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Simultaneous Flow Cytometric DNA and Volume Measurements of Bone Marrow Cells as Sensitive Indicator of Abnormal Proliferation Patterns in Rat Leukemias

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Simultaneous flow cytometric DNA and volume analysis of normal rat bone marrow cells shows three populations of nucleated cells with different mean volume. Each of these populations proliferates in a distinct cell cycle (α, β, γ). Normally the α-cell cycle has the highest amplitude, the β-cell cycle is intermediate, and the γ-cell cycle is low. The α-cell cycle was very significantly depressed and the β + γ-cell cycle was increased in three different rat leukemias (L5222, Shay, BNML), growing on three different rat strains (BDIX, Holtzmann, Brown Norway). The two parameter analysis further revealed that cells of the β + γ-cell cycle were slightly hyperdiploid and hypertetraploid in leukemic animals. The decrease of the α-cell cycle and the hyperploidies were more sensitive indicators for the abnormal proliferation pattern than the analysis of one parameter DNA distributions which remained within normal limits in all three leukemias.

Flow cytometric measurements of DNA distributions are used to quickly characterize the proliferative state of bone marrow cells (6). A practical application of this method is to follow the therapeutic affects of cytostatic drugs specifically affecting a particular phase of the cell cycle. Application of Adriamycin, Daunomycin, Vincristine or Cytosine Arabinoside has been observed to cause an accumulation of cells in the G2/M phase of the cell cycle (2, 5, 8). Another important aim of DNA distribution measurements is to recognize abnormal proliferation patterns as a diagnostic indicator for the presence of leukemic cells (8, 9), as has been shown in strongly aneuploid leukemias (1, 12). Such measurements are more difficult to interpret in slightly aneuploid leukemias because aneuploidy is not recognized in the DNA histogram and in addition the quantitative ranges of cells in the S and G2/M phase of the cell cycle in leukemic and normal individuals overlap (8, 9).

Previous work in this laboratory has shown that three individual cell cycles (α, β, γ) can be distinguished in the bone marrow of normal rats by their different cell volume (15). Similar results were recently obtained by others using preparative velocity sedimentation and free flow electrophoresis (17). Each cell cycle has its own G0/G1, S and G2/M phase. The α-cell cycle originates from the volume peak of small nucleated cells. It contains mainly mature normoblasts and small lymphocytes. The $\beta + \gamma$ -cell cycles originate from the volume peak of large nucleated cells which contain granulocytes, large lymphocytes, proerythroblasts, monocytes and promyelocytes (15). If the cell volume and the cell DNA are measured simultaneously in a flow cytometer, the resulting two parameter histogram contains nine different cell clusters. The aim of this study was to investigate if the two parameter histogram was a more sensitive indicator of abnormal proliferation patterns in bone marrow cell suspensions of leukemic rats as compared to a one parameter DNA distribution analysis.

MATERIALS AND METHODS

Animals and leukemia transplantation: The L5222 leukemia (4, 10) and the Shay-chloroleukemia (13) were used as experimental models. The leukemias were transplanted into healthy rats by i.p. injection of cell suspensions from leukemic animals. Single cell suspensions of the spleen of L5222 leukemia bearing female BD IX rats (Freiburg, Germany) of 120 g weight were prepared by mincing the organ and syringing the tissue pieces several times through needles with an inner diameter of 1.0, 0.8 and 0.6 mm in a 0.95% NaCl solution buffered with 5 mM Tris/HCl to pH 7.4 (NaCl/Tris buffer). Cells, 1×10^7, were injected per animal. The animals died on day 8 or 9 after innoculation. The Shay-chloroleukemia was grown on male Holtzmann rats (Ciba-Geigy AG, Basel, Switzerland) by injecting 0.3 ml of blood of a leukemic animal i.p. into a healthy recipient between days 10–15 after birth.

At day 6 (L5222) or day 8 (Shay) after innoculation, the animals were bled under ether anesthesia through the abdominal aorta until respiratory arrest. One femur was then removed and single cell suspensions of the bone marrow were prepared and fixed in 70% ethanol (v/v) (15).

Flow cytometric measurements: The fixed cells were stained with Mithramycin (3) at a final concentration of 100 μg/ml in NaCl/Tris buffer containing, in addition, 15 mM $MgCl_2$. The cell volume and the Mithramycin fluorescence of the bone marrow cells were then measured simultaneously in a Fluvo-Metricell flow cytometer (11) at a rate of 800–1000 cells/sec.

Data processing: All cell volume pulses were gated by the fluorescent signal. Only volume signals were accepted which were associated with a fluorescence (DNA) signal above threshold. The fluorescence threshold was usually set between 0.1–0.5 times the signal height of the G0/G1 phase fluorescence signals. The two parameter histogram was stored in a 64 × 64 multichannel analyzer array. The histogram data were transferred for further analysis onto magnetic

tape. The data on the tape were analyzed by a FORTRAN computer program (200 kbyte core memory in a Siemens 4004/150 computer (Siemens Corp., Iselin, N.J.)) (15). The program plots three dimensional views of the histogram from different angles and calculates the percentage of particles in limited areas. It further plots the projection of the cumulated class contents of each area on either the volume or the DNA axis. In addition, DNA/volume ratios are calculated and the ratio distribution is plotted. The projected curves and the ratio distributions can be approximated by linear or logarithmic normal distribution for further data reduction (14).

RESULTS

The DNA distribution curves of the bone marrow cells of leukemic animals were qualitatively (Fig. 1b,c) and quantitatively (Table I) undistinguishable from those of normal rats (Fig. 1a, Table I), however, the volume distribution curves of normal (Fig. 1d) and leukemic bone marrow cells (Fig. 1e,f) were different. Usually the amplitude of the volume peak B is 100% and that of peak D between 30–80% of the maximal amplitude (Fig. 1d). In the leukemic animals the amplitude of volume peak B were diminished and of peak D increased (Fig. 1e,f). The two parameter histograms (Fig. 2a–c) show that proliferation of normal bone marrow cells starts mainly from volume peak B (α-cycle), whereas in leukemic animals it originates mainly from the cells in the volume peak D which corresponds to the β-cell cycle. This is also visible in the isoamplitude plot (Fig. 3a–c) where the areas of the G0/G1, S and G2/M phase for the cell cycle α and for the cell cycles β and γ are limited by lines. The $\beta + \gamma$-cell cycles were taken together for the quantitative calculation (Table I) because the γ-cell cycle was not clearly distinguishable in leukemic animals. The most impressive alteration in both leukemias was that the α-cell cycle was greatly diminished (15–40% of maximal amplitude) and the $\beta + \gamma$-cell cycle increased (100% of maximal amplitude, Fig. 2b,c). Similar changes also occurred with respect to the quantitative contribution of the cells of the α and $\beta + \gamma$-cell cycle to the whole histogram (Table I).

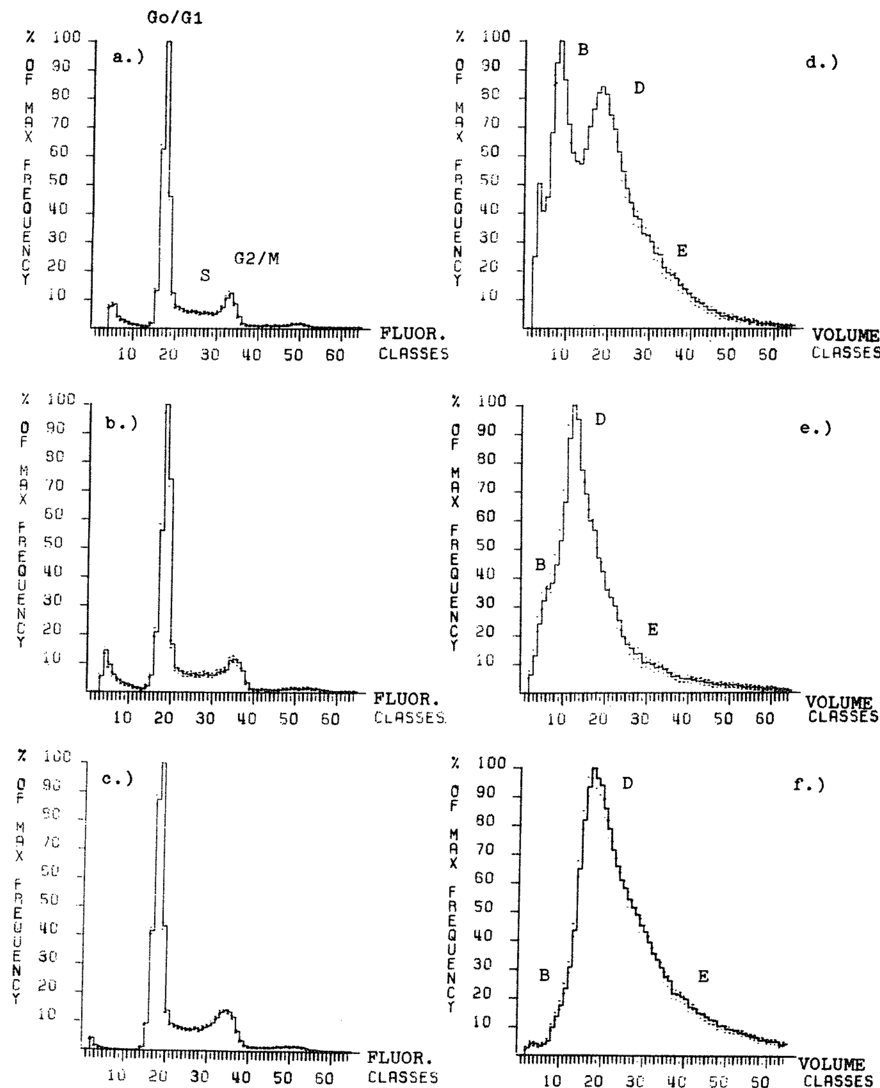

FIG. 1. DNA (a-c) and volume (d-f) distribution curves of bone marrow cells of normal rat (a,d), and of L5222 (b,e) and Shay chloroleukemia (c,f) rats 6 and 8 days after inoculation.

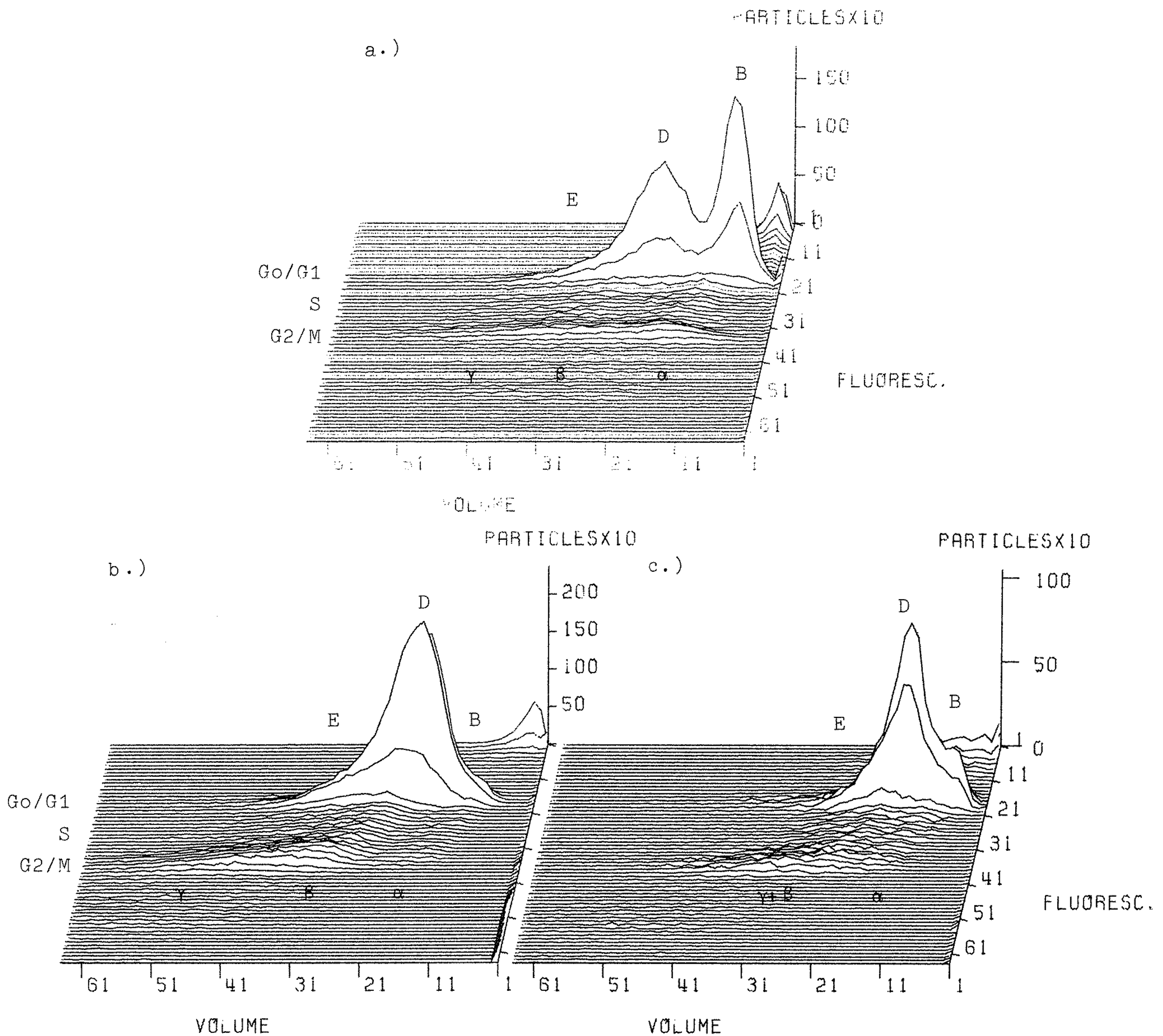

FIG. 2. Three dimensional plot of the cell volume *vs* cell DNA histograms of bone marrow cells of normal rats (a) and rats with L5222 (b) and Shay chloroleukemia (c) 6 and 8 days after inoculation.

The cells of the $\beta + \gamma$-cell cycle were hyperdiploid and hypertetraploid in both leukemias. The hyperploidy was not very pronounced (1.05–1.09 times the DNA in normal cells) and would have been missed in the one parameter DNA-distributions (Fig. 1b,c). The two parameter histograms were divided in a next evaluation step into five different areas which represent the G0/G1 phase, the early (S1), intermediate (S2), and late (S3) S phase, and the G2/M phase of the cell cycle. The cumulated fluorescence channel contents of each volume channel in these five areas of the two parameter histogram were plotted. These curves represent the volume distribution curves of cells in the different cell cycle phases (Fig. 4a–c). The comparison shows that the form of the volume distribution curves of the S1, S2, S3 and G2/M phase are similar to the curve in the G0/G1 phase, but changes in the relative height of the peak amplitudes occurred which probably indicates differences of the cell cycle phase transit times between cells of the α or the $\beta + \gamma$-cell cycle.

A comparison of the normal bone marrow cells (Fig. 4a) with those of leukemic animals (Fig. 4b,c) shows more clearly than the original histograms (Fig. 2a–c, 3a–c) how much the α-cell cycle is reduced in both leukemias despite the normal DNA distribution curves in the one parameter analysis (Fig. 1a–c). The two parameter histogram is therefore a more sensitive indicator of the abnormal proliferation patterns in leukemic rats than the one parameter DNA distribution.

DISCUSSION

The main result of this study is that leukemic cells of the L5222 and the Shay-chloroleukemia are slightly hyperploid

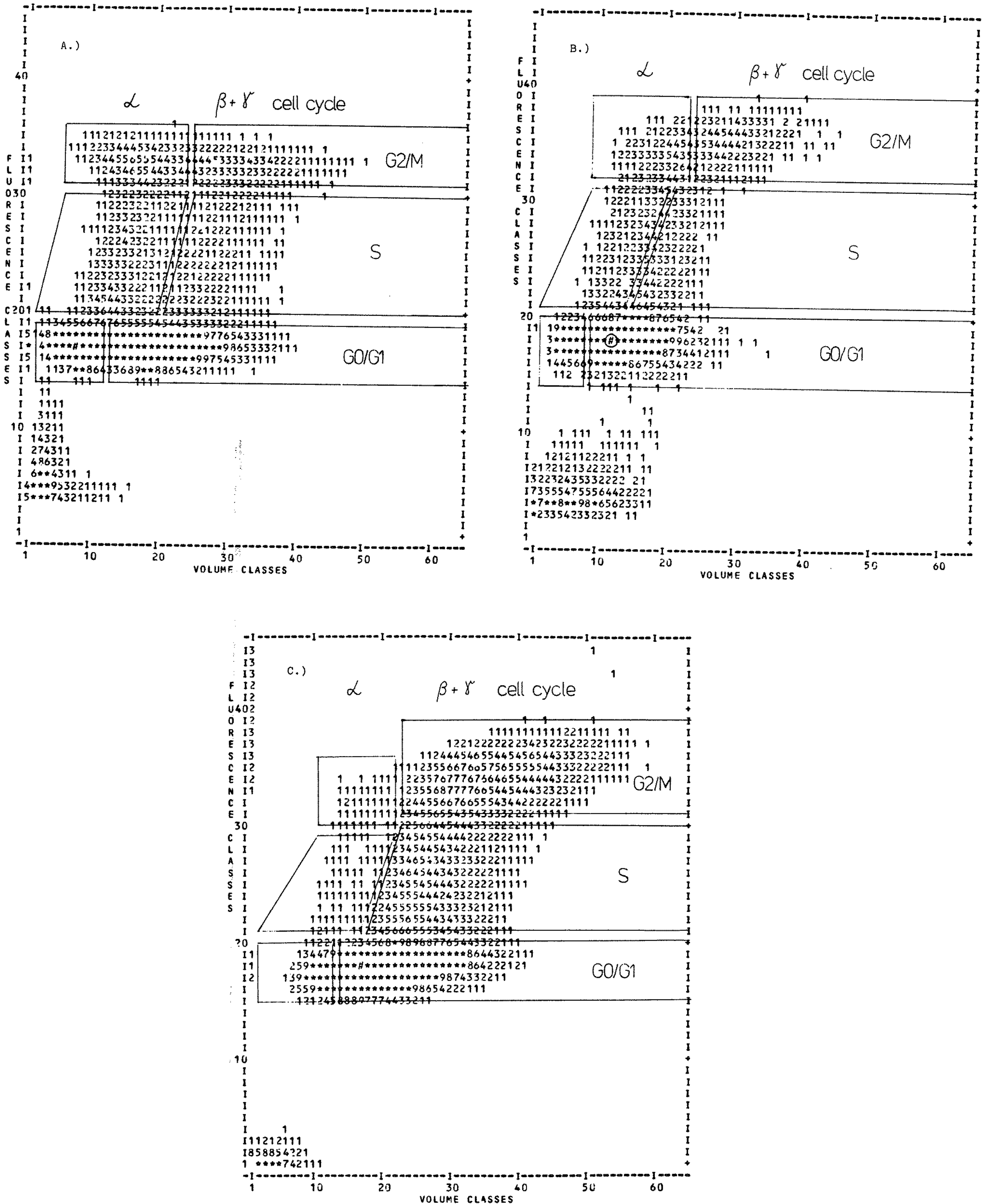

FIG. 3. Isoamplitude plot of the two parameter histograms of Figure 2 for bone marrow cells of a normal rat (a) (94691 cells), and of L5222 (b) (44629 cells) and Shay leukemia (c) (141843 cells) bearing animals. The plots are normalized to their maximal amplitude (#). The amplitude range between 0-10% is divided into 10 steps with 1% stepsize (numbers 0-9). Channel contents between 0-0.99% of the maximum amplitude (number 0) are suppressed in the printout. Channel contents of more than 10% of the maximum amplitude are indicated by *. The lines in the plot were used to limit the different cell cycle phases for quantitative analysis (Tab. I). The outer lines of the areas were positioned where the channel contents decreased below 1% of the maximum amplitude. The S phase limiting lines were drawn where the steep decrease of the G0/G1 and G2/M peaks of the DNA distribution (Fig. 1a-c) changed into the horizontal part of the curve between both peaks. The limiting lines between the α and the $\beta + \gamma$ -cell cycle were positioned at the minimum between the peaks of the volume distribution curves of the G0/G1, S1, S3 and G2/M phases (Fig. 4a-c).

TABLE I

Quantitative Cell Cycle Analysis of the Cell Volume vs Cell DNA Histograms of Figure 3a–c

Cycle Phase	% of All Cells in Cycle			% Cells of Each Cycle in Cycle Phase	
	α	$\beta+\gamma$	$\alpha+\beta+\gamma$	α	$\beta+\gamma$
Normal bone marrow cells					
G0/G1	31.0	36.9	67.9	69.2	66.8
S	9.3	9.7	19.0	20.8	17.6
G2/M	4.5	8.6	13.1	10.0	15.6
Total	44.8	55.2	100	100	100
L5222 leukaemia					
G0/G1	11.2	56.9	68.1	44.8	75.9
S	7.9	9.1	17.0	31.6	12.1
G2/M	5.9	9.0	14.9	23.6	12.0
Total	25.0	75.0	100	100	100
Shay-chloroleukaemia					
G0/G1	7.0	56.8	63.8	66.0	63.4
S	2.2	14.8	17.0	20.7	16.7
G2/M	1.4	17.8	19.2	13.3	19.9
Total	10.6	89.4	100	100	100

and proliferate predominantly in the β and γ-cell cycle of the bone marrow while the α-cell cycle is euploid and relatively diminished (Table I). The apparently normal contribution of S and G2/M phase cells in the one parameter DNA distribution histogram (Fig. 1b,c) resembles the situation in human leukemias (8, 9). If one considers the distribution of cells in the different cell cycle phases for the α or the $\beta+\gamma$-cell cycle individually, the number of S + G2/M phase cells has a tendency to increase in the α-cell cycle and to decrease in the $\beta+\gamma$-cell cycle (Table I). In addition to the L5222 and the Shay leukemia we have recently measured bone marrow cells of a BNML leukemia (7, 16). Similar proliferation patterns and cell cycle characteristics were observed as in the other two leukemias. The conclusion from these results is that the number of cells in the $\beta+\gamma$-cell cycle is similarly increased and that the cells are slightly hyperploid in three different leukemias growing in three different rat strains. It will be of interest if human bone marrow cells show similar distribution patterns. If so, it is thought that the two parameter measurement is especially useful as early indicator for leukemic relapses. Although it is evident from the results that the two parameter method is more sensitive than the one parameter DNA distribution measurement, one may ask how specific both parameters are to prove the presence of leukemic cells. The increase of the cells in the $\beta+\gamma$-cell cycle of the two parameter histograms is not strictly specific for leukemic cells. Preliminary studies on rapidly proliferating bone marrow cells of rats during the first days after birth and during repopulation of bone marrow after depletion of cells by 600 R total body x-irradiation indicate that the $\beta+\gamma$-cell cycle also increases but not to the same extent as in leukemias. The more specific indicators for leukemia are the hyperdiploid and hypertetraploid cells in the G0/G1 and G2/M phase of the $\beta+\gamma$-cell cycle. Such cells are not observed during normal bone marrow

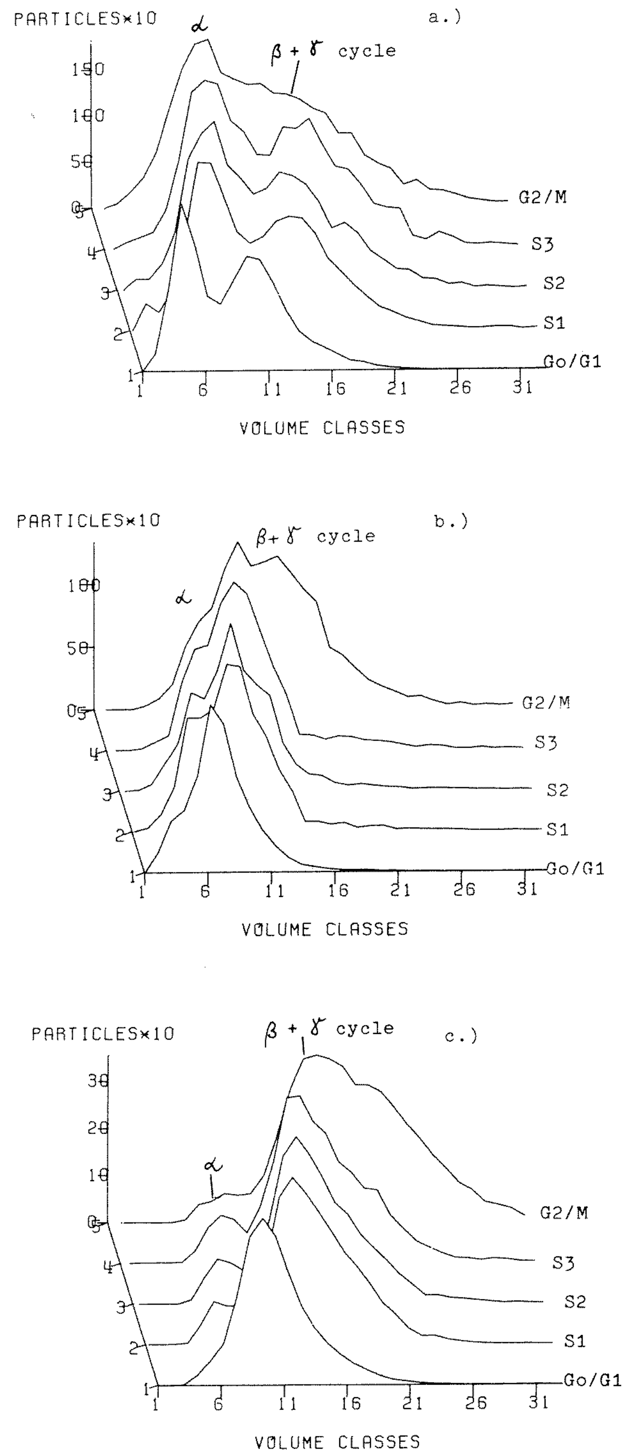

FIG. 4. Volume distribution curves of the G0/G1, S1, S2, S3 and G2/M phase of normal rat bone marrow cells (a), of L5222 leukemia (b) and of Shay chloroleukemia (c). Each of the volume distribution curves is normalized to its maximum for better comparison.

proliferation. To make this a more objective criterion, one can calculate the relative frequencies of cells in the G2/M phase of the α and the $\beta+\gamma$-cell cycles above a certain DNA threshold. A cell sample would be suspicious if the relative

number of cells in the $\beta + \gamma$-cell cycle above the threshold is significantly higher than in the α-cell cycle which contains the remaining normal cells. More resolution could be obtained if the leukemic cells would quantitatively differ from normal cells in a cell cycle dependent additional parameter. One would then obtain a separate cell cluster for the leukemic cells in a simultaneous three parameter analysis. The advantage would be that such a measurement allows a specific recognition of leukemic cells, although, none of the three parameters alone is strictly specific for leukemia detection. More work, especially measurements of human bone marrow biopsies, correlation between histology and flow cytometric results and measurements of premixed samples of normal and leukemic cells are needed to evaluate the significance of this method for therapy control and diagnosis of human leukemias.

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