TECHNICAL NOTE

Immediate bromodeoxyuridine labelling of unseparated human bone marrow cells *ex vivo* is superior to labelling after routine laboratory processing

P. Ø. Jensen, B. T. Mortensen,* I. J. Christensen and J. K. Larsen

*Finsen Laboratory and *Haematological Department, Finsen Center, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark*

(Received 15 May 1997; revision accepted 15 December 1997)

Abstract. It is important to evaluate the proliferation of bone marrow cells in several disease conditions and during treatment of patients with for example cytokines. Labelling with bromodeoxyuridine (BrdUrd), immunocytochemical staining with anti-BrdUrd antibody and analysis by flow cytometry provides a reliable and reproducible technique for estimation of the fraction of cells that incorporated BrdUrd into DNA during S-phase. We have compared immediate BrdUrd labelling of unseparated bone marrow cells with the previously used labelling in the laboratory after routine separation of the mononuclear cells. Bone marrow aspirates from seven lymphoma patients without bone marrow involvement were studied with these two methods. We found higher BrdUrd labelling indices (LI) in the mononuclear cells, when cells were labelled immediately. A large variation in LI was found between patients. Our results suggest that *ex vivo* BrdUrd labelling of bone marrow cells should be performed immediately after aspiration and before separation, because these data are closer to values reported from *in vivo* labelling with BrdUrd.

The investigation of the proliferating cells in human bone marrow is of interest in a variety of conditions, for example in leukaemias in connection with chemotherapy and cytokine treatment. To study cell proliferation in the bone marrow, flow cytometric multiparameter analysis of bromodeoxyuridine (BrdUrd) incorporation in combination with DNA content (Begg *et al.* 1985) or expression of phenotypic markers of leucocyte subsets (Carayon & Bord 1992) might provide important insights. For ethical reasons, *in vivo* labelling of patients with BrdUrd is often not feasible due to the carcinogenic and mutagenic risk (Napalkov *et al.* 1989). Therefore, BrdUrd labelling *ex vivo* is an attractive alternative. In search of an optimal procedure for *ex vivo* BrdUrd labelling of the mononuclear cell fraction of normal human bone marrow aspirates, we have compared immediate BrdUrd labelling of unseparated bone

Correspondence: Peter Østrup Jensen, Finsen Laboratory, Rigshospitalet, Department of 8621, Strandboulevarden 49, DK-2100 Copenhagen, Denmark.

marrow cells with the previously used procedure of labelling after storage on ice and separation of the mononuclear cells by gradient centrifugation.

MATERIALS AND METHODS

BrdUrd labelling of bone marrow cells

After informed consent, bone marrow cells were obtained from seven lymphoma patients who underwent staging procedures showing no bone marrow involvement. The cells were aspirated from the *spina iliaca posterior superior* with heparin-coated syringes. The first 8 ml of aspirated cells were divided into two equal portions for labelling with BrdUrd before (method I) and after separation (method II). The density centrifugation for separation of mononuclear cells (MNC) was carried out simultaneously for the two methods.

Method I

Immediately after aspiration, portions of 1 ml aspirate were transferred to four tubes with 1 ml RPMI 1640 medium and 100 units of heparin. Three tubes additionally contained 20 μ M BrdUrd (5-bromo-2'-deoxyuridine, Sigma, St Louis, MO). The tubes were incubated before and after sample addition at 37°C in a block heater, and were shielded from light. The cells were harvested from tubes containing BrdUrd after 30, 60 and 120 min of labelling, and from the tube without BrdUrd after 120 min. After labelling, the cells were cooled on an ice-bath until separation of all cell samples at the end of the 120 min labelling period. The MNC were separated by density centrifugation using Lymphoprep (Nycomed, Oslo, Norway), then cooled in an ice-bath, washed with PBS (Dulbecco's phosphate buffered saline without calcium and magnesium, pH 7.2), and the sedimented cells were fixed by suspension in cold 70% ethanol and kept at -20°C.

Method II

The other half of the bone marrow aspirate was kept on ice for 120 min until separation of MNC in parallel with method I. The MNC were then divided in four aliquots. The aliquots were incubated as above with 20 μ M BrdUrd for 30, 60 and 120 min, and without BrdUrd for 120 min. The cell samples were finally cooled on ice and fixed as above.

Flow cytometry

Using a previously described method, optimized for small samples of leukaemia cell lines (Jensen *et al.* 1994), the fixed samples were stained for flow cytometric analysis of incorporated BrdUrd and DNA content. In brief, cells were pretreated with pepsin/HCl, incubated on ice with monoclonal mouse anti-BrdUrd antibody (M-744, DAKO, Glostrup, Denmark) and then with polyclonal FITC-conjugated rabbit antimouse antibody (F-313, DAKO). Finally, the DNA was stained with a solution of propidium iodide containing RNase, in order to monitor the cell cycle distributions of labelled and unlabelled cells. Flow cytometric analysis of the stained samples was performed as previously described (Jensen *et al.* 1994), either using a FACS IV or a FACS Vantage (Becton Dickinson Immunocytometry Systems, San José, CA). Debris and aggregates were excluded according to the PI fluorescence. The LI of a sample was calculated as the percentage of cells with a FITC fluorescence intensity higher than the unspecific background level indicated by the sample incubated without BrdUrd. The reproducibility of the LI estimates was calculated from double determination on samples that were stained and flow cytometrically analysed on different days (SD=2.6%). Two-way analysis of variance was used to test the hypothesis that

the means of the two methods are equal for each of the three durations of incubation with BrdUrd.

RESULTS

An example of the flow cytometric BrdUrd/DNA dual parameter analysis of mononuclear cells from normal human bone marrow aspirates is shown in Fig. 1, comparing cells immediately labelled with BrdUrd (method I) with cells labelled after routine cell separation (method II).

The LI values of method I were significantly higher compared to method II for all chosen durations of incubation (for 30 min, $P=0.004$; for 60 min, $P<0.0001$; and for 120 min, $P=0.004$), see Table 1. The difference in the LI of method I and method II increased with time for two patients (patients 1 and 2) and was relatively constant for the other five patients. There was a considerable variation between patients. In addition to individual variations, the interpatient variation may also include large sampling variations due to the compartmental structure of the bone marrow and possible admixture of blood into the aspirate. These variations were not included in our estimate of the reproducibility of the flow cytometric assay.

The flow cytometric BrdUrd/DNA dot plots indicated a large degree of heterogeneity with regard to the BrdUrd labelling intensity of the cells with S-phase DNA content, which is not revealed by the simple distinction between positive and negative BrdUrd labelled cells in Table 1. Considering the cells with a DNA content typical for the middle part of the S-phase, we found in five patients that relatively many cells were weakly labelled with method I (Fig. 1, region A) and very few cells were unlabelled (Fig. 1, region B). The opposite was found with method II in 4 patients, very few weakly labelled cells and many unlabelled cells. This pattern was observed at all durations of BrdUrd incubation (30, 60 and 120 min).

With both methods, but most pronounced with method II, we found that the LI was significantly lower than the fraction of cells with S-phase DNA content (SPF) (Table 1). Thus, samples immediately labelled with BrdUrd also contained a significant fraction of cells with S-phase DNA content that were not active in DNA synthesis. The size of SPF, in itself, did not indicate a difference between the methods or between the durations of incubation with BrdUrd.

DISCUSSION

The results of the present study suggest that *ex vivo* BrdUrd labelling for the purpose of measuring the cell proliferation in the mononuclear cell fraction of human bone marrow aspirates must be done immediately after bone marrow aspiration, and not after a period of cooling followed by density centrifugation, which clearly leads to a systematic underestimation of the LI. In the samples incubated with BrdUrd after separation, the occurrence of a significant subpopulation with a DNA content typical for the middle part of the S-phase, but showing no incorporation of BrdUrd, indicates that the cell cycle progression has been perturbed due to unfavourable conditions for S-phase progression.

This finding of a decreased LI, due to delay by laboratory procedures before incubation with BrdUrd, is supported by the experience of other investigators from studies of other cell systems. In a paper on tumour cells and stimulated blood lymphocytes, Stout & Suttles (1988) comment that the cells should not be chilled prior to the BrdUrd pulse, because bringing the cells back to 37 °C will not ensure a restoration of the original rate of DNA synthesis per cell. Therefore, refrigerated centrifugation of specimens prior to BrdUrd

Figure 1. Flow cytometric dual parameter dot plots of BrdUrd incorporation (log FITC fluorescence) vs. DNA content (propidium iodide fluorescence) for BrdUrd labelled and unlabelled mononuclear bone marrow cells of patient 6. The BrdUrd LI was estimated for cell samples incubated with BrdUrd for 30, 60 and 120 min either immediately (method I) or after cell separation by density centrifugation (method II). The large regions indicate nuclei that are BrdUrd positive (R3) and negative (R2). $LI = 100 \times R3 / (R2 + R3)$. The small regions A and B delimit in the one dimension the middle region of the S-phase according to DNA content, and in the other dimension, respectively, a region of weakly BrdUrd labelled cells supposed to be slowly DNA replicating (A), and a region of BrdUrd unlabelled cells supposed to have ceased but not completed the DNA replication (B).

Table 1. Bromodeoxyuridine labelling index (BrdUrd LI) and DNA S-phase fraction (DNA SPF)^a of mononuclear cells of bone marrow aspirates from seven lymphoma patients without bone marrow involvement, estimated by flow cytometry in cells incubated with BrdUrd for 30, 60 and 120 min either immediately (method I) or after routine cell separation (method II)

Patient	Parameter	BrdUrd labelling period								
		30 min			60 min			120 min		
		Method I	Method II	Difference	Method I	Method II	Difference	Method I	Method II	Difference
1	BrdUrd LI (%)	9.0	7.3	1.7	9.0	6.4	2.6	14.8	5.7	9.1
	DNA SPF (%)	11	10	1	9	25	−16	9	13	−4
2	BrdUrd LI (%)	12.8	7.1	5.7	16.1	7.7	8.4	24.3	8.2	16.1
	DNA SPF (%)	20	13	7	20	12	8	26	12	14
3	BrdUrd LI (%)	14.0	6.3	7.8	14.5	9.0	5.5	15.7	9.5	6.2
	DNA SPF (%)	16	15	1	18	18	0	17	15	2
4	BrdUrd LI (%)	6.3	1.0	5.3	7.7	1.6	6.1	7.8	2.0	5.8
	DNA SPF (%)	10	6	4	12	7	5	12	7	5
5	BrdUrd LI (%)	2.3	1.6	0.7	2.7	2.0	0.7	3.4	2.1	1.3
	DNA SPF (%)	5	6	−1	5	5	0	4	7	−3
6	BrdUrd LI (%)	4.6	3.8	0.8	6.5	3.3	3.3	6.2	6.2	0.0
	DNA SPF (%)	7	14	−7	9	11	−2	10	14	−4
7	BrdUrd LI (%)	13.4	15.9	−2.5	16.2	13.8	2.5	12.2	15.0	−2.8
	DNA SPF (%)	16	18	−2	16	12	4	11	14	−3
Mean	BrdUrd LI (%)	8.9	6.1	2.8	10.4	6.2	4.1	12.0	7.0	5.1
	DNA SPF (%)	12.1	11.7	0.4	12.7	12.9	−0.1	12.7	11.7	1.0
SD	BrdUrd LI (%)	4.7	5.0	3.6	5.3	4.4	2.6	7.1	4.5	6.3
	DNA SPF (%)	5.4	4.6	4.5	5.5	6.8	7.8	7.0	3.4	6.7

^aCalculated from the DNA distribution, as described by Vindeløv & Christensen (1990), using a 2° polynomial for fitting the S-phase fraction.

pulsing should be avoided. In addition, in a recent study of rat thymocytes and using *in vivo* BrdUrd labelling as a control, Carbajo-Pérez *et al.* (1995) found that immediate labelling *ex vivo*, after the sample was obtained, resulted in a slight decrease of the proportion of S-phase cells analysed either according to DNA content or to BrdUrd immunolabelling, and that keeping the samples for 15 min or more before BrdUrd incubation, regardless of storage temperature, led to a further decrease in the S-phase fraction. Thus, the results from others together with the present experience with human bone marrow cells indicate it to be a general rule that cells for BrdUrd labelling should be kept for a minimum of time at temperatures lower than 37 °C before labelling. Furthermore, the separation of MNC before labelling could remove valuable cells and cytokines with a resulting decrease of LI. The effect of the time kept on ice vs. the labelling before and after separation cannot be addressed in this study as these two factors are confounded.

For ethical reasons, it has not been possible in our study of normal human bone marrow to include a comparison with *in vivo* BrdUrd labelling. Our estimate of a mean LI for the MNC of 10.4% (range 2.7–16.2%) obtained by immediate BrdUrd incorporation *ex vivo* for 60 min, may be compared with the autoradiographical finding of Lord *et al.* (1989) of 12% and 15% in MNC LI from two patients by *in vitro* labelling with ³H-TdR for 30 min in aspirates from the posterior iliac crest. In another experiment Lord *et al.* (1994) found by *in vivo* labelling with ³H-TdR for 60 min a LI of ≈18% in aspirates from the posterior iliac crest of four patients. Riccardi *et al.* (1989) found a mean LI of 15.4% (range 12.4–25.4%) by *in vivo* BrdUrd labelling for 6 h in an analysis of sternal aspirates from cancer patients with normal (uninvolved) bone marrow. They used a similar flow cytometric methodology with correction for the contribution from divided labelled cells. The deviance between these results could be explained by the different techniques and limited number of patients.

In the study of human bone marrow cells, sampling variations due to tissue heterogeneity and the unintended admixture of blood into the aspirate are further complications that may bias the results and limit the comparability of results from different studies. Furthermore, microenvironmental conditions in the bone marrow that may inhibit the uptake of BrdUrd *in vivo*, cannot be evaluated *ex vivo*. The LI of the entire MNC fraction of the bone marrow may have limited clinical applicability, because it does not differentiate for cell differentiation and maturity, however, this might be achieved by applying a combined analysis of BrdUrd incorporation and immunophenotype using, e.g. the staining method of Carayon & Bord (1992).

In conclusion, for estimating the cell proliferation in the human bone marrow immediate *ex vivo* BrdUrd labelling must be preferred to labelling after storage on ice and cell separation, because the obtained LI is higher, approaching the LI level of *in vivo* labelling, and increasing with incubation time as expected. For many purposes, as in normal bone marrow and in allogeneic bone marrow transplantation, immediate BrdUrd labelling of bone marrow aspirates is an alternative to *in vivo* labelling and is without any ethical complications.

ACKNOWLEDGEMENTS

This study was supported by the Danish Cancer Society, Danish Medical Research Council and Simon Spies Foundation.

REFERENCES

- BEGG AC, McNALLY NJ, SHRIEVE DC, KÄRCHER H. (1985) A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* **6**, 620.

- CARAYON P, BORD A. (1992) Identification of DNA-replicating lymphocyte subsets using a new method to label the bromo-deoxyuridine incorporated into the DNA. *J. Immunol. Method.* **147**, 225.
- CARBAJO-PÉREZ E, CARBAJO S, CIUDAD J, LÓPEZ A, DOLBEARE F, ORFAO A. (1995) *In vitro* bromodeoxyuridine-labelling of single cell suspension: effects of time and temperature of sample storage. *Cell Prolif.* **28**, 609.
- JENSEN PØ, LARSEN JK, CHRISTENSEN IJ, VAN ERP PEJ. (1994) Discrimination of bromodeoxyuridine labelled and unlabelled mitotic cells in flow cytometric bromodeoxyuridine/DNA analysis. *Cytometry* **15**, 154.
- LORD BI, BRONCHUD MH, OWENS S *et al.* (1989) The kinetics of human granulopoiesis following treatment with granulocyte colony-stimulating factor *in vivo*. *Proc. Natl. Acad. Sci. USA* **86**, 9499.
- LORD BI, TESTA NG, BRETTI S *et al.* (1994) Haemopoietic progenitor and myeloid cell kinetics in humans treated with interleukin-3 and granulocyte/macrophage colony-stimulation factor in combination. *Int. J. Cancer* **59**, 483.
- NAPALOV NP, ANISIMOV VN, LIKHACHEV AJ, TOMATIS L. (1989) 5-Bromodeoxyuridine-induced carcinogenesis and its modification by persistent estrus syndrome, unilateral nephrectomy, and X-irradiation in rats. *Cancer Res.* **49**, 318.
- RICCARDI A, DANOVA M, DIONIGI P *et al.* (1989) Cell kinetics in leukaemia and solid tumours studied with *in vivo* bromodeoxyuridine and flow cytometry. *Br. J. Cancer* **59**, 898.
- STOUT R, SUTTLES J. (1988) Problems and applications of cell cycle analysis: distinguishing G₀ from G₁ and G₁ from S phase. *Cytometry (Suppl. 3)*, 34.
- VINDELØV LL, CHRISTENSEN IJ. (1990) A review of techniques and results obtained in one laboratory by an integrated system of methods designed for routine clinical flow cytometric DNA analysis. *Cytometry* **11**, 753.