Laboratory techniques

Assessment of DNA content and cell cycle distribution of erythroid and myeloid cells from bone marrow

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SUMMARY A method is described for the measurement of DNA index and cell cycle distribution in purified erythroid and myeloid populations from human bone marrow. Erythroid cells were prepared after complement mediated lysis of non-erythroid marrow cells. Myeloid cells were obtained by fluorescence activated cell sorting by forward and wide angle light scatter. Mononuclear marrow cells were prepared with a density gradient. Nuclei prepared from the separated populations were stained with propidium iodide. Myeloid cells had a higher DNA index than erythroid cells, and the mononuclear preparation had an intermediate value. There were more erythroid than myeloid cells in the S and G_2M phases of the cell cycle. These lineage differences are particularly relevant when considering data derived from unseparated bone marrow cells, and further experiments are needed to determine the origin of these anomalies.

A number of methods exist for fluorochrome staining of nucleic acids for quantitation of cellular DNA content by flow cytometry and cell cycle measurements.[1] The information is extracted from DNA histograms which represent the distribution of DNA contents for a population of cells throughout the cell cycle. This ranges from those which in normal cells have a diploid DNA content (G_0/G_1) to those with a tetraploid content (G_2M), any cells with intermediate DNA contents being allocated to S phase. The position of the G_0/G_1 peak may also be compared with a known standard to derive the DNA index (DI)[1] that enables minor changes in the position of the G_0/G_1 peak to be detected. This can arise from true aneuploidy (cells with a modal DNA content differing from that of diploid cells) or from changes in the permeability and stainability of various cell types to DNA stains.

These methods have been applied to clinical material from both solid tumours and bone marrow malignancies. In solid tumours abnormalities are often gross with extra peaks, or pronounced skewdness, or broadening of the G_0/G_1 peak.[1] With bone marrow malignancies such as acute leukaemia or myelodysplastic syndrome, abnormalities of DNA content commonly affect only small amounts of

chromosomal material, either translocations or deletions, or addition of a single chromosome.[2] The ability of flow cytometry to detect slight changes in cellular DNA content with respect to a reference standard is theoretically totally dependent on the sensitivity of the instruments and the magnitude of the karyotype abnormality. This, however, assumes a stoichiometric association between DNA and the fluorochrome in the staining process. Vindelov *et al*[3] have shown differences in staining between nuclei from cells of different tissues in the mouse, and between human peripheral blood mononuclear cells and granulocytes.

It seems likely that DNA staining may vary among other human cell types. Darzynkiewicz *et al*[4] showed differences in the accessibility of DNA for fluorochrome staining in proliferating undifferentiated erythroleukaemia cells and those that were fully differentiated. Their data suggested that before treatment a large proportion of DNA was unavailable for staining and that interaction with dyes depended largely on the histone content of the nucleus and chromatin structure. Koch *et al*[5] suggested that although precise and reproducible estimations of cellular DNA content may be obtained in most amniotic cell cultures, this is not always possible. They drew attention to the importance of nuclear pyknosis, presumably as a terminal event, in producing chromatin compaction and a consequent reduction in dye penetration of the nucleus.

Table *DNA index (DI) and cell cycle analysis of total marrow mononuclear cells and separated erythroid and myeloid cells (mean and coefficient of variation (CV))*

	DNA index			Cell cycle (%)			
	No of samples	DI	CV(%)	No of samples	G_0/G_1	S	G_2M
Total mononuclear cells	21	1·015**†	2·55	19	75·1***	20·0***	5·2***†
Erythroid	14	1·005	2·26	14	59·5	31·1	9·5
Myeloid	19	1·032***	2·38	14	70·4***	22·6*	7·0*

Significant difference from p values when compared with erythroid cells *p ≤ 0·01, **p ≤ 0·001, and ***p ≤ 0·0001; when compared with myeloid cells †p ≤ 0·01.

Despite these potential complications in the interpretation of DNA index measurements on mixed cell populations, a number of groups have reported measurements on samples of human bone marrow in both normal subjects and in various diseases[6-9] and have related these to clinical states. We have prepared purified populations of freshly obtained normal bone marrow erythroblasts and myeloid cells to compare the DNA index and DNA distribution within the cell cycle of these lineages with data obtained from infractionated populations.

Material and methods

Samples of bone marrow were obtained by curette from the sternum of patients undergoing cardiac bypass operations. All patients had given their fully informed consent, and had normal haematological measurements. Samples of marrow were collected into 10 ml Eagle's minimal essential medium (Wellcome Reagents Ltd) buffered with 20 mmol/l hydroxyethylpiperazine ethane sulphonic acid (HEPES) (Gibco) to pH 7·4 at 37° and containing 15 units/ml preservative free heparin (Evans Medical Ltd). The osmolality was 280–300 mOsm/kg water. Single cell suspensions were prepared from the samples,[10] and divided appropriately for preparation of the different cell fractions. Marrow mononuclear cells were prepared by Ficoll Hypaque (Sigma) separation. Enriched erythroblast populations were prepared using a complement mediated lysis technique.[11] TG-1, a monoclonal antibody reactive against myeloid cells, and a polyclonal rabbit antihuman mononuclear cell antibody, were added to lyse non-erythroid cells, using AB compatible human plasma as the source of complement. Enriched myeloid populations were sorted using a combination of forward angle light scatter and 90° light scatter measurements[12] with a FACS III (Becton Dickinson). Cytocentrifuge preparations were made on the enriched populations and stained with Jenner-Giemsa. Four hundred cell differential counts were carried out using conventional morphological criteria for scoring. Myeloid cells were scored as myeloblasts, myelocytes, metamyelocytes,

and polymorphonuclear cells, and the results converted to percentages. The polymorphs are representative of peripheral blood contamination and, because they are non-cycling cells, they are all in the G_0/G_1 fraction. Subtracting the percentage of polymorphs from the G_0/G_1 peak and rescaling the resulting cell cycle to a total of 100% eliminates this source of contamination.

All cells were washed in citrate buffer[13] adjusted to a concentration of $2·5 - 3·5 \times 10^6$ cells/ml and frozen in 200 μl aliquots at −70°. The nuclei are stable in this condition for at least a year.[14]

Nuclei were stained for DNA with propidium iodide (PI) by the method of Vindelov *et al.*[13] Flow cytometry was by a 488 nm excitation wavelength, the emission being analysed through a 620 nm long pass filter. In each case the G_0/G_1 DNA content was determined using chicken and trout erythrocyte nuclei as internal standards.[15] Peripheral blood mononuclear cells obtained by Ficoll Hypaque separation and treated in the same way were run in duplicate in each assay as a standard to minimise variation within batches.[16] Ten thousand nuclei were analysed/sampled. The DNA index (DI)[17] of each marrow sample was calculated by relating the G_0/G_1 DNA content of each sample to that of the human peripheral blood mononuclear cell standard. Separate samples omitting chicken and trout nuclei were used for cell cycle analysis. Two × 10^4 nuclei were analysed and the histogram was resolved into G_0/G_1, G_2M, and S-phase compartments by a modification of the method of Dean.[18] Normal ranges were calculated using the mean (1·96 SD) for each measurement, and the Mann-Whitney U test was used for statistical analysis of the results.

Results

The results are summarised in the table. The mean purity of the erythroblast preparations was 91% (range 88–98%) and the myeloid preparation was 97% (range 89–100%). The contaminating cells were usually a mixture of monocytes, lymphocytes, and plasma cells. Mean recovery of erythroblasts was 58% (SE 6%). These recovered populations have similar maturity distributions to the erythroblast population
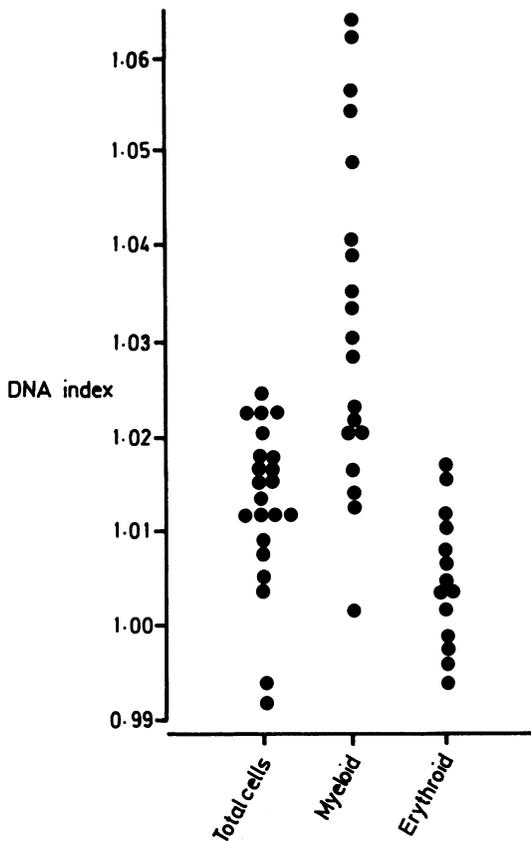
Fig 1 *Distribution of DNA index of total marrow mononuclear cells and separated erythroid and myeloid cells.*

in whole marrow,[19] suggesting that there is no selective loss of any stage of erythroblast maturity. By carefully selecting the myeloid population by forward and wide angle light scatter, over 95% of the total myeloid cells can be included in the window, and therefore both myeloid and erythroid preparations reflect the population structure in the original samples. A correction factor of 0·9825 was applied to the DI results from female patients to allow for the difference in DNA content between the X and Y chromosomes.[20] Myeloid cells had a greater DI range than that of erythroid cells (fig 1) with a significantly higher mean (p $\leq$ 0·0001). The total mononuclear cells formed an intermediate population with a mean DI lower than that for myeloid cells (p $\leq$ 0·01) and higher than that for erythroid cells (p $\leq$ 0·001). There were no significant differences among the coefficients of variation (CV) of the G_0/G_1 peaks for the three groups. Compared with the total mononuclear population, the erythroid cells had a smaller percentage in G_0/G_1 and more in both G_2/M and S phase (p $\leq$ 0·0001 in all cases). A similar

difference was apparent between myeloid and erythroid cells (p $\leq$ 0·0001 for G_0/G_1 and p $\leq$ 0·01 for the G_2M and S phases). The only significant difference between myeloid and total mononuclear cells was for the G_2M component, which was higher for the myeloid cells (p $\leq$ 0·01).

Discussion

DNA flow cytometric analysis of human bone marrow is potentially useful in the investigation and characterisation of haematopoietic disorders and in monitoring drug treatment. The clinical value of this technique, however, may be limited by the heterogeneity of the bone marrow. More specific information can be obtained by physical separation of different cell lineages. Our results have shown that erythroid and myeloid cells have a significantly different DI when compared with the same reference cells, and there are normally differences in the distribution of cells within the cell cycle in these two lineages. The large numbers of erythroblasts in samples of marrow with low DIs and of immature myeloid precursors in those with a high DI have previously been reported.[8 21] They suggested that the degree of compactness of chromatin affected the ease of intercalation of fluorochrome dyes and the present results are compatible with this hypothesis. With only two exceptions, erythroid cells had a lower DI than myeloid cells taken from the same patient, suggesting that erythroid chromatin is less easily accessible to PI than myeloid chromatin. In the mixed group obtained using Ficoll Hypaque interface cells, the percentage of each cell type may be a factor in determining the DI measurement. In two patients with abnormal erythroid hyperplasia, Percoll fractionation of the erythroid cells into subpopulations of varying maturity did not show a significant difference in DI between fractions (unreported data).

In the present study myeloid cells comprised a mean of 65% of the total mononuclear cells (range 52–81%) and erythroid cells only 16% (range 8–31%). In abnormal marrow samples, however, the myeloid: erythroid ratio might deviate significantly from normal and influence the final DI measurement. In an earlier study no significant correlation was found between DI and either the percentage of myeloid cells or of erythroblasts in marrow samples from patients with myelodysplastic syndrome,[20] though in that instance the correlation was made with cell counts on the original marrow preparation rather than the Ficoll interface layer. In a later study[9] mixed marrow populations from patients with primary acquired sideroblastic anaemia and erythroid hyperplasia were commonly found to have a DI significantly greater than that of normal marrow, suggesting that even in a heterogeneous cell population a decreased myeloid: erythroid
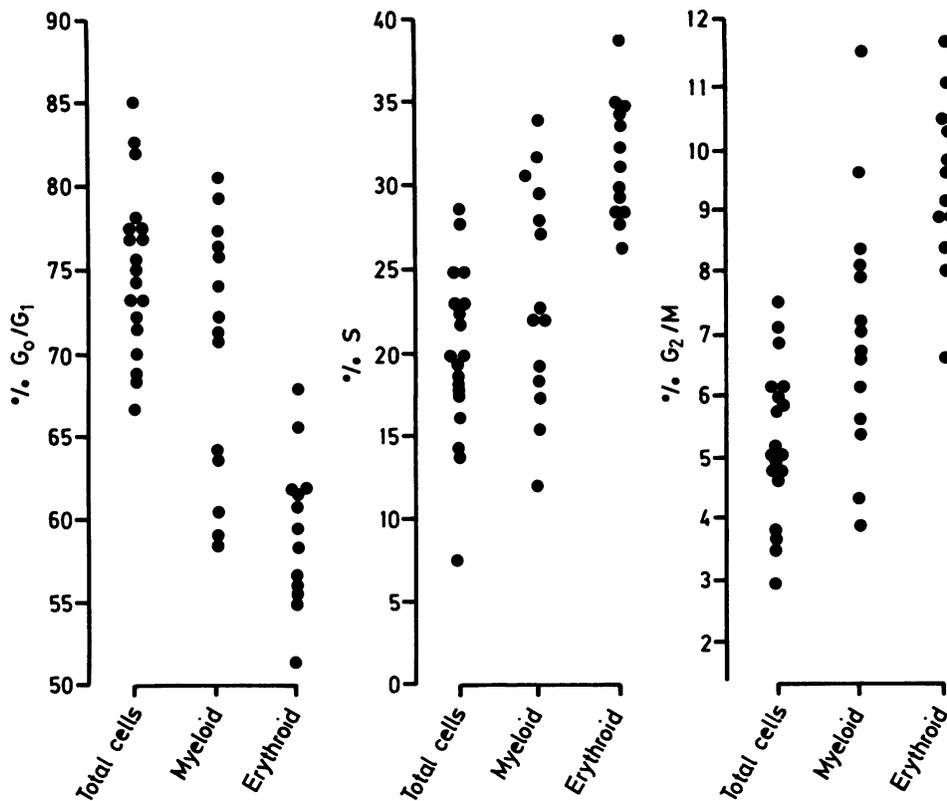
Fig 2   *Cell cycle phase distribution of total marrow mononuclear cells and separated erythroid and myeloid cells.*

ratio does not necessarily obscure a change in DI due to an increase in the content of DNA.

In the present investigation the coefficient of variation of DI measurements was minimised by careful alignment of the flow cytometer and compares favourably with those reported by other authors using the same staining technique.[8 20 21] The acquisition of normal ranges for separate cell lineages should enable small deviations to be detected. The finding that in normal marrow more erythroblasts are found in the S and G_2M phase of the cell cycle than myeloid cells is in agreement with studies using tritiated thymidine.[22] The clinical importance of the DNA index in disease such as acute lymphoblastic leukaemia[7] or myelomatosis[6] or the value of cell cycle analysis in acute myeloblastic leukaemia,[23 24] is associated with observations made on comparatively homogeneous populations of primitive malignant cells. In myelodysplastic syndrome, in which a mixture of inadequately differentiated lineages are present and remnants of normal haemopoiesis may remain, Clark *et al*[9] found that a low DNA index indicated a poor prognosis and Montecucco *et al*[8] found prolonged survival in patients

with the highest proportion of cells in the S and G_2M phases. It may be that all lineages are usually abnormal in this disease but recent observations[25] suggest that this is not necessarily so and it would be useful to carry out lineage specific measurements to obtain greater precision and define the progression of the disease in more detail.

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