Reduced uptake of (^{3}H) thymidine by PHA-responsive human peripheral blood lymphocytes as a function of storage

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Phytohaemagglutinin (PHA)-responsive lymphocytes from human peripheral blood samples, either irradiated or unirradiated, showed a reduced frequency of (^{3}H) thymidine-labelled cells (detected by autoradiography) as a function of storage duration. The study indicated that storage of blood samples could lead to a delay in the initiation of cell cycle progression and may impart additional source of variability when these cells are used for experimental or clinical purposes.

PERIPHERAL blood lymphocytes (PBL) of human are widely used for clinical and experimental purposes. One of the most widespread uses of PBL is in environmental mutagenesis studies. In such studies, these non-dividing cells are stimulated to divide by mitogens such as phytohaemagglutinin (PHA), under *in vitro* conditions, in order to analyse chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) from individuals occupationally or accidentally exposed to mutagenic agents. In such studies, CAs and SCEs are analysed respectively from cells which have divided once (first division or M1 cells) or twice (second division or M2 cells) in culture following PHA-stimulation[1]. It is well known that under *in vitro* conditions, a large number of factors influence the cell cycle kinetics of these cells. It is essential to recognize and take into account such factors when biological dosimetry and genetic risk evaluation are made. In this context, it is not always possible to use the blood samples immediately after their collection. A delay, therefore, is inevitable between collection of blood samples and setting up of the cultures especially when they are to be transported from distant places. Only a few reports are available on the effect of storage duration on cell cycle progression of these PHA-responsive cells.

Introduction of fluorescence plus Giemsa (FPG) staining technique[2] has facilitated the identification of cells which have divided once or more[3]. This technique as a prerequisite requires thymidine analogue, 5-bromodeoxyuridine (BrdU), when cells are grown in cultures. Using this technique, increased proportion of M1 cells and subsequent reduction in the frequency of other division cells was shown from the cultures of stored blood samples compared to fresh ones which were irradiated prior to setting up the cultures[4]. In another study on unirradiated blood samples, an increase in the proportion of M1 cells was also noticed as a function of storage duration[5]. It was suggested that when stored blood samples are used for cultures either there is a delay in cell cycle progression[4,5] or a failure in the differential uptake of Giemsa stain by the chromosomes of M2 and subsequent division of cells, leading to misidentification of M2(+) cells as M1 cells[5]. It was also recommended that when blood samples are stored for a day or more, increased amount of BrdU should be added in the cultures for proper differential staining of the chromatids[5]. We, however, found that PHA-responsive lymphocytes, either irradiated or unirradiated, show increased frequency of M1 cells as a function of storage duration. Increasing BrdU concentrations slowed down the cell cycle progression and its higher concentration was not required to elicit proper sister chromatid differential staining when cultures were set up from stored blood samples[6]. The study therefore suggested that storage condition delays the cell cycle progression of these PHA-responsive cells.

In order to check if there is any change in the programme commitment of these cells for the initiation of proliferation due to storage condition which could be responsible for increased proportion of M1 cells, in the present study, an attempt was made to analyse (^{3}H) thymidine uptake by these cells (as a function of storage duration) following mitogen stimulation. Autoradiographic technique was used to analyse (^{3}H) thymidine uptake by lymphocytes from both unirradiated and irradiated blood samples to corroborate our earlier cytogenetic findings[6]. Needless to say, this sensitive technique is being widely used in regulatory and scientific studies to identify mutagenic hazard by measuring unscheduled DNA synthesis (UDS). In these studies, radiolabelling is determined by counting the number of silver grains which develop over the cells[7,8].

Blood samples were collected from two healthy donors using heparnised syringes. Each sample was divided into three equal parts as per storage requirement (0, 1 and 3 days). The fresh blood samples were irradiated with a dose of 2 Gy of X-ray (110 kV 4 mA, @1 Gy min^{-1} at 37°C). Irradiation was performed within 2 h of collection under aseptic conditions at normal atmospheric pressure in 25 ml glass beakers (in the presence of atmospheric oxygen). After 2 h incubation at 37°C, the irradiated blood samples were used for culture. Other two parts of the sample were stored at room temperature (22 ± 2°C) in a biochemical oxygen demand (BOD) incubator and used for cultures after 24 and 72 h (1 and 3 days) respectively. Unirradiated blood samples from both the donors were also used parallel to the irradiated blood samples. Cultures were initiated by adding 0.30 ml of irradiated or unirradiated blood in 5 ml of

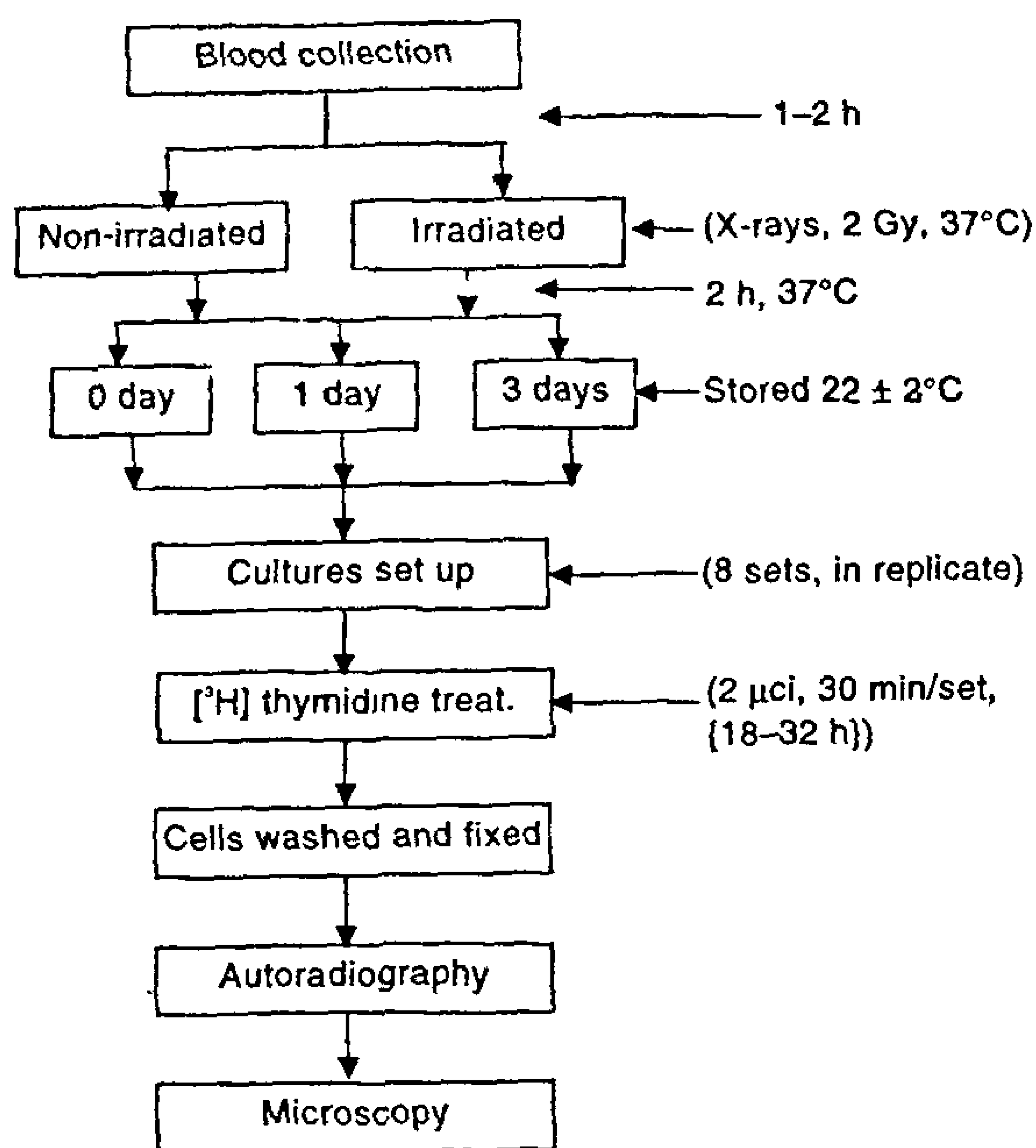

Figure 1. Experimental protocol to study the effect of storage duration on (^{3}H) thymidine uptake by phytohaemagglutinin (PHA)-responsive peripheral blood lymphocytes (PBL).

Eagles minimum essential medium, supplemented with antibiotics, 20% heat-inactivated human AB$^+$ serum and 0.15 ml of PHA (HA 15 Wellcome Reagent Ltd, UK), as described elsewhere in detail[6]. The experimental protocol has been summarized in Figure 1.

As a pilot autoradiographic study, to check the origin time of incorporation of (^{3}H) thymidine in our culture conditions, 12 cultures (in duplicate) were set up from freshly collected blood sample. A 30 min (^{3}H) thymidine pulse (2 µCi ml^{-1}; specific activity 6 Ci Mm^{-1}; BARC, India) was given to these cultures at hourly intervals beginning from 12 h of PHA stimulation up to 24 h. The cells were then fixed in methanol:acetic acid (3:1) followed by very brief washing (2 min) with cold hypotonic (0.56% KCl). Air-dried slides were prepared and processed for autoradiography. The slides were treated with 10% chilled trichloroacetic acid for 5 min, rinsed twice in ethanol and kept under running water for 10 min. After coating with 1:1 dilution of Ilford L4 nuclear emulsion in safe light, the slides were left for exposure in light proof boxes at 4°C for 20 days. The slides were developed in Kodak D 19b developer for 10 min at 10°C and fixed in acid fixer. After drying, they were stained lightly in 2% Giemsa stain and observed under light microscope for the frequency of labelled nuclei. It was found that grains start appearing in cultures 20 h after the PHA stimulation, indicating that initiation of DNA synthesis starts after about 20 h of PHA stimulation in normal lymphocyte cultures.

Table 1. ANOVA for per cent (^{3}H) thymidine-labelled cells from cultures of unirradiated and irradiated blood samples of different individuals after varying hours of PHA stimulation. The cultures were set up on the same day of blood collection or after 1 and 3 days of storage.

Source of variation	Degree of freedom	Sum of squares	Mean of square	Variance ratio	Probability <
Individual	1	13.67	13.67	3.31	ns
Irradiation	1	51.86	51.86	12.56	0.005
Hours after PHA stimulation	6	6762.47	1127.08	272.90	0.005
Storage day	2	268.67	134.34	32.53	0.005
Irradiation × hour	6	31.01	5.17	1.25	ns
Storage day × hour	12	201.53	16.79	4.06	ns
Error	41	169.33	4.13		

ns = not significant.

Therefore, in the subsequent experiments, cultures from both unirradiated and irradiated blood samples after 0, 1 and 3 days of storage were given a 30 min (^{3}H) thymidine pulse at 2 h interval in the same way as mentioned above between 18 and 32 h of PHA stimulation. Eight sets of cultures (in duplicate) for 18–32 h post-PHA-stimulation period, for both irradiated and unirradiated conditions for each storage time and each individual were set up (Figure 1). Slides were prepared, processed for autoradiography as mentioned above, and observed for frequency of labelled nuclei. At least 200 nuclei were scored for each point. Percentage of labelled nuclei was statistically analysed using two-way analysis of variance (ANOVA), as it was considered to be the most appropriate method to check the influence of storage duration on the (^{3}H) thymidine incorporation in the unirradiated and irradiated blood samples used from two different individuals.

The ANOVA for (^{3}H) thymidine-labelled cells in both unirradiated and irradiated samples from two different individuals at different hours of pulse treatment is summarized in Table 1. The two individuals did not show any significant difference for the yield of labelled cells (Figure 2). The results showed that the irradiated samples have significantly less frequency of labelled cells compared to unirradiated ones, in relation to storage duration and advancing hours after PHA-stimulation (Figure 2; Table 1). With the increasing storage duration, independent of advancing hours of pulse treatment, a trend of gradual decrease in the frequency of labelled cells compared to fresh blood samples was also noticed in both unirradiated and irradiated conditions (Figure 2). This difference was found to be significant (Table 1).

Using liquid scintillating analysis, it has been shown that (^{3}H) thymidine incorporation into cellular DNA starts only 16–20 h after PHA-stimulation, and the (^{3}H) thymidine incorporation and activity of DNA polymerase increase linearly as a function of time up to 30 h (refs. 9, 10). This is in broad agreement with the

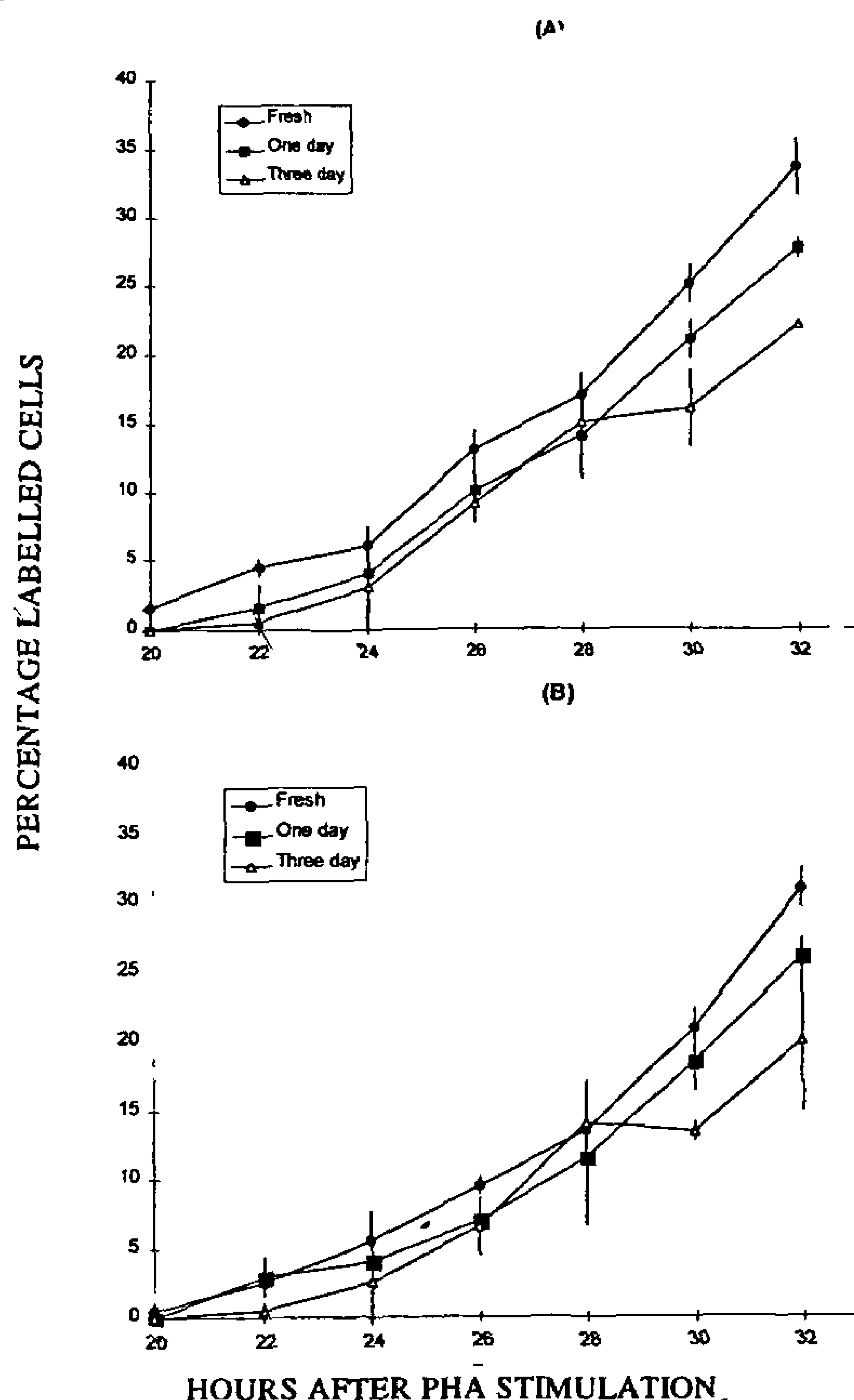

Figure 2. Frequency of (^{3}H) thymidine-labelled lymphocytes in fresh, one day or three day stored unirradiated (*a*) and irradiated (*b*) blood samples after various hours of PHA stimulation.

present autoradiographic study where no significant cellular uptake of (^{3}H) thymidine was observed up to 18 h and then it increased linearly from 20 to 30 h irrespective of storage duration and radiation dose. The pattern for cellular uptake of exogenous deoxyribonucleoside triphosphates (dNTPs) differs in different synchronously or asynchronously dividing cell types and their levels inside the cells are regulated by many factors[11]. Although in the present study, the statistical analysis (ANOVA) did not show any significant inter-individual variation for the frequency of (^{3}H) thymidine-labelled cells, such an inter-individual differences for the response of lymphocytes towards PHA has been a well-documented phenomenon[3]. Significant inter-individual variation for the yield of (^{3}H) thymidine-labelled cells has also been observed when peripheral blood samples from six individuals were labelled between 28 and 34 h following PHA-stimulation[12]. It is therefore likely that independent of the influence of storage condition and radiation treatment, an inter-individual variation would have been observed, if blood samples from more individuals were used in the present study. It will be worth mentioning that in cytogenetic assays using peripheral blood lymphocytes (e.g. micronucleus assay), more variation among the individuals than among repeated experiments on the same individual has been reported[13]. While such inter-individual and flask-to-flask variability for cultured mammalian cells exist, it has been suggested that it has no practical effect on the test outcome[14].

The variability in the (^{3}H) thymidine uptake as a function of storage duration clearly indicates that programmed commitment of cells for the onset of DNA synthesis is altered. This altered commitment for proliferation in turn would influence the cell cycle progression and therefore arrival of the cells at the metaphase stage as observed in our earlier cytogenetic study[6]. A delay in progression from G1 to S, S to G2 or G2 to M phases of the cell cycle may account for an over all effect on cytogenetic dosimetric analysis. In case of X-ray-irradiated lymphocytes, a low dose as 2 cGy has been shown to induce significant variations in cell cycle progression and arrival of the cells at the metaphases[12]. It will be important to mention that there is accumulating evidence indicating that cellular and genetic instability can manifest multiple generations after exposure of cells to agents which either directly or indirectly interact with DNA[15]. It is therefore also possible that the cells might be proportionately delayed in the subsequent cell cycle in the present study. Since the objective of the study was to check whether the appearance of first division metaphases following PHA stimulation as a function of storage is really due to change in the programmed commitment of cells for initiation of proliferation, the cells were fixed at 32 h post-PHA stimulation time. At this time, the first set of metaphases are likely to appear when the cells are processed for chromosomal analysis. With the present experimental approach, it was therefore not possible to verify if there was any delay in the subsequent cell cycle as a function of storage duration.

Since the complexities of cellular growth and division are not precisely clear and products of thousands of genes are required for the normal growth and reproduction of cells[16], it is difficult to assign any specific reason for the delay in cell cycle progression as a function of storage duration. It has been reported that lymphocytes from both pig and human proliferate more slowly in plasma lymphocyte culture than in parallel whole blood culture[17]. Isolated mononuclear leucocytes also exhibit a slower cell cycle progression than whole blood culture[18]. It is well documented that after storage or preservation, changes take place in different components of the blood. These include gradual increase in the osmotic fragility of red blood cells (RBCs), their spontaneous lysis leading to rise in plasma haemoglobin levels besides changes in the nucleotides (including ATP) and phosphorylated carbohydrates[19]. Use of heparin as an

anti-coagulant has also been shown to have no effect on RBC preservation as it lacks dextrose. On storage, its anti-coagulant effect is neutralized by thromboplastic and anti-heparin materials liberated by the cellular elements of the blood[20]. Platelets are considered to be another labile component of blood. It has been suggested that blood stored for more than two days should be considered to be free of viable platelets[21]. The lymphocytes, on the other hand, can be stored as long as four days at room temperature for immunological sub-population analysis[22,23]. These lymphocytes in the absence of optimum blood components like erythrocytes and platelets may progress slowly after PHA-stimulation in culture as observed in plasma lymphocyte or isolated mononuclear leucocyte culture[17,18]. In fact, it has been shown that when rat RBCs are added to purified rat leucocyte cultures, the cell cycle progression is considerably faster. It has been suggested that lymphocytes are released from inhibition to growth and proliferation by the presence of RBCs[24]. In this context, it will be also important to mention that mitogen-stimulated lymphocytes are particularly susceptible to undergo the phenomenon of apoptosis (programmed cell death) either under stressful culture conditions such as serum deprivation or after the induction of damage to cellular DNA[25]. Since storage condition leads to change in the biochemical and physiological properties of the different components of the blood, it is likely that under such stressful condition, the lymphocytes may undergo the phenomenon of apoptosis, resulting into reduced frequency of labelled cells. Parallel to this, the irradiated cells would have probability to undergo DNA damage-induced apoptosis and would show a further reduction in the frequency of (^{3}H) thymidine-labelled cells.

In conclusion, the storage of peripheral blood samples at room temperature delays the cell cycle progression and may impart additional source of variability when used for experimental purposes. It would be important to understand how the interaction of component cells of blood influences the rate of proliferation and the duration of the cell cycle. It would be also interesting to precisely identify different storage labile factors and then by manipulating them, prevent or modify the proliferative response. Such factors, besides other components of the blood may include sub-cellular metabolites, cytosolic and membrane-bound receptors and enzymes.

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3.55 Ga old zircon from Singhbhum–Orissa Iron Ore Craton, eastern India

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^{207}Pb/^{206}Pb ages of zircons from the Older Metamorphic Group (OMG), the oldest recognized rock unit from the Singhbhum–Orissa Iron Ore Craton, eastern India, have been determined by ion microprobe.