACT

Counterflow centrifugation (elutriation) was used to separate an exponentially growing lymphoblastic cell line (RPMI 1788) in subpopulations, enriched with cells in a distinct phase of the cell cycle. The subpopulations were subsequently recultured for 18 hours, during which the proliferation kinetics was monitored with DNA-flow cytometry (FCM), two parameter DNA/RNA-FCM, ^{3}H-thymidine incorporation and cell counting.

Analysis of the flow cytometric data was performed by plotting the sequential changes in specific cell cycle compartments of the histograms.

The results indicate that counterflow centrifugation provides means to obtain unpertubred sunchronized subpopulations of cells in different phases of the cell cycle. Flow cytometric analysis of such cell-cohorts, during reculturing, permits the estimation of cell cycle phase durations.

INTRODUCTION

Counterflow centrifugation (elutriation) of cell suspensions, render cell populations, different in cell size (1). Because, during cell-cycle traverse, cells gradually increase in size and mass up to mitosis (2), elutriation provides means to obtain cell populations enriched with cells in various phases of the cell-cycle. Reculturing of these fractions subsequently results in cellkinetic profiles, similar to synchronized cell populations obtained with stathmokinetic agents or metabolic manipulations (5).

The present study was designed to determine the feasibility of combined counterflow centrifugation and flow cytometry (FCM) for the analysis of the cell cycle.

MATERIALS AND METHODS

Cells

A human lymphoblastic cell line (RPMI 1788) was maintained in RPMI-1640 containing 2 mM glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and 10 % (v/v) fetal calf serum. Cells were studied during the exponential growth phase at a temperature of 37°C in humidified air with 5 % carbon dioxide.

Counterflow centrifugation (elutriation)

For elutriation a Beckmann J2-21C centrifuge and JE-6 elutriator rotor was employed.

The rotor, separation chamber and connecting tubes were sterilized before cells were injected,by pumping 70 % ethanol through the apparatus during 30 minutes. Ethanol was removed by flushing the apparatus with 50 ml of sterile, phosphate buffered saline (PBS). Then PBS was replaced by growth medium (RPMI-1640) containing 10 % fetal calf serum before the addition of cells. Ten ml of the cell suspension (10^8 cells/ml) was injected slowly (0.3 ml/min) into the connecting tubes and subsequently pumped at 5 ml/min into the elutriation rotor. Elutriation was carried out at 37°C and initiated at a rotorspeed of 2200 rpm and at a flow rate of 9 ml/min. The procedure was accomplished by stepwise decrease of the rotorspeed. Cells in the effluent tube were monitored by an electro optical scatter unit (Technicon), allowing control of cell number and cell size during the elutriation procedure (6). The rotorspeed was decreased when the cell output became less than 100 cells/sec.

Six fractions were obtained, which were centrifuged and resuspended to a cell concentration of about 1 5 x 10^8 cells/ml Approximately 90 minutes were required to collect the fractionated cells from the elutriator rotor

Reculturing

The six fractions were diluted in RPMI to 10^6 cells/ml and each fraction distributed, over 25-30 tissue culture dishes (3 ml/dish) Culture conditions were identical to those of the unfractionated cells Cytokinetic parameters of the recultured fractions were monitored over a 18-hour period by analysis of the cellular content of one culture dish of each fraction after various time intervals with ^{3}H-thymidine incorporation, DNA-FCM, two parameter DNA/RNA-FCM and cell counting.

^{3}H-thymidine incorporation

Aliquots of 1 ml cellsuspension at half hourly or hourly intervals were incubated with 2 µCi ^{3}H-thymidine (Radiochemical Centre Amersham specific activity 5 Ci/mmol) for 60 minutes at 37°C. ^{3}H-thymidine uptake was arrested by adding 2 ml cold-medium (°C) with an excess of unlabeled thymidine. The cells were collected on glass fibre filters (Schleicher and Schull No 9), washed with water and precipitated with 1M perchloric acid The total amount of radioactivity in the perchloric acid precipitable fraction was counted in a liquid scintillation counter (LKB 81000).

Cell staining and fluorescence measurement

Staining for DNA was performed by adding 0.5 x 10^6 cells to 5 ml hypotonic ethidium bromide (0 1 % trisodium citrate, 25 mg ethodium bromide), supplemented with 0 2 ml of 0 05 % Nonidet-P40 (7,8) The relative fluorescence of 10^5 cells was measured with an ICP-11 pulsecytophotometer (Phywé, Gottingen, Germany) at a flow rate of 400 cells/sec.

Simultaneous staining for DNA and RNA was performed according to Traganos and Darzynkiewicz (9) The cell suspension (0 2 ml) was mixed with 0.4 ml of a solution containing 0.1 % (v/v) Triton X-100, 0.08 N HCl and 0 15 M NaCl. After 30 sec 1.2 ml Acridine Orange (15 µg/ml in 10^{-3} M EDTA, 0.15 M NaCl, 0.1 M phosphate-citrate buffer pH 6 0) was added resulting in a final dye concentration of 2 1 x 10^{-5}M Acridine Orange Stained cells in the reagent solution were analyzed in an ICP-11 modified for two parameter analysis The

green fluorecence (DNA) and red fluorescence (RNA) emissions were separated optically by a dichroic mirror and measured by separate photomultipliers. The electric signals were accumulated in dual parameter displays and stored on a magnetic tape for computer analysis and copying (PDP 11/45). The data from each DNA/RNA histogram are based on the analysis of 1.5×10^4 cells per sample.

Histogram analysis

Analysis of time sequences of DNA and DNA/RNA histograms was performed by selecting certain "windows" of DNA or RNA content and plotting as a function of time the relative cell numbers present in these compartments (FP_i-analysis according to Zietz 10).
In DNA histograms with the G_1- and G_2 + M-peak respectively in channel 31 and 62, cells in early S-phase were assumed to be in channel 38-43, and cells late in S-phase in channel 50-55 (Figure 1). Traverse of cells from the early to late S-phase compartment (12 channels) was estimated to take about 0.4 part of the total S-phase time (30 channels).

In two parameters DNA/RNA histograms a distinction was made between G_1 cells with low RNA (G_1 A) and G_1 cells with higher RNA-content (G_1 B), according to a line drawn at a minimal RNA-content of S-phase cells (Figure 2)(11). The percentages of cells in these two compartments at various time intervals during reculturing were plotted, showing the time evolution of changes in these compartments (FP_i analysis).

Estimation of percentages of cells in G_2 + M- and S-phase in synchronous growing cell populations was performed according to Barlogie et al (12).

RESULTS

A distribution profile of DNA and RNA values in individual cells from the exponentially growing cell culture is shown in Figure 2. The intensity of the green fluorescence is proportional to the DNA-content per cell and permits to subclassify the cells into G_1, S and G_2 + M-phase cells. The DNA-profiles obtained with this two parameter method are identical to those, obtained by the one parameter DNA-staining with ethidium bromide. Analysis of the red fluorescence pattern indicates a progressive increase in RNA during the cell cycle, so that average G_2 + M cells have nearly twice as much RNA as average G_1 cells. The heterogeneity of G_1 cells with recpect to their RNA-content, together with the high RNA-content of S-phase cells, suggest that only cells with RNA-values above the marked threshold are entering the S-phase directly

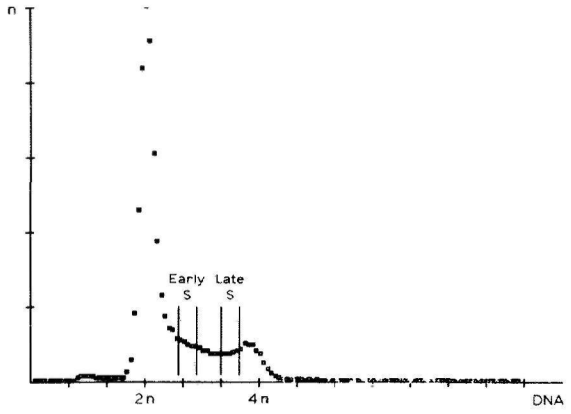

Figure 1. DNA-profile of an exponentially growing lymphoblastic cell-line (RPMI 1788). An early S-phase and a late S-phase compartment were located respectively in channel 38-43 and channel 50-55 of minimal distance of G_1- respectively G_2 + M-peak of two times the standard deviation of G_1 and G_2 + M fluorecence intensity.

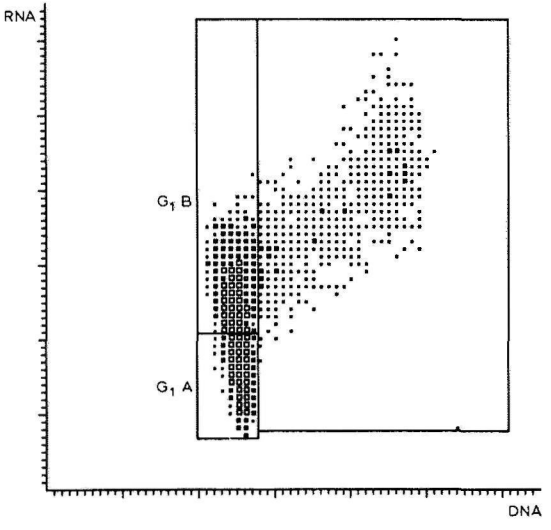

Figure 2. DNA-RNA histogram of an exponentially growing lymphoblastic cell-line (RPMI 1788). G_1 cells with low RNA content (G_1A cells) were distinguished from G_1 cells with high RNA content (G_1B cells) according to a line drawn at minimal RNA content of S-phase cells.

(figure 2). G_1 cells with low RNA content (G_1 A cells) apparently first increase their RNA-content during G_1 phase before entering the S-phase.

	G_1A %	G_1B %	S %	G_2M %
Fraction 1	60 7	32 6	6 0	0.8
2	34 0	48 9	14 8	2 3
3	16 8	50 4	25 0	7 8
4	8.1	40 1	39 9	11 9
5	11 6	27 1	36 3	25 0
6	18 8	28 0	26 9	26 3

Table 1. Percentages of cells in distinct cell cycle phases as measured with FCM in six subpopulations obtained by elutriation of an exponentially growing lymphoblastic cell-line (RPMI 1788).

Table 1 shows the percentage of cells in the various cell-cycle phases in the six fractions with elutriation of the RPMI 1788 cell-line. G_1 A cells are maximally enriched in fraction I; G_1 B cells in fraction 2 and 3; S-phase cells in fraction 4 and 5, and G_2 + M-phase cells in fraction 5 and 6. Reculturing of these six fractions show sequential changes in the various cell cycle windows, as depicted in figure 3.

Fraction 1 shows that an initial decrease of G_1 B cells parallels an increase in early S, followed by a decrease in G_1 A, which is caused probably by a continuous flow of cells from G_1 A to G_1 B and S-phase. The fraction of cells in late S-phase steadily increases to a maximum value at about 13 hours. The FP_i curves of *fraction 2* are essentially similar to those of fraction 1, although they show a phase shift of about 3 hours to the left i.e. the maximum value in late S-phase is reached after about 10 hours. *fraction 3* shows accumulation of cells in early S-phase after two hours and in late S-phase after 6 hours, indicating a traverse of cells from early S-phase to late S-phase in 4 hours. Assuming a constant DNA-synthesis rate, the S-phase might be estimated to be about 10 hours minimally (4:0.4 = 10 hours). The FP_i curves of *fraction 4* show, that the maximum percentage of cells in late S-phase is reached already after 5 hours. Oscillations of the relative cell-numbers in early and late S-phase in this figure suggest a total cycle time

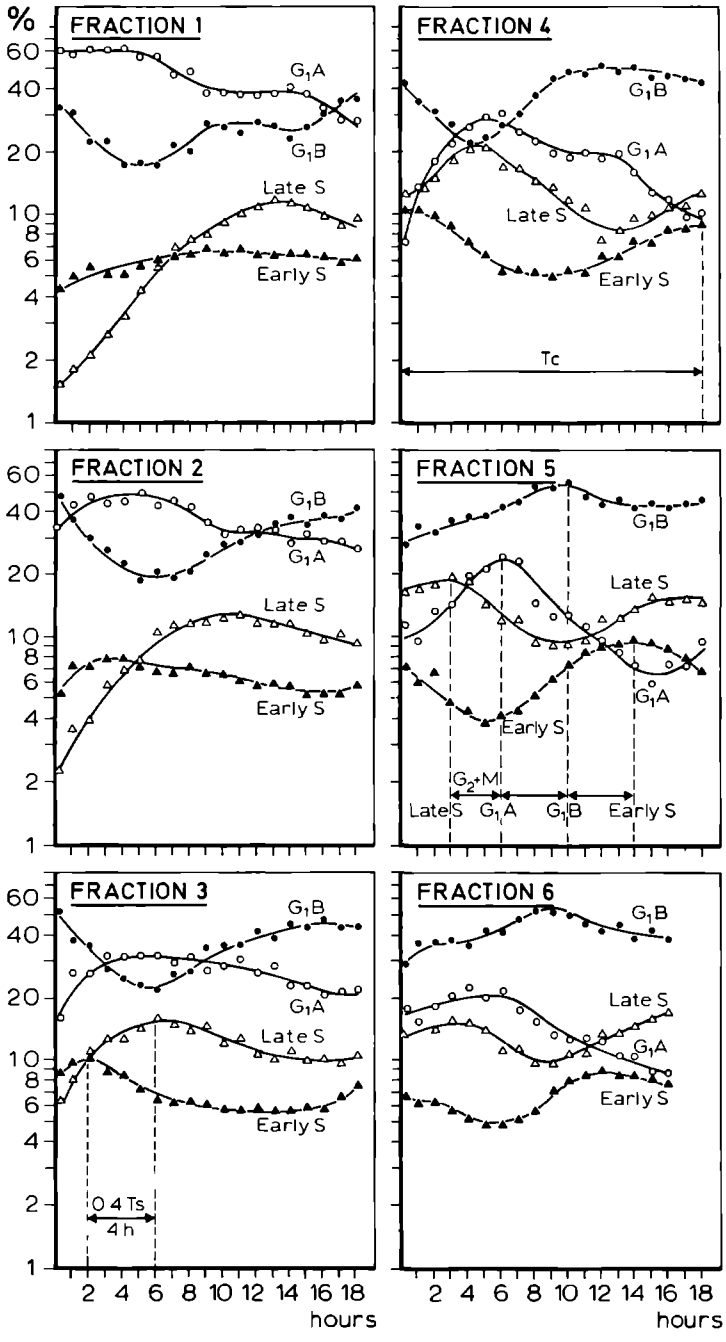

Figure 3. FP_i-curves (fraction of population in compartment i) of six synchronized subpopulations of RPMI 1788 cells (see text).

of about 18 hours. The FP_i curves of *fraction 5* permis to estimate the duration of cell cycle traverse from G_1 A to G_1 B respectively G_1 B to early S-phase. Both time periods are about 4 hours, whereas the time duration of the late S-phase to the peak value in the G_1 A compartment suggest a G_2 + M-phase duration of about 3.0 hours. The FP_i curves of *fraction 6* are essentially similar to those obtained in fraction 5. The sum of the cycle phase durations (i.e. G_1 A, S and G_2 + M-phase is about 17 hours and corresponds with the estimated cell cycle time in fraction 4. In table 2 the estimated cell cycle phase durations are summarized.

S-phase	= ± 10.0 hours
G_2+M-phase	= ± 3.0 hours
G_1A to G_1B	= ± 4.0 hours
Cell cycle time	= ± 18.0 hours

Table 2. Estimated cell cycle phase durations of an exponentially growing lymphoblastic cell-line (RPMI 1788).

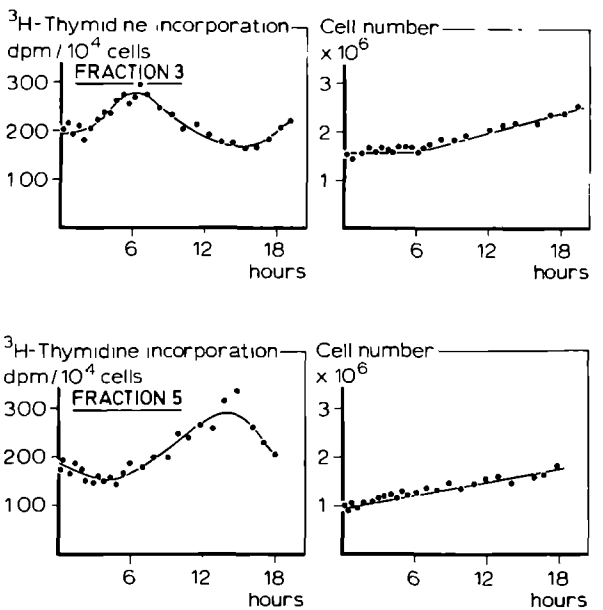

Figure 4. ^{3}H-thymidine incorporation and cellnumbers during reculturing of fractions 3 and 5.

In figure 4 the results are shown of the ^{3}H-thymidine incorporation in fractions 3 and 5 during reculturing. Oscillations in the ^{3}H-thymidine uptake is accordant with estimation of the cell cycle time as determined with DNA-FCM. The doubling time of the cellnumber in these two fractions is about 18 hours as well.

DISCUSSION

Cell synchronization with counterflow centrifugation offers the advantage, that one obtains with a simple procedure cell populations, which in one time are partially synchronized at different phases of the cell cycle. This effect is attained without use of metabolic inhibitors, temperature changes, nutrient deprivation and without removing cells from their culturing conditions (5). Reculturing of the fractions combined with FCM, subsequently disclose the traverse of cells through the cell cycle and allow with FP_i analysis of the histograms to estimate the various cell cycle phase durations. As shown, the cell kinetic data, obtained in the various fractions are in some way complementary, which permits to unravel the composition of the entire cell cycle. Results obtained with ^{3}H-thymidine uptake were shown to correlate well with FCM-data.

The estimated cell cycle phase durations in this preliminary study must be considered inaccurate yet, as the fractions obtained with elutriation were only partially synchronized in order to obtain an adequate cellnumber in each fraction. Moreover, the assumption of a constant DNA synthesis rate for calculation of the S-phase duration is perhaps not correct (13,14). On the other hand cell traverse from the G_1 A to the G_1 B compartment prior to entering the S-phase, as demonstrated by Darzynkiewicz et al (10), was confirmed by the FP_i curves obtained in fraction 5 and 6, showing accumulation of cells successively in G_1 A, G_1 B and early S-phase.

Elutriation combined with FCM, at least seems a promising method for cell cycle analysis, especially with regard to the determination of the duration of cell cycle phases. Consequently, it may be expected, that the application of this method will contribute to the understanding of the effects of chemotherapeutic drugs on the various cell cycle phases (15).

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Variation of the phosphorylation of Ara-C during the cell cycle of human leukemic cells (in preparation).

In *Chapter I* a short review is presented of the development of flow cytometry as applied in clinical hematology, whereas also the objective of the study is outlined in this chapter.

In *Chapter II* some fundamental concepts of cellkinetics are described. Moreover, some cell kinetic data of normal and leukemic bone marrow in man are summarized.

In *Chapter III* the applied flow cytometric procedures are outlined. The instruments, the measuring procedure, the methods for histogram analysis and the cell staining methods are successively described.

In *Chapter IV* a method is described allowing the quantification of peripheral nucleated cell admixture in bone marrow aspirates. The method is based on the observation(with ^{51}Cr-labeled erythrocytes and ^{125}I-labeled albumin) that nearly all erythrocytes (or hemoglobin) in bone marrow aspirates are derived from peripheral blood.

In *Chapter V* the size and the prognostic significance of the S-phase compartment in bone marrow (% S_{bm}) in adult patients with acute leukemia is reported. The median % S_{bm} in 31 patients with acute non-lymphocytic leukemia (ANLL) at diagnosis and in 14 patients with ANLL at relapse did not differ significantly from the median % S_{bm} in normal bone marrow. On the other hand in 12 out of 23 patients with acute lymphocytic leukemia (ALL) at diagnosis and in 6 out of 11 patients with ALL at relapse, the % S_{bm} was much increased compared to normal.
Patients with ALL at diagnosis in whom the % S_{bm} was high appeared to have a bad prognosis with regard to remission duration and survival, compared with those having a normal or low % S_{bm}.

In *Chapter VI* the size of the S-phase compartment in blood (% S_{pb}) and bone marrow (% S_{bm}) is reported as measured in patients with chronic myelogenous leukemia (CML) at different disease stages.
At diagnosis and at "remission" the % S_{bm} in these patients appeared to be of the same order of magnitude as in normal bone marrow. However, at malignant metamorphosis the % S_{bm} appeared higher than normal in 3 out of 4

patients in whom this percentage could be determined reliably Aneuploidy was demonstrated in 4 out of 11 patients with CML at this disease stage. Peripheral nucleated cell admixture in bone marrow aspirates was shown to be considerable, especially in CML patients with a high white blood cell count (> 100 x 10^9/l).

In *Chapter VII* changes in % S_{bm} after intravenous push injections of Ara-C in 11 patients with ANLL are described. DNA-flow cytometry revealed a slight decrease of the % S_{bm} at 7 hours after the injection but showed subsequently an increase to a maximum value exceeding the pretreatment value at about 30 hours after the Ara-C injection
Labeling Indices (determined with ^{3}H-thymidine autoradiography) reached, after an initial decrease, pretreatment levels at about 24 hours after injection. ^{3}H-thymidine incorporation increased after an initial inhibition to about twice the pretreatment levels at 30-40 hours after injection.

The perturbation of the cell cycle appeared identical with the three different Ara-C dosages used (i.e. 100 mg/m^2, 380 mg/m^2 and 1000 mg/m^2).

The observations suggest that with regard to the cell kinetics,24-hour scheduling might be optimal for Ara-C given by push-injections

In *Chapter VIII* a simplified method is described for simultaneous DNA and protein staining of human hematopoietic cells, with propidium iodide (PI) and fluorescein isothiocyanate (FITC)

In contrast to previous protocols, all reagents are added sequentially to ethanol fixed cells through which no centrifugation steps are required, and cell clumping is prevented. The staining time is reduced to 10 minutes as compared to 3 hrs by previous methods.

In *Chapter IX* it is shown, that plasma cell enrichment with counterflow centrifugation of bone marrow in patients with multiple myeloma or benign monoclonal gammopathy, augments the discriminatory potential of flow cytometry with regard to cell kinetic profiles and ploidy abnormalities of plasma cells.

In *Chapter X* is demonstrated that with counterflow centrifugation of cultured cells, subpopulations are obtained enriched with cells at well defined cell cycle phases. Reculturing of these synchronized fractions enabled flow cytometric analysis of various cell cycle phase durations.

SAMENVATTING

In *Hoofdstuk I* wordt een beknopt overzicht gegeven van de ontwikkeling van flow cytometrie binnen de klinische hematologie terwijl tevens in dit hoofdstuk het doel van het onderzoek wordt uiteengezet.

In *Hoofdstuk II* worden enkele basis begrippen van de celkinetiek besproken en wordt een overzicht gegeven van de celkinetische gegevens betreffende het normale en leukemische beenmerg bij de mens.

In *Hoofdstuk III* worden de in dit onderzoek gebruikte flow cytometrische methoden uiteengezet. De apparatuur, de feitelijke meetprocedure, de methoden voor de analyse van de verkregen histogrammen en de celkleuringsmethoden worden achtereenvolgens beschreven.

In *Hoofdstuk IV* wordt een methode beschreven welke de mogelijkheid biedt de mate van bijmenging van perifere kernhoudende cellen in beenmergaspiraten te kwantificeren. Deze methode berust op de waarneming (verricht met Cr^{51}-gelabelde erythrocyten en I^{125}-gelabeld albumine) dat nagenoeg alle erythrocyten (of hemoglobine) in een beenmergaspiraat afkomstig zijn van het perifere bloed.

In *Hoofdstuk V* wordt verslag gedaan van een onderzoek naar de grootte, én de prognostische betekenis van het S-fase compartiment in het beenmerg (% S_{bm}) bij volwassen patienten met akute leukemie.

Het mediane % S_{bm} by 31 patienten met akute niet lymfatische leukemie (ANLL) ten tijde van de diagnose en bij 14 patienten met ANLL ten tijde van het recidief verschilde niet significant van het mediane % S_{bm} zoals dat werd gevonden in het beenmerg van gezonde kontrole personen. Daarentegen werd bij 12 van de 23 patienten met akute lymfatische leukemie (ALL) bij diagnose, en bij 6 van de 11 patienten met ALL tijdens het recidief een beduidend hoger % S_{bm} gemeten dan normaal.
Patienten met ALL met een hoog % S_{bm} bleken een significant slechtere prognose te hebben wat betreft remissieduur en overleving, dan patienten met een normaal of laag % S_{bm}.

In *Hoofdstuk VI* wordt het onderzoek beschreven naar de grootte van het S-fase compartiment in bloed (% S_{pb}) en beenmerg (% S_{bm}) van patienten

met chronische myeloide leukemie (CML), in verschillende stadia van de ziekte. Ten tijde van diagnose en bij "remissie" bleek het % S_{bm} niet significant te verschillen van normalen.
Tijdens "maligne metamorfose" echter, bleek het % S_{bm} significant hoger dan normaal althans bij 3 van de slechts 4 patienten waarbij dit percentage betrouwbaar kon worden bepaald. Aneuploidie kon worden aangetoond bij 4 van de 11 patienten met CML in deze ziekte-fase.

Bijmenging van perifere kernhoudende cellen in beenmergaspiraten bleek vaak aanzienlijk te zijn bij CML patienten met een hoog perifeer leukocyten aantal (> 100 x 10^9/l).

In *Hoofdstuk VII* worden de veranderingen gerapporteerd in het percentage S-fase cellen van het beenmerg (% S_{bm}) na een intraveneuze bolus injectie van Ara-C.

Met DNA-flow cytometrie werd 7 uur na de injectie een geringe daling van het % S_{bm} gemeten, terwijl vervolgens dit percentage steeg tot een maximale waarde die ongeveer 30 uur na injectie het uitgangsniveau overschreed.

De labeling indices (gemeten met ^{3}H-thymidine autoradiografie) bereikten, na een initiële daling, ongeveer 24 uur na injectie het uitgangsniveau.

^{3}H-thymidine incorporatie tenslotte, steeg 30-40 uur na Ara-C injectie (na een aanvankelijke remming) tot een waarde van ongeveer twee maal het uitgangsniveau.

De celcyclus veranderingen zoals die ontstonden na toediening van drie verschillende doses Ara-C (te weten 100 mg/m^2, 380 mg/m^2 en 1000 mg/m^2) bleken identiek.

De resultaten suggereren dat celkinetisch, éénmalige toediening van Ara-C per etmaal, althans bij intraveneuze bolus injecties van dit cytostaticum, de aangewezen methode is.

In *Hoofdstuk VIII* wordt een vereenvoudigde methode beschreven voor de gelijktijdige kleuring van menselijke hematopoietische cellen op DNA en eiwit. Hierbij wordt gebruik gemaakt van de fluorochromen propidium iodide (PI) en fluoresceine isothiocyanaat (FITC).

In tegenstelling tot eerder beschreven kleuringsmethoden worden bij deze methode achtereenvolgens alle reagentia toegevoegd aan met alkohol gefixeerde cellen, waardoor tussentijds centrifugeren niet langer noodzakelijk is en celklontering wordt vermeden.

De beschreven kleuringsmethode is tevens aanmerkelijk korter dan eerder beschreven methoden (10 minuten i.p.v. ± 3 uur).

In *Hoofdstuk IX* wordt aangetoond dat tegenstroom centrifugatie (of cel elutriatie) een geschikte methode is om bij patienten met multipel myeloom (MM) of benigne monoclonal gammopathie (BMG) beenmerg fracties te verkrijgen die verrijkt zijn met plasma cellen.

Flow cytometrisch onderzoek (DNA-flow cytometrie of DNA/eiwit-flow cytometrie) van deze met plasma cellen verrijkte fracties verschaft meer informatie dan flow cytometrisch onderzoek van het totale (niet gescheiden) beenmerg. Dit betreft vooral gegevens betreffende enkele celkinetische parameters, en afwijkingen in het DNA-gehalte van plasma cellen.

In *Hoofdstuk X* wordt aangetoond dat d.m.v. tegenstroom centrifugatie (of cel elutriatie) van cellen in kweek, fracties kunnen worden verkregen, verrijkt met cellen die verkeren in een bepaalde fase van de celcyclus. Door deze gesynchroniseerde celpopulaties opnieuw in kweek te brengen wordt de mogelijkheid geschapen met behulp van flow cytometrie onderzoek te doen naar de tijdsduur van de onderscheiden celcyclus-fasen.

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De auteur van dit proefschrift werd geboren op 9 augustus 1946 te Oosterhout (N.B.). In 1963 behaalde hij het diploma HBS-B aan het Constantijn College te Amersfoort. Daarna studeerde hij geneeskunde aan de Katholieke Universiteit te Nijmegen. Na het artsexamen in 1971 startte hij met de opleiding tot internist in de Universiteits Kliniek voor Inwendige Ziekten van het Binnengasthuis te Amsterdam (Hoofd: Prof.Dr.A.M.van Leeuwen en wijlen Prof.Dr.J.G.G.Borst). Registratie als Internist vond plaats in 1976. Sinds augustus 1976 is hij werkzaam in de Universiteits Kliniek voor Inwendige Ziekten van het St. Radboud Ziekenhuis te Nijmegen (toenmalig hoofd: Prof. Dr.C.L.H.Majoor). Sedert november 1977 is hij binnen deze kliniek verbonden aan de afdeling Hematologie (Hoofd: Prof.Dr.C.Haanen).

STELLINGEN

1

Kwantitatieve gegevens omtrent celeigenschappen in beenmergaspiraten hebben alleen waarde indien hierin de mate van bijmenging met perifere kernhoudende cellen gemeten is.

dit proefschrift

2

Het percentage cellen in S-fase van het beenmerg bij acute niet-lymfatische leukaemie verschilt niet wezenlijk van dat in normaal beenmerg.

dit proefschrift

3

Een hoog percentage cellen in S-fase van het beenmerg bij acute lymfatische leukaemie heeft een zeer slechte prognostische betekenis.

dit proefschrift

4

Het DNA-gehalte van plasma cellen bij het multipel myeloom is meestal in belangrijke mate afwijkend van normaal.

dit proefschrift

5

De verlaagde "Labeling Index" van leukaemische blasten kan men beschouwen als uiting van een gestoorde differentiatie.

6

Bij het ontstaan van Thrombotische Thrombocytopenische Purpura spelen progestativa en oestrogenen een belangrijke rol.

eigen waarneming

7

De gelijkenis van Thrombotische Thrombocytopenische Purpura en (pre)eclampsie is soms zo treffend, dat een gemeenschappelijke pathogenese waarschijnlijk moet worden geacht.

8

Indien bij patienten met multipel myeloom een stabiele remissie is verkregen lijkt continuering van chemotherapie niet zinvol.

Alexanian R., Gehan E., Haut A., Saiki J.,
Weick J. Blood *51*, 1005, 1978
Durie BGM., Russell DH., Salmon SE.
Lancet II: 65, 1980

9

De opvatting, dat tijdens hypoglycaemie bij insuline-afhankelijke diabeten een zodanige stimulering van de secretie van contraregulerende hormonen optreedt, dat hierdoor een hyperglycaemie ontstaat (het zogenaamde Somogyi effect), is onjuist.

Gale EAM., Kurtz AB., Tattersall RB.
Lancet II: 279, 1980

10

Bij een afsluiting van de arteriae iliaca externa en femoralis communis dient de half-gesloten thromboëndarteriëctomie met behulp van de gemodificeerde ringstripper als reconstructieve ingreep overwogen te worden.

Donders, H.P.C., v. Dongen RJAM
VASA *8*, 343, 1979

11

De mening, dat "Jogging" bescherming biedt tegen het krijgen van hart-vaatziekten, berust niet op overtuigende feiten.

Rennie D., Hollenberg NK.
N. Engl. J. Med. *301*, 103, 1979

12

Bij, door het parlement voorgestelde wijzigingen van departementale begrotingen dient het totaal kader van de Miljoenennota opnieuw ter discussie te staan.

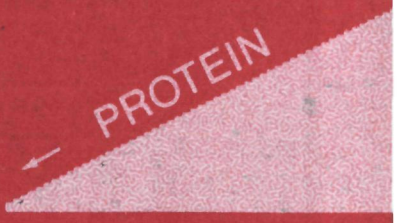
Le Blanc LJCM NRC-Handelsblad 18-7-1981.

13

Hollanditis is geen "Veteranen-ziekte".

R.S.G. Holdrinet

Nijmegen, 2 october 1981



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