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# Computer Analysis of Two Parameter Histograms of Rat Bone Marrow Cells : Cell Volume to DNA Relationship Measured by Means of Flow Cytometry

G. VALET, G. HANSER, G. RUHENSTROTH-BAUER

*Abteilung für Experimentelle Medizin, Max-Planck-Institut für Biochemie, Martinsried (München), F.R.G.*

## Summary

*Rat bone marrow cells were analysed by simultaneous electrical sizing and by axial epi-illumination DNA fluorescence measurement. Three distinct cell cycles α, β and γ were found in the bone marrow of normal rats. A Fortran-computer program is discribed which performs the quantitative analysis of the nine different cell cycle phases. The program further draws perspective and isoamplitude plots or single volume and fluorescence histograms. The histograms can be fitted by linear or logarithmic Gaussian normal distributions. Various parameters of the distributions such as mode, median, mean value, coefficient of variation and the percent contribution of each Gaussian distribution to the total histogram are calculated.*

## Introduction

Hematopoietic tissues consist of a complex mixture of different cells (1). They contain a wide variety of differentiating and mature cells of the erythrocytic, lymphocytic, granulocytic and thrombocytic cell lines besides a small number of hematopoietic stem cells. It has become increasingly important to separate cells of the hematopoietic system for analytical or preparative purposes into well defined subpopulations in order to understand the mechanism of differentiation. Flow cytometry is an efficient tool for analytical studies. High numbers of cells can be processed, data can be quickly analysed by a computer, comparatively few cells are needed and the overall time of measurement and analysis is short.

One parameter histograms of DNA (Deoxyribonucleic acid) (2, 3) or cell volume distributions (4, 5, 6, 7) have yielded valuable information for the characterisation of cells of the hematopoietic system. Changes in those histograms may indicate pathological alteration of hematopoiesis (2, 3, 7, 8, 9). In view of the complexity of the hematopoietic tissues the resolution of a one parameter analysis is often limited. The

simultaneous two parameter analysis is a more powerful tool for the separation of different populations of cells with similar properties, in analogy to the two dimensional chromatograms in protein or peptide chemistry. Two parameter analyses of cells of the hematopoietic system have been performed for the combination DNA/RNA (Ribonucleic acid) content (10, 11, 12), DNA/volume (13) and two angle light scatter (14). Light scatter is, however, a rather complex parameter (10, 15, 16, 17) because the signals include information on the cell volume, the cell membrane and the cell interior. We were preferentially interested in cell volume distributions of haematopoietic cells since the cell volume provides important information on changes in hematopoiesis as shown earlier (4, 5, 6). The cell volume was determined by electrical sizing. The electrical sizing process is well understood and accurate measurements of the cell volume and volume distribution can be obtained with the hydrodynamic focusing technique (7, 18). The volume analysis was combined with a simultaneous DNA measurement for cell cycle analysis. DNA fluorescence was measured by axial epi-illumination of the particles (19) while they were streaming through the electrical sizing orifice (20).

## Methods

*a. Cell suspensions*

A single cell suspension of bone marrow cells was obtained by syringing the bone marrow tissue of the femurs of 120-130 g female Wistar rats several times through a number 1, 12 and 14 needle (0.90, 0.70, 0.65 mm diameter and 40-50 mm length) into 5 mM Tris (trishydroxymethylaminomethane) buffered 0.95% NaCl solution pH 7.4. The cell suspension was filtered through a sieve of V2A steel wire (100 $\mu$m mesh distance), washed twice by centrifugation for 10 min at 500 g with NaCl/Tris buffer and resuspended in the same buffer at a final concentration of 5-6 x $10^6$ cells/ml. All preparative procedures were performed at 0-4° C. The cells were fixed by dropwise addition of the cell suspension into absolute ethanol until a final ethanol concentration of 70% (v/v) was reached. The ethanol cell suspension was continuously stirred at medium speed with a magnetic stirrer during the dropwise addition of the cells. The cells were pelleted after 2h fixation by 2 min centrifugation at 8000 g in an Eppendorf model 3200 centrifuge and resuspended in NACl/Tris buffer containing 100 $\mu$g/ml Mithramycin and 15 mM $MgCl_2$. In some experiments the bone marrow cells were separated by volume with a Beckman elutriator centrifuge using NACl/Tris buffer at 4°C as suspending medium. The cells were pelleted on a slide with a cytocentrifuge, stained with the Pappenheim method and differentiated microscopically.

*b. Fluorescence and volume measurements*

The cells were analysed with a Fluvo-Metricell flow cytometer (20 after 30-90 min staining with Mithramycin at room temperature. A HBO100 mercury lamp was used as exciting light source. The fluorescent light was separated from the exciting light by a barrier filter with a cut off point for light below 520 nm wave length. The orifice for electrical sizing was cylindrical with a diameter of 110 $\mu$m and a length of approximately 100 $\mu$m. The cells were sized in NACl/Tris buffer at 25°C (63 $\Omega$ cm) with a current of 0.90 mA at a rate of 150-250 particles/sec to keep the possibility for a coincident passing of the particles through the orifice low. The volume and fluorescence signals were amplified, converted in an analog digital converter and stored in a multichannel analyser within a 64 x 64 array. An electronic coincidence circuit assured that the volume and fluorescence pulses were only stored if both signals came from the same particle.

*c. Data analysis*

The data in the multichannel analyser were transferred to a magnetic tape. A Fortran computer program was developed which displays the 64 x 64 channel array of the multichannel analyser as a rectangular perspective plot with the volume on the x-axis, the fluorescence on the y-axis and the number of particles per channel in the z-axis. The plot can be rotated from its original position by 90 (Fig. 2b), 180 (Fig. 2a) and 270 degrees to visualise areas of the plot which are hidden without rotation. The plot can be drawn in addition with a variable shift between the individual curves of the plot to the right (Fig. 2a, b) or left side. Individual curves or groups of volume (Fig. 3b, c) or fluorescence distribution curves (Fig. 3a) of the 64 x 64 array can be analysed by fitting linear or logarithmic Gaussian distributions to the histograms with an earlier developed program (5).
The gap phases ($G_0$, $G_1$, $G_2$), the DNA synthesis phase (S) and the mitotic phase (M) are distinguished within the cell cycle. It is important to have normalised isoamplitude plots of the 64 x 64 channel array for a rapid comparison of different histograms. Beginning in the x-y plane a variable part of the maximal amplitude in the z-axis is divided into ten equal parts. The computer checks the contents of all channels and according to their content, a number between 0-9 is assigned to each channel. The ten step matrix is then printed out. The calculation of the absolute number of particles in a rectangular or in an obliquely limited area of the isoamplitude plot gives quantitative values for the particle distribution in the two parameter histogram.

sistance of the cell membrane. This diminishes the apparent electrical cell volume.

The perspective plots (Fig. 2a, b) show that the majority of the bone marrow cells are in the $G_0/G_1$ phase of the cell cycle. Only few cells were in the S or $G_2$/M phase. Aggregates of two or three nucleated cells were present in fixed and unfixed cell suspensions. They were in the order of 2-4% of the total cells when counted microscopically. The

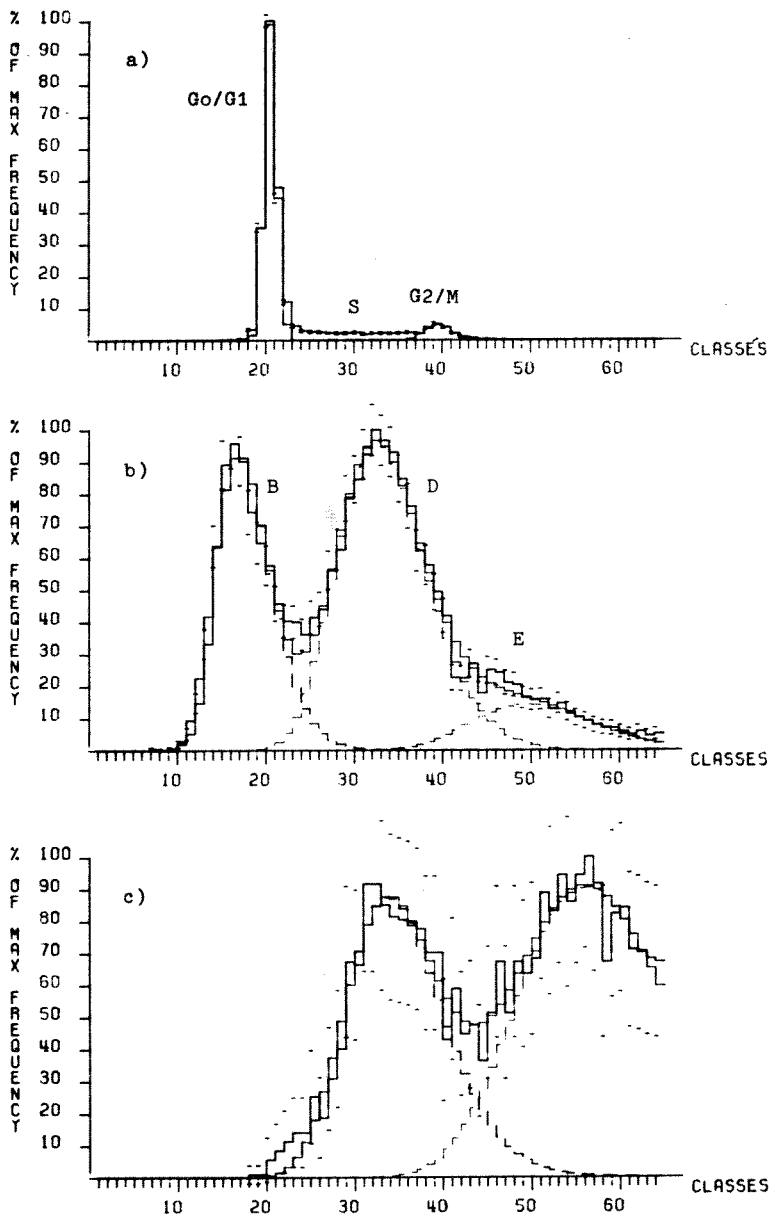


Fig. 3. DNA Distribution of all bone marrow cells (a) and volume distribution curves of the cells in the G0/G1 (b) and G2/M phase of the cell cycle (c). The curves b, c represent the sum of the volume distribution curves of fluorescence channels Nr. 17-22 for the G0/G1 phase (30178 cells) and of Nr. 37-42 for the G7/M phase (2974 cells) of Fig. 4. The coefficients of variation for the DNA distributions are 3.8 and 4% for the G0/G1 and G2/M phases (a). The volume distribution of the cells in G0/G1 is approximated with logarithmic normal distributions with coefficients of variation of 19.1, 15,7 and 14.0% (b) and of 16.3 and 15.4% for the G2/M cells (c).

aggregates were often quite loose. It seems possible that some of them disintegrate during the acceleration and flow through the sizing orifice. It is unlikely that the cell populations at the $G_2$/M location in Fig. 2, 3c are only due to aggregates of cells of the volume peak B and D (Fig. 3b) because 7.7% of all cells are found in the $G_2$/M phase of the cell cycle (Tab. I) as compared to maximally 4% cell aggregates. Pulses registered in the S phase region of Fig. 2 and 4 could be artificially generated by particle flow rates over 2000 particles/sec by a partial overlap of the electronic pulses due to coincident passing of the particles through the orifice. Such effects are below 1% at flow rates of 150-250 particles/sec as they were used in the present experiments (Tab. I.). The conclusion of these considerations is that the S and $G_2$/M regions of the histograms are real and not artificially generated during the measurement. The $G_2$/M region is, however, over-estimated by 2-4% due to cell aggregates. The DNA and volume distribution curves of all cells (Fig. 3a) or of the cells in the $G_0/G_1$ and $G_2$/M phase of the cell cycle (Fig. 3b, c) were approximated by Gaussian normal distributions. The DNA distribution follows the typical pattern for dividing cells (Fig. 3a) with 78.6% of the cells in the $G_0/G_1$ phase of the cell cycle (Tab. I). The volume distribution curves of the cells in the $G_0/G_1$ phase is well approximated by three logarithmic distributions (Fig. 3b), and the volume distribution of the $G_2$/M cells by two logarithmic distributions (Fig. 3c). The coefficients of variation for the DNA distribution (Fig. 3a) are 3.8 and 4% and for the volume distributions (Fig. 3b, c) between 14-19% for the various cell populations (Fig. 3). The isoamplitude plot with 1% stepsize indicates the presence of three cell cycles, $\alpha$, $\beta$ and $\gamma$ in the normal bone marrow (Fig. 4).

*Table I*

*Quantitative computer analysis of the isoamplitude plot (Fig. 4) of rat bone marrow cells*

| cycle phase | % of nucleated bone marrow cells in cycle $\alpha$ | $\beta$ | $\gamma$ | $\alpha+\beta+\gamma$ | % of cells in different phases for each cell cycle cycle $\alpha$ | $\beta$ | $\gamma$ |
|---|---|---|---|---|---|---|---|
| $G_0/G_1$ | 25.2 | 43.2 | 11.1 | 79.6 | 72.9 | 80.8 | 87.6 |
| S | 5.0 | 6.0 | 1.6 | 12.6 | 17.1 | 11.2 | 12.3 |
| $G_2$/M | 3.4 | 4.3 | a) | 7.7 | 9.9 | 7.9 | a) |

a) $G_2$/phase of cycle $\gamma$ outside of Fig. 4 plot

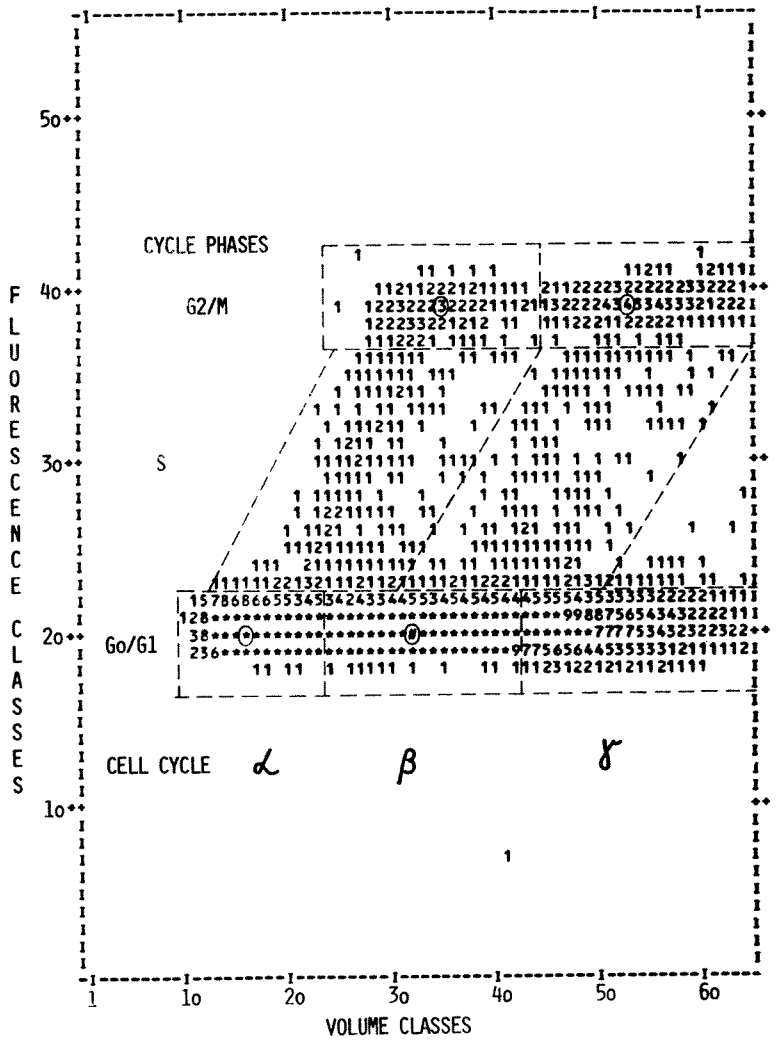


Fig. 4. Isoamplitude print out of the volume/DNA plot of Fig. 2a,b. The range 0-10% of the maximal amplitude (#) is divided into ten steps of 1% stepsize. The steps are indicated by numbers 0-9 according to the channel content. All channels with number 0 (0-0.99% of the maximal amplitude) are suppressed in the printout for better visibility of the cell clusters. Channels containing more than 10% of the maximal channel content are indicated by * .
Three cell cycles $\alpha$, $\beta$ and $\gamma$ with the respective G0/G1, S and G2/M cell cycle phases can be distinguished. The G2/M phase and parts of the S phase of the $\gamma$ cycle are outside the plot. The dashed lines are the limits of the compartments for the calculation of the absolute number of cells in the different cycle phases (tab. 1). The circles indicate the position of the modes of the volume distribution curves of Fig. 3b,c.

## Discussion

The nucleated bone marrow cells can be separated by volume distribution analysis into three distinct populations of nucleated cells with different modal volume (Fig. 3b peaks B, D and E). These cell populations are heterogeneous since volume peak B contains mature poly- and orthochromatic erythroblasts and small lymphocytes, peak D proerythroblasts, myeloblasts, mature granulocytes and large lymphocytes and

peak E promyelocytes, macrophages and monocytes. Similar results have been obtained by others using 1g sedimentation and preparative free flow electrophoresis (21) instead of elutriation. It is of interest that there is a relative monotony of the cell volume distribution in the bone marrow in spite of the large variety of cells in many steps of differentiation and development. To each of the cell volume populations belongs one S and one $G_2$/M phase. This constitutes a total of three separate cell cycles which are simultaneously operative in the normal rat bone marrow. Cells of a given line may change from large to small volume during differentiation (e.g. proerythroblasts into mature erythroblasts) or inversely grow from one differentiation step to the next (e.g. small stem cells to myeloblasts, or myeloblasts to promyelocytes). The volume shift of cells from one cell cycle into another could occur during the $G_0/G_1$ or $G_2$/M phase of the cell cycle or by assymetric cell divisions. Such shifts could cause the right skew of the volume peaks B and D of the $G_0/G_1$ cells (Fig. 3b).

The simultaneous two parameter volume/DNA measurement yields more information than the one parameter DNA histograms because it spreads the bone marrow into nine clusters of cells (Fig. 4) instead of only three distinct areas ($G_0/G_1$, S and $G_2$/M) in the conventional DNA histogram (Fig. 3a) (2, 3). In view of the leukemic transformation of the hematopoietic tissues it seems possible that one of the three cell cycles is preferentially involved in the multiplication of leukemic cells. The two parameter histograms would then be a more sensitive and specific tool for the diagnostic or therapeutic screening than a one parameter DNA analysis. The two parameter histogram analyses should also provide more information on the mechanism of the maturation of the hematopoietic system in the young mammalian organism. It is of importance to know there to what degree the sudden changes of differentiation in the erythropoietic and leukopoietic system during maturation (4, 5, 6) are accompanied by changes in the proliferative activity of the different hematopoietic cell cycles in the bone marrow.

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