

## Effects of Colcemid Concentration on Chromosome Aberration Analysis in Human Lymphocytes

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### Chromosome aberration/Colcemid/Human lymphocytes/X-ray

As a part of technical improvements of chromosome aberration analysis on human peripheral lymphocytes for biological radiation dosimetry, we examined the optimal conditions for the use of colcemid in chromosome preparation in order to obtain enough number of cells at metaphase in the first cell division. When treated with colcemid at concentrations below 0.01  $\mu\text{g}/\text{ml}$  from the beginning of culture, cultures harvested at 48 hours had low mitotic indices. Colcemid treatment at 0.025 to 0.05  $\mu\text{g}/\text{ml}$  during 48 hours resulted in high mitotic indices (8 to 15%) and almost of the mitotic cells remaining in the 1st cell division, suggesting that this range of colcemid concentration was appropriate for continuous treatment with colcemid. We further examined the effect of colcemid concentration on the quantitative consistency of the yields of radiation-induced chromosome aberration. Repeated experiments showed that the yield of dicentrics and centric rings in the culture having colcemid at 0.025  $\mu\text{g}/\text{ml}$  concentration were larger than that at 0.05  $\mu\text{g}/\text{ml}$ . These data indicate the importance of assuring the accuracy of colcemid concentration in the lymphocyte culture for cytogenetic radiation dosimetry.

### INTRODUCTION

For application of chromosome aberration analysis to radiation dosimetry, it is essential to score aberrations of only the first post-irradiation mitotic division in order to maintain the quantitative response. IAEA<sup>1)</sup> recommended for this purpose the treatment of lymphocytes with bromodeoxyuridine (BrdU) followed by fluorescence plus Giemsa (FPG) staining<sup>2)</sup>, which enables to distinguish metaphases in the first division from the ones in later divisions. Although the FPG staining has been widely used, the chromosome images are rather obscure in this method in comparison with Giemsa staining alone. There is another disadvantage in the FPG

staining that BrdU has the cell toxicity which induces mitotic delay<sup>3</sup>). One alternative to overcome these difficulties is to treat lymphocytes with colcemid during the whole culture period in order to collect only metaphases in the first division<sup>4,5</sup>).

It was reported that the chromosome preparations made from a purified lymphocyte culture were superior to those from the conventional whole blood culture<sup>6</sup>). Recently, the purified lymphocyte culture has become an easy technique by using the LeucoPREP tube (Becton Dickinson Co., Ltd.)<sup>6</sup>). In this study, we examined the effect of colcemid treatment during the whole culture period and tried to establish the optimal concentration in the purified lymphocyte culture system. A phenomenon was observed that the frequency of radiation-induced aberration increased at lower concentration of colcemid, suggesting the importance of assuring the accuracy of colcemid concentration in the lymphocyte culture for cytogenetic radiation dosimetry.

### MATERIALS AND METHODS

The human peripheral blood was obtained from healthy individuals by a heparinized syringe and kept at 37°C until the time of irradiation. Immediately after irradiation *in vitro* by X-rays (250 kV, 20 mA, 1–3 Gy delivered at 0.65–0.7 Gy/min), the blood sample was placed into a water bath and incubated at 37°C for 3 hours. In the case of non-irradiation, a blood sample was used just after it was drawn from the donor. Purified lymphocytes were cultured according to the procedure by Hayata *et al.*<sup>6</sup>). In brief, mononuclear cells, which were mainly lymphocytes, were separated with a LeucoPREP tube (Becton Dickinson Co., Ltd.) from the peripheral blood, and washed once with Hank's solution supplemented with 2% fetal calf serum. The number of mononuclear cells was counted with an automatic blood cell counter, and the cells were cultured in RPMI 1640 medium with 20% fetal calf serum at the concentration of  $5 \times 10^5$  cells/ml. Colcemid (Demecolcine, SIGMA Chem. Co.), of which concentration was carefully adjusted to the prescribed level, and 2% phytohaemagglutinin HA15 (Wellcome Foundation, Ltd.) were added to the medium at the beginning of the culture. The cultures were kept in 5% CO<sub>2</sub> atmosphere in an incubator at 37°C. After the hypotonic treatment with 0.075 M KCl for 20 minutes at 37°C, cultured cells were fixed with methanol-acetic acid (3:1). Chromosome preparations were made by dropping 13  $\mu$ l of cell suspension onto a slide which was sloped slightly under the warm (29–32°C) and humid (70–80%) atmosphere condition<sup>7</sup>).

The mitotic index was examined on non-irradiated lymphocytes which were cultured for 48 hours at various concentrations of colcemid. The slide preparation for scoring mitotic index was made by dropping 5  $\mu$ l of cell suspension onto the center of a slide glass which was placed horizontally. After the staining with Giemsa, we counted the numbers of metaphase and interphase cells along the vertical and horizontal lines crossing the center of sample on the slide. Scoring of mitotic index was made on more than 1000 cells for each point of observation. Following the same experimental procedures, effect of colchicine on mitotic index in cultured lymphocytes was also examined and compared with the effect of colcemid.

The ratio of the first and the second division metaphases was determined in the sample cultured for 48 or 54 hours at the colcemid concentration of 0.025 or 0.05  $\mu$ g/ml after the

irradiation at 1 Gy. BrdU (10  $\mu\text{g/ml}$ ) was added in the medium at the initiation of the culture. Air-dried slide preparations were processed by the FPG staining method<sup>8)</sup>, and 250 cells were analyzed for each experimental group.

The frequency of dicentrics and centric rings was examined in the lymphocytes irradiated at 1 or 3 Gy which were cultured with 0.025 or 0.05  $\mu\text{g/ml}$  of colcemid for 48 hours. The preparations were stained conventionally by Giemsa and 100 to 200 metaphase cells were analyzed in each group. The experiments were repeated 5 times using lymphocytes irradiated at 1 Gy.

## RESULTS

Figure 1 shows the mitotic indices in the specimens cultured with 0.001, 0.0025, 0.005, 0.01, 0.025 and 0.05  $\mu\text{g/ml}$  of colcemid or colchicine. Very few metaphases were accumulated in the cultures having colcemid concentrations below 0.01  $\mu\text{g/ml}$ . On the other hand, a remarkable number of metaphase cells was observed in the cultures having colcemid concentrations of 0.025 and 0.05  $\mu\text{g/ml}$ , which ensured sufficient mitotic indices for analysis (8 and 15%). Although colchicine exhibited a considerable effect of mitotic block at the lower concentrations than colcemid did, the overall efficacy of colchicine was very low as compared by the maximal levels in dose-response curves (Fig. 1).

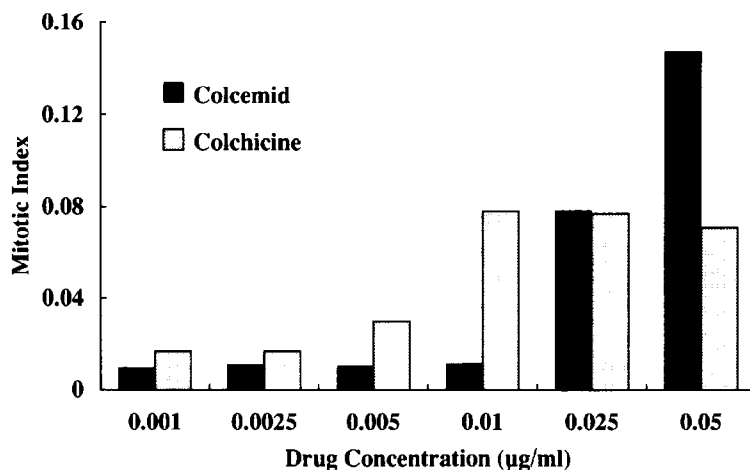
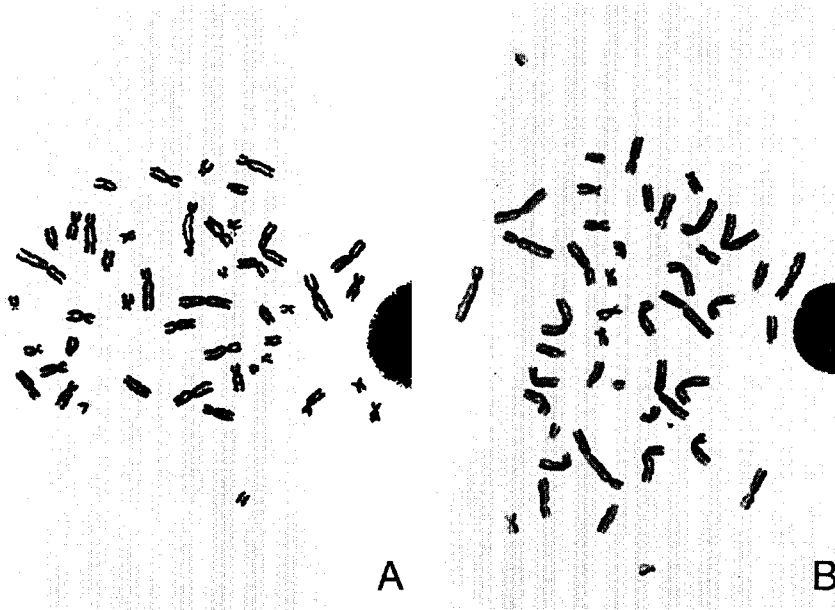


Fig. 1. The effects of concentrations of colcemid (■) and colchicine (□) on mitotic index measured for human lymphocytes which were cultured for 48 hours.

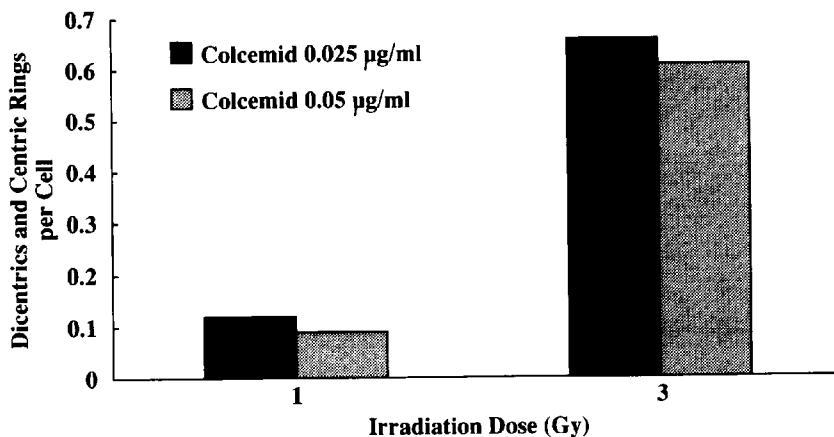
The division cycle of the metaphases was determined by FPG staining (Fig. 2). The 48 hour culture with 0.025  $\mu\text{g/ml}$  of colcemid showed that the ratios of the first and the second division metaphases were 97.6% and 2.4%, respectively. The 48 hour culture at the concentration of 0.05  $\mu\text{g/ml}$  of colcemid showed that 99.6% of metaphase were in the first division. On the other hand, 54 hour cultures at the colcemid concentrations of 0.025 and 0.05  $\mu\text{g/ml}$  showed that



**Fig. 2.** (A) A metaphase cell in the first cell cycle. Sister chromatids are not differentially stained by fluorescence plus Giemsa staining.  
(B) A metaphase cell in the second cell cycle. Sister chromatids are differentially stained by fluorescence plus Giemsa staining.

16.8% and 1.6% of metaphases were in the second division, respectively.

Figure 3 shows frequencies of dicentrics and centric rings of lymphocytes irradiated at 1 or 3 Gy. In the specimen irradiated at 1 Gy, the frequencies of aberrations per cell in the cultures with 0.025 and 0.05  $\mu\text{g/ml}$  of colcemid were 0.12 and 0.09, respectively. In the specimen irradiated at 3 Gy, the aberration yields per cell with 0.025 and 0.05  $\mu\text{g/ml}$  of colcemid were



**Fig. 3.** The effect of colcemid concentration on the frequency of dicentrics and centric rings in human lymphocytes. The lymphocytes were separated from the blood which was irradiated at 1 or 3 Gy *in vitro*, and cultured for 48 hours with 0.025 (■) and 0.05 (▨)  $\mu\text{g/ml}$  of colcemid.

0.66 and 0.61, respectively. The aberration yields at the concentration of 0.025  $\mu\text{g/ml}$  seemed to be higher than those at the concentration of 0.05  $\mu\text{g/ml}$  in both 1 Gy and 3 Gy groups. Aberration frequencies at two colcemid concentrations were examined repeatedly using the lymphocytes which were irradiated at 1 Gy. The aberration frequency in the culture with 0.025  $\mu\text{g/ml}$  of colcemid was larger than that with 0.05  $\mu\text{g/ml}$  of colcemid in 4 out of 5 experiments (Fig. 4). Wilcoxon signed-ranks test was applied to compare aberration yields obtained from paired samples which were cultured with 0.025 and 0.05  $\mu\text{g/ml}$  of colcemid in 7 series of experiments in total (Figs. 3, 4), showing a significant difference ( $p=0.05$ ).

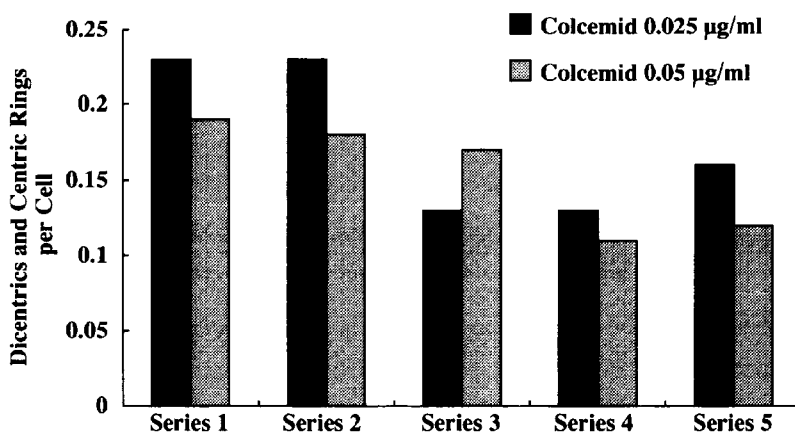


Fig. 4. The effect of colcemid concentration on the frequency of dicentric and centric rings in human lymphocytes. The lymphocytes were separated from the blood which was irradiated at 1 Gy *in vitro*, and cultured for 48 hours with 0.025 (■) and 0.05 (▨)  $\mu\text{g/ml}$  of colcemid. The same experiments were repeated 5 times.

## DISCUSSION

The phenomenon that the change in colcemid concentration influenced on the aberration yield was confirmed by repeating the experiment. One possible explanation for this phenomenon is that the fraction of escaping cells from arrest may be higher in normal cells than in aberrant cells in the culture with 0.025  $\mu\text{g/ml}$  of colcemid, whereas at 0.05  $\mu\text{g/ml}$  of colcemid mitoses are completely blocked both in normal and aberrant cells. Consequently, the ratio of normal metaphases could be lower in the culture at the lower colcemid concentration. Another hypothesis could be that the colcemid exerts a certain inhibitory effect on the repair or replication of some kind of latent lesions of DNA during S phase which are expressed as aberrations at metaphase, and that the higher the colcemid concentration the less the resultant aberrations.

The continuous treatment of cells with colcemid in combination with the use of the purified lymphocyte culture in the present study resulted in the improved chromosome preparations

which contain at least 10 times more metaphases per slide than traditional preparations obtained by whole blood culture. One of the reasons is that the sample of purified lymphocyte culture contains little debris such as broken red blood cells which interfere with spreading the cells on a glass slide. Accordingly, a concentrated sample can be placed on the slide so that enough number of metaphases can be observed in a small visual field. Another reason is that mitotic index itself is considerably higher in the purified lymphocyte culture than in the whole blood culture, since cells which do not respond to PHA are excluded in the purified lymphocyte culture. This improved slide preparation is also suitable for chromosome analysis by such modern technique as chromosome painting method both from technical and economical point of view.

IAEA reported the practical protocol for performing chromosome aberration analysis in 1986<sup>1)</sup>. According to the protocol, whole blood cells are cultured for 48 hours and 0.45  $\mu\text{g}/\text{ml}$  of colcemid is added to medium at 3 hours before fixation. The range of colcemid concentration used in the present study was from 0.025 to 0.05  $\mu\text{g}/\text{ml}$  which was less than one-ninth of the concentration described in IAEA<sup>1)</sup>. This concentration range is thought to be optimal for the 48 hour treatment of colcemid on purified lymphocytes in order to accumulate a sufficient number of first mitotic metaphases for analysis. The concentration higher than 0.05  $\mu\text{g}/\text{ml}$  is thought to be inappropriate for the continuous treatment with colcemid, since it tends to increase over-condensed chromosomes which interfere with the analysis<sup>8)</sup>. In addition, it was concluded that colchicine could not be substituted for colcemid, since the continuous treatment of colchicine did not obtain as high mitotic index as colcemid treatment did.

There is one another important point to note regarding the procedures to ensure quantitative response in chromosome aberration analysis. It has been a commonly accepted practice in preparing the cell specimens for radiation dosimetry as well as congenital abnormalities analysis that the volume of colcemid to be added to the culture are measured by counting the number of drops of colcemid solution. It was found, however, that the change in colcemid concentration within the range between 0.025 and 0.05  $\mu\text{g}/\text{ml}$  influenced on the yield of chromosome aberration. Control of concentration within this range can not be achieved by droplet counting. It is necessary therefore to measure the amount of colcemid accurately by use of micropipette. Especially when a highly precise aberration yield is required, for instance, in the case of low-dose estimation, much attention should be paid on the purity of colcemid used and the means for measuring the amount of colcemid both in preparing the stock solution and in applying it to the culture medium in order to maintain a defined culture conditions used in a series of experiments. In addition, it should be noted that the data obtained from experiments which used different colcemid concentrations should not be pooled when analyzing the results.

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