# The effect of blood storage on differential chromosome staining of human lymphocytes[1]

J. S. Prosser and J. E. Moquet

*National Radiological Protection Board, Chilton, Didcot, Oxon OX11 0RQ (England), March 18, 1983*

*Summary.* Human blood was stored for up to 3 days before 66-h culture with 3 concentrations of bromodeoxyuridine (BrdU) (10, 20 and 40 μM). In cultures containing 10 or 20 μM BrdU, the number of cells staining as first division metaphases (M1) increased with storage time. In addition, as the storage time was increased the staining quality deteriorated although this was less pronounced in cultures containing 40 μM BrdU. The implications of these results for routine fluorescence plus Giemsa staining as part of a cytogenetic radiation dosimetry service are discussed, with the recommendation that for accurate identification of M1 cells, the concentration of BrdU should be increased in cultures set up from blood that has been stored for more than 2 days.

For accurate cytogenetic radiation dosimetry it is important to ensure that only metaphases from first division cells (M1) are analyzed because of the progressive elimination of unstable chromosome aberrations during successive cell divisions in culture[2]. Over the past few years it has become evident that even in the 48-h lymphocyte cultures which are routinely used to provide M1 cells for analysis a variable number of second (M2) or even later mitoses may be present[3]. The proportion of each generation of cells observed at 48 h is dependent on the precise conditions of culture employed[4]. Donor variability in the speed of response of lymphocytes to phytohaemagglutinin stimulation and the rate of progression through the cell cycle to metaphase is also important in this respect[3].

The application of the fluorescence plus Giemsa (FPG) staining technique should improve the accuracy of chromosome dosimetry, because it allows unequivocal identification of cells from M1, M2 or later mitoses. However, FPG staining requires the addition of a small quantity of bromodeoxyuridine (BrdU) to each culture which, in addition to altering the staining properties of newly synthesized chromosomal DNA, also slows mitotic activity[5]. After exposure to radiation, lymphocytes suffer an average mitotic delay of about 1 h per gray[6]. If an additional delay due to BrdU incorporation is superimposed, the standard 48-h culture time may become too short. This problem becomes of particular importance for the analysis of the mixed population of cells which results from a partial body exposure involving a high radiation dose to a proportion of the circulating lymphocytes. To reduce the mitotic delay to a minimum, a BrdU concentration as low as possible is necessary and a concentration of 10 μM was chosen for use in this laboratory, based on published techniques[3,7] and trial experiments.

Unforeseen problems with the FPG staining technique first became apparent during a study of sister chromatid exchange induction involving mailed-in samples of blood that were in transit for 1 or 2 days. A large proportion of preparations failed to stain differentially whereas no problems were encountered with parallel control cultures from fresh blood. In addition, better contrast was observed in the FPG stained chromosomes of control preparations. Thus it seemed possible that for a biological dosimetry service working from mailed-in blood samples the use of a minimal BrdU concentration determined by studies on fresh blood might result in a failure to detect all the M2 cells in cultures from posted blood, and an investigation of the influence of BrdU concentration and blood storage on the differential staining of chromosomes was undertaken.

In the experiment described here, 3 concentrations of BrdU have been compared for their effectiveness in permitting the correct identification of M1, M2 and M3 cells in

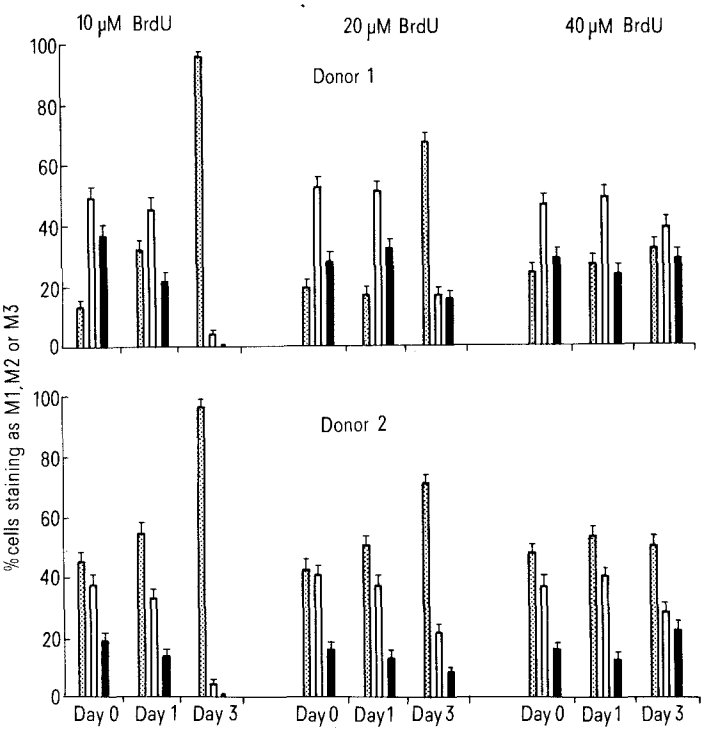


Figure 1. The percentages of metaphases ± SE staining as M1 ▩, M2 □ and M3 ■ observed in 66-h cultures of lymphocytes from 2 donors set up with 3 concentrations of BrdU from fresh, 1-day- and 3-day-old blood specimens.

lymphocyte cultures set up immediately, or 1 or 3 days after sampling.

*Material and methods.* Two healthy male donors provided samples of venous blood which were kept in sterile lithium-heparin tubes for 0, 24 and 72 h at room temperature. Standard whole blood cultures[8] were set up and cultured in the dark for 66 h in the presence of 10, 20 or 40 μM BrdU. The same stock of medium was used for all cultures and coded slides were stained in 1 batch 1 week later. The culture time of 66 h was chosen in order to obtain significant numbers of M2 cells.

In each category, 200 cells per donor, 50 from each of 4 replicate cultures, were scored. For all data points the 4 replicates agreed and were therefore pooled. The data in each category were assumed to obey a binominal distribution and the SE were calculated using the expression

$$SE(N_x) = \sqrt{N_x\left(\frac{N_t - N_x}{N_t}\right)}$$

where $N_t$ is the total number of cells sampled and $N_x$ is the number of cells in a particular cell cycle category. Tests of significance between categories were performed by a simple difference method, a probability of 0.05 or less being regarded as significant.

*Results and discussion.* Figure 1 shows, for each donor, the percentages of cells staining as M1, M2 or M3 for days 0, 1 and 3 at each of the 3 BrdU concentrations. For cultures set up immediately after sampling, the percentages of cells identified as M1 were compared for each BrdU concentration. With donor 1 a small but significant difference between the values for cultures containing 10 μM and 40 μM BrdU was observed ($p = 0.005$). The M1 yield in cultures containing 20 μM BrdU was intermediate and therefore suggestive of a trend and there was a corresponding decrease in the percentage of cells identified as M3. In contrast, results for donor 2 showed no significant differences in the M1 yields ($p \geq 0.549$). Taken together, the results for both donors indicate that any influence of BrdU concentration on the speed of the cell cycle is small under these experimental conditions.

These results are in broad agreement with a number of studies which have examined the effect of increasing BrdU concentration on the speed of cell division in vitro. Using lymphocytes cultured from freshly sampled blood, significant slowing of the cell cycle has been observed at BrdU concentrations above 10 μM[9], 16.3–32.5 μM[10], 35 μM[5] or 50 μM[11].

In the case of blood samples which have been stored before culture, an increase in the proportion of M1 cells with increasing storage time has been reported[12] and this was attributed to a delay in cell cycle progression. Figure 1 shows that at 10 μM BrdU both donors exhibited a significant increase in the number of cells staining as M1 if culture initiation was delayed for 3 days ($p = 0.0001$). In the case of donor 1 this increase was significant even by day 1 ($= 0.0001$). There was a corresponding decrease in cells identified as M2 and M3, the numbers of which were reduced to nearly zero in cultures from both donors set up on day 3. A similar, smaller but still significant increase by day 3 in the proportion of metaphases not staining differentially was observed in cultures from both donors containing 20 μM BrdU ($p = 0.0001$). However, at a BrdU concentration of 40 μM, no significant increase in the numbers of cells staining as M1, was observed even with a storage time of 3 days ($p = 0.366$ or 0.617).

These results are consistent with the view that in cultures containing 10 or 20 μM BrdU either there is a delay in cell cycling time after storage as concluded by Sharma and Das[12] or the differential staining method leads to an overestimate of the numbers of M1 as storage time is increased. From published results (referred to above) obtained using freshly taken blood specimens it would be expected that the highest M1 frequency would be observed in cultures containing higher concentrations of BrdU – not the reverse. This lends support to the suggestion that because of a failure in differential uptake of the stain, a BrdU concentration of 10 or 20 μM is insufficient to allow M2 and later cells to be correctly identified; and that for adequate differential staining of newly synthesized DNA a BrdU concentration above 20 μM is required in cultures set up from blood sampled a day or more previously. The implication for cytogenetic dosimetry is that if the usual minimal BrdU concentration of 10 μM is used in such cultures the number of chromosome aberrations ascribed to M1 cells will be underestimated. This is due to the inclusion of M2 cells in the analysis that have not stained differentially and have therefore not been recognized. In those laboratories where standard 48-h cultures of fresh blood can yield up to 50% M2 cells such misidentification could be a serious source of error.

A further possible source of error that has been examined is the quality of the staining. This can have a marked effect on both the correct identification of the cell cycle and on the ease and accuracy of the analysis for unstable chromosome aberrations. Therefore a subjective analysis of the quality of FPG staining was carried out on the coded slides by a single scorer using the degree of contrast between

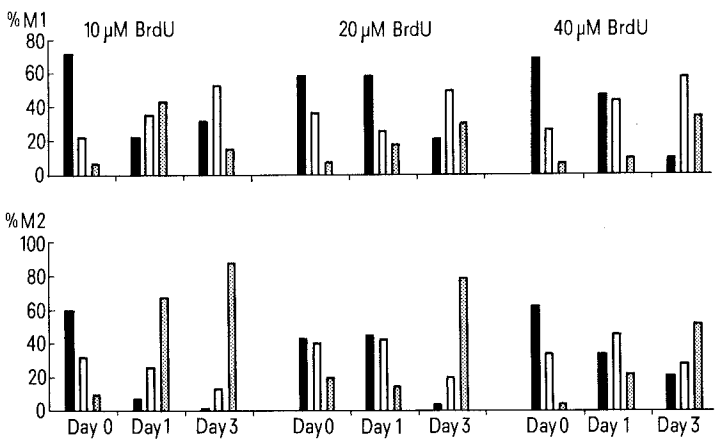


Figure 2. The percentage of good ■, medium □, or poor ▩ quality FPG stained metaphases observed in cultures of lymphocytes containing 3 BrdU concentrations from fresh, 1-day- and 3-day-old blood specimens. Data from 2 donors combined.

sister chromatids to designate the staining of M2 or M3 cells as good, medium or poor. The same 3 categories were accorded to M1 cells based on the general clarity and definition of the chromosomes. No difference in these results was observed between the 2 donors and therefore the data have been combined in figure 2. Data for M3 cells have not been included as the results were essentially the same as those for M2.

A statistical analysis of these data was not considered appropriate owing to their subjective basis and the indeterminate nature of their associated errors. For lymphocytes cultured on day 0 the quality of chromosome preparations does not differ over the range of BrdU concentrations examined and most were considered 'good' or 'medium'. Delay for 1 day has some adverse effect on the quality of M1 chromosome staining but this is not consistent. However, for M2 (and M3) cells, even this short delay in setting up cultures has a marked effect on the staining quality. To some extent this appears to be dependent upon BrdU concentration – 10 μM BrdU giving poorer results than the higher concentrations. After a 3 day delay the majority of cells stained poorly at all BrdU concentrations.

*Conclusions.* It is suggested that the correct identification of M1 and M2 cells in cultures set up from stored blood requires a higher concentration of BrdU in the medium than is necessary in cultures of fresh material. When a concentration of 10 or 20 μM BrdU is used in the culture medium the numbers of cells staining as M1 increases in relation to the storage time due to a failure in differential uptake of Giemsa stain. In addition, as delay is increased the quality of the stained chromosomes becomes poor to a point where identification of M1 and M2 cells is unreliable. However, this can be offset in part by increasing the concentration of BrdU to 40 μM. The need to use this higher concentration does not influence the quality of the staining. It is concluded that no single BrdU concentration is ideal for stored blood but a relationship exists between the time of delay and the optimum BrdU concentration. It is now our practice to increase to 40 μM the BrdU concentration added to cultures of blood that has been delayed in transit for longer than 2 days.

1 This work was partly supported by Euratom Contract 171-76-1 BIO-UK.
2 Buckton, K.E., and Pike, M.C., Int. J. Radiat. Biol. *8* (1964) 439.
3 Crossen, P.E., and Morgan, W.F., Exp. Cell Res. *104* (1977) 453.
4 Purrott, R.J., Vulpis, N., and Lloyd, D.C., Radiat. Prot. Dosimetry *1* (1981) 203.
5 Tice, R., Schneider, E.L., and Rary, J.M., Exp. Cell Res. *102* (1976) 232.
6 Purrott, R.J., Vulpis, N., and Lloyd, D.C., Mutation Res. *69* (1980) 275.
7 Perry, P., and Wolff, S., Nature, Lond. *251* (1974) 156.
8 Lloyd, D.C., and Prosser, J.S., NRPB Memorandum M-70 (1983).
9 Kolin-Gerresheim, J., and Bauchinger, M., Mutation Res. *91* (1981) 251.
10 Scott, D., and Lyons, C.Y., Nature, Lond. *278* (1979) 756.
11 Lambert, B., Hansson, K., Lindsten, J., Sten, M., and Werelius, B., Hereditas *83* (1976) 163.
12 Sharma, T., and Das, B.C., Int. J. Radiat. Biol. (1983) in press.
13 White, C.M., NRPB R-23, Appendix 1. National Radiological Protection Board, Harwell 1974.

0014-4754/83/070778-03$1.50 + 0.20/0


## Interaction of abscisic acid and kinetin on the growth of *Haworthia* callus in vitro

T.S. Kochhar

*Department of Biology, Kentucky State University, Frankfort (Kentucky 40601, USA), January 25, 1983*

*Summary.* Abscisic acid (ABA) in combination with kinetin substantially enhanced the growth of callus obtained from inflorescence segments of *Haworthia* cultured in vitro. This enhancement was noticed when relatively low concentration of these growth regulators were included in the medium. Neither compound was as effective when used alone. Synergistic effect of ABA and kinetin on the in vitro growth of a monocotyledonous tissue is reported.

Abscisic acid (ABA), though generally regarded as growth inhibiting phytohormone[1-3] has been shown to enhance the growth of certain plant tissues when supplied in the presence of other growth hormones. Aspinall et al.[4] reported that ABA interacts synergistically with $GA_4$ and $GA_7$ in enhancing the growth of cucumber hypocotyls. The growth of soybean callus is similarly enhanced by combination of kinetin with ABA and with an ABA-like compound naturally occurring in avocado fruits[5]. Recently this investigator reported that ABA alone or in the presence of indole-3-acetic acid (IAA) plus α-naphthaleneacetic acid (NAA) promoted the growth of callus obtained from anther-derived tobacco plants[6].

These studies indicate that both in vivo and in vitro, ABA can act synergistically with major categories of growth regulators such as auxins, gibberellins and cytokinins. However, such action of ABA has been demonstrated mostly on the growth of dicotyledonous tissues. The present study describes the influence of ABA plus kinetin on the in vitro growth of callus obtained from a monocotyledonous species.

*Materials and methods.* Callus used in the present study was obtained by culturing surface-sterilized segments of the inflorescence of *Haworthia mirabilis* Haw on modified Murashige and Skoog's medium[7], supplemented with NAA (0.2 mg/l) and kinetin (0.2 mg/l). The callus was propagated upon a similar medium at about 8-week intervals. Experiment involving four concentrations (0, 0.02, 0.2 and 2.0 mg/l) each of ABA and kinetin was conducted to determine the growth of callus. All possible combinations were tested (table). The plant growth regulators used were purchased from Sigma Chemical Co., St. Louis, Mo. For each treatment calli weighing about 130 mg were planted on 15 ml of modified Murashige and Skoog's solid medium in 25 × 150 mm culture tubes. The pH of the medium was adjusted to 5.8 before sterilization by autoclaving at 1.24 bar for 20 min. For every combination at least 12 replicate