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■論 文■

Acridine Orange Stained Micronucleus Assay in Human B and T-lymphocytes after Low Dose ϒ-irradiation

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아크리딘 오렌지 형광염색법을 이용한 저선량 감마선 유도 말초혈액 B와 T-림프구 미소핵 분석

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Abstract - Firstly, we compared the two staining techniques, Giemsa and Acridine orange, to determine micronuclei on samples of cultures of five healthy human peripheral blood lymphocytes after ϒ-irradiation (^{137}Cs) in dose ranges of 0 to 800cGy. It was found that the Acridine orange staining method gives more reliable results than the usual Giemsa staining method in micronucleus tests. Moreover, the frequency of micronuclei in cytokinesis-blocked human B-lymphocytes was studied after in vitro irradiation in dose ranges of 0 to 50cGy. After sorting and separating the B-lymphocytes, the frequency of radiation-induced micronuclei were observed as the end-point markers for the low-dose radiation dosimetry after staining with Giemsa and Acridine orange dyes. The micronuclei frequency in B-lymphocytes was significantly elevated from 10 to 30cGy ϒ-irradiation. The determination of micronuclei in B-lymphocytes after staining with Acridine orange was higher than that of Giemsa. The frequency of micronuclei in B-lymphocytes was observed to be at least two times higher than those of T-lymphocytes Giemsa in dose increasing. Therefore, the determination of low-dose radiation-induced micronuclei in B-lymphocytes after staining with Acridine orange is likely to have the greatest potential in the estimation of low dose radiation exposure.

Key words : T/B-lymphocytes, acridine orange, Giemsa, low dose radiation, dosimetry

요약 - 방사선에 의해 유도되는 사람 말초혈액 림프구 미소핵 관찰빈도를 높이면서 생물학적 선량평가법으로서 활용 가능성을 확인하고자 본 연구를 수행하였다. 우선 5명의 건강한 사람으로부터 혈액을 제공 받아 선량영역을 0에서 800cGy로 하여 감마선(^{137}Cs)을 조사 한 후 김사와 아크리딘 오렌지 형광 염색하고 미소핵 출현빈도를 비교하였다. 아크리딘 오렌지 염색법을 이용하여 미소핵을 관찰하였을 때, 김사 염색법에 비교하여 적갈색의 비특이성 과립과 녹황색의 DNA가 붉은색의 세포질을 배경으로 명확히 구별되었을 뿐만 아니라 선량이 증가하면서 검출율도 높았다.

아울러, 말초혈액 T와 B-림프구에 대하여 선량영역을 0에서 50cGy로 하여 방사선을 조사한 후 미소핵 출현빈도를 아크리딘 오렌지 염색하여 김사염색과 비교한 바, B-림프구에서 선량이 증가하면서 적어도 2배 이상 높게 관찰되었다.

본 실험 결과, 사람 말초 혈액 B-림프구를 대상으로 한 아크리딘 오렌지 형광염색 미소핵 분석법은 저선량 방사선 인체영향 평가나 과피폭 선량추정시 활용이 가능 할 것으로 생각된다.

중심어 : T/B-림프구, 아크리딘 오렌지와 김사 염색법, 저선량 방사선, 선량평가

INTRODUCTION

The micronucleus test is a method devised primarily for screening chemical materials for chromosome-breaking effects [1,2]. Cytokinesis block micronucleus assay in human lymphocytes has been adopted worldwide as a sensitive, reliable method for assessing chromosome damage [3,4]. It has been applied both to monitoring chromosome loss and breakage in the human population as well as for the in vitro testing of genotoxicity by radiation exposure [5,6]. This testing procedure has a number of important advantages over the analysis of human peripheral blood metaphase chromosomes. All these properties render the micronucleus test highly suitable for routine screening of radiological dose estimation [7]. Unfortunately, however, artifacts that are barely distinguishable from micronuclei do sometimes occur. During the last decade, May-Grunwald-Giemsa [8], or Giemsa single staining, has been routinely used for the micronucleus test. The Giemsa test, however, has disadvantages because not only micronuclei, but also some cell inclusions containing RNA and other acidic materials are stained dark blue by Giemsa [9]; it is occasionally difficult to identify micronuclei from these inclusions. Recently, some reports have shown that an Acridine orange staining technique can distinguish true micronuclei from basophilic granules in rat bone marrow [10]. However, concerning the radiation response of lymphocytes with respect to cytogenetic damage after Acridine orange staining for true micronuclei differentiates are available a few publications. Using the fluorescent DNA-binding dye Acridine orange [11] herein is described an application of this staining method to biological dosimetry using human peripheral lymphocytes, because human blood is a mixture of mainly T-lymphocytes, B-lymphocytes and natural killer cells. Some of previous reports agree upon the high radiosensitivity of B-lymphocytes when compared to the other subpopulations, and this with respect to different end-points such as apoptosis and proliferation capacity [12-17]. These early data indicated the availability of B-lymphocytes as a biological radiation dosimeter [18,19]. In this study, we investigated whether the validation of the micronucleus method by Acridine orange staining supports its use for human lymphocyte micronuclei for low-dose radiation biological dosimetry.

MATERIALS AND METHODS

Sampling and radiation exposure

Whole blood samples were drawn by venepuncture from five healthy volunteers ranging in age from 24 to 45 years. One sample of each donor served as a control for determining the spontaneous micronucleus frequency. The others were exposed to ^{137}Cs ϒ -ray. For determining the dose response of T-lymphocytes for each individual, doses of 10, 50, 75, 100, 200, 400 and 800cGy were delivered at a dose rate of 0.8Gy/min. On the other hand, B-lymphocytes were irradiated with dose ranges of 10, 20, 30 and 50cGy.

Preparation of T and B-lymphocytes and micronuclei assay

For the cultivation of T-lymphocytes, anaphase spreads were prepared following a standard protocol [5]. After irradiation, whole blood cultures were prepared by adding 0.5ml of blood to 10ml ofculture medium consisting of RPMI 1640 with HEPES buffer and 20% fetal bovine serum supplemented with antibiotics. The ϒ-irradiated cells together with the non-irradiated controls were then cultured. Phytohemagglutinin (PHA) at a concentration of 5㎍/ml was used to stimulate the division of lymphocytes in culture. Cultured lymphocytes were harvested after 72 hours following 28 hours of cytochalasine-B (4㎍/ml) treatment. At the end of the culture period, the PHA-stimulated cells were centrifuged, given a hypotonic shock (75mM KCl, 4℃) for 10 min, and fixed three times in methanol/acetic acid (3:1) to obtain cells with a well-preserved,

clearly stained cytoplasm. In the cultures with PHA, although no sorting for T-lymphocytes was performed, the binucleate cells can be considered as T-lymphocytes because only this lymphocyte subpopulation is adequately stimulated by PHA in the described culture conditions.

For the isolation of the B-lymphocytes, the Pokeweed mitogen (PWM)-stimulated cultures were centrifuged and then washed in degassed PBS (pH, 7.4) with 2mM EDTA, 0.5% BSA. An appropriate amount ($10^7/10\mu\ell$) of CD-20 magnetic microbeads (MACS, MiltenyiBiotec, Germany) was added to cell pellets and incubated for 15 min at 4℃. The cells were then washed again in PBS buffer and re-suspended in a $250\mu\ell$ buffer. The cell suspension was applied to MACS separation column and then rinsed three times with PBS buffer at room temperature. The magnetically labeled B-lymphocytes were retained in the column and separated from the unlabelled cells that passed through. After removal of the column from the magnetic field, the retained fraction of magnetically labeled B-lymphocytes was eluted. After fixation, cells were dropped onto dry slides, air-dried, and stained with Acridine orange (Wako, Japan) and Giemsa. The micronucleus test was performed after staining with Acridine orange according to the method described in the summary report by Hayashi et al [10]. The micronucleus test with lymphocytes was carried out by the conventional method with Giemsa staining.

Slide scoring and analysis

The identification of micronuclei was done according to the criteria summarized by Fenech [9]. For the each culture, at least 1,000 binucleate cells with preserved cytoplasm were scored. A statistical analysis was carried out using the INSTAT GRAPHPAD program (Version 3.0).

RESULTS

An illustration of human peripheral blood lymphocytes stained with Acridine orange and Giemsa stains is shown in Figure 1A and B. Cytoplasm of peripheral blood was clearly identified by its color of red fluorescence. Micronuclei were round in shape and exhibited a strong yellow-green fluorescence, compared to the dark blue of Giemsa. These results show that the staining characteristics of micronuclei marked with Acridine orange versus those marked with Giemsa showed a more precise counting of micronucleated lymphocytes.

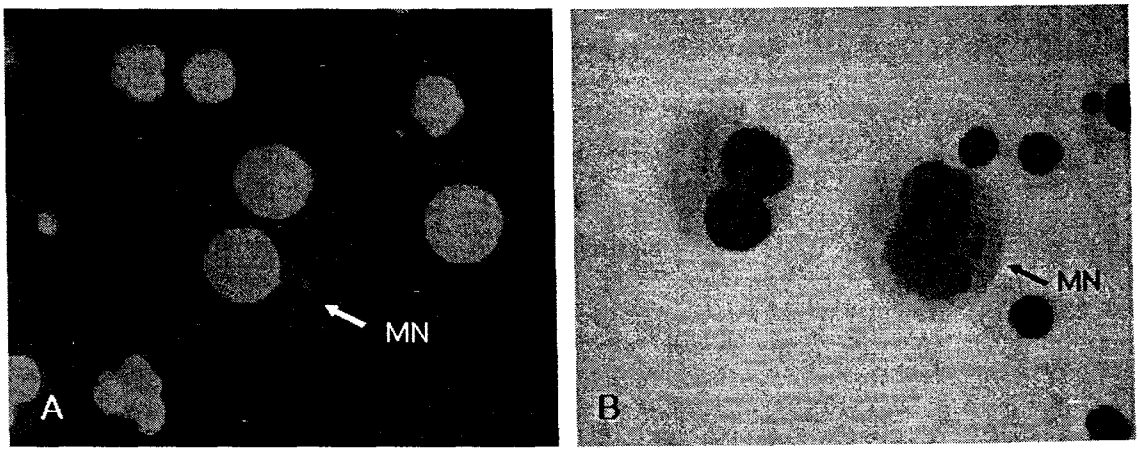

Fig. 1. Comparison of typical appearance of micronuclei (MN) in binucleated human lymphocytes after Acridine orange (A) and Giemsa (B) stain.

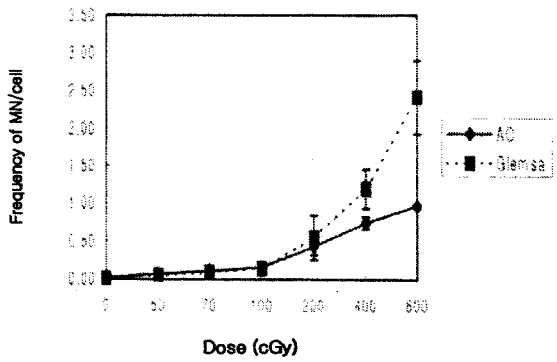

Fig. 2. Frequency of micronuclei in human lymphocytes after stain with Acridine orange (AO) and Giemsa.

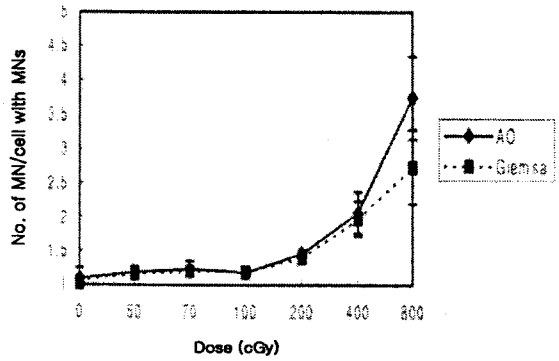

Fig. 3. Number of micronuclei per cell with micronuclei in human lymphocytes after stain with Acridine orange (AO) and Giemsa.

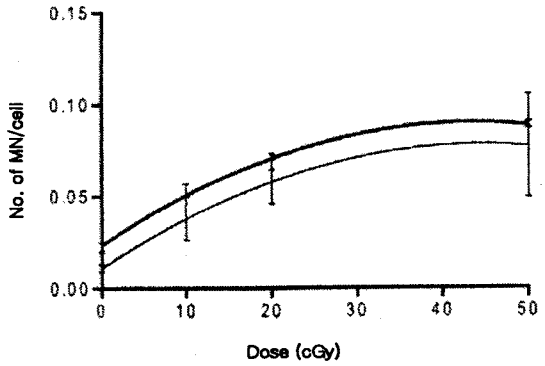

Fig. 4. Frequency of micronuclei in B-lymphocytes after with Acridine orange(▬▬) and Giemsa (——).

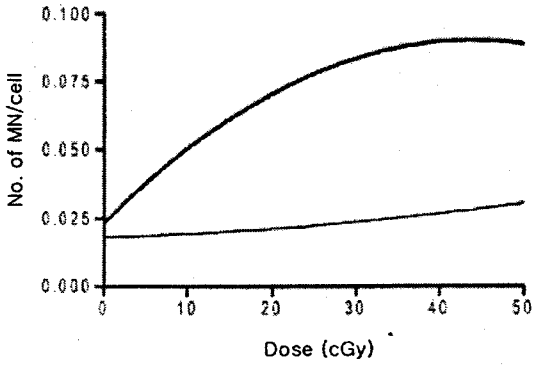

Fig. 5. Comparison of radiation induced micronuclei between human B (▬▬) and T-lymphocytes (——) after Acridine orange staining.

Dose-response data were obtained in our study by scoring micronuclei after staining with Giemsa and Acridine orange in cytokinesis- blocked cells of five donors. The micronucleus frequency after staining with both dyes in peripheral blood lymphocytes was significantly elevated from 50cGy ϒ-irradiation. Figure 2 further shows that micronucleus values after staining with Giemsa were generally higher from 100cGy irradiation than those stained with Acridine orange. When analyzed by a linear-quadratic model, the line of best fit after staining with Giemsa was $y=-0.029+2.19\times10^{-3}D-1.41\times10^{-6}D^2$ ($r^2=0.93$, y= frequency of micronucleus/scored cells, D= radiation dose in cGy). The mean dose response curve for micronuclei obtained after staining with Acridine orange shows a somewhat different pattern ($y=-0.0204+2.48\times10^{-3}D-1.55\times10^{-6}D^2$, $r^2=0.96$). The results obtained from this experiment show that there was a significant difference in micronucleus determination dependant on the stain method, and the trend of micronuclei per cell with micronuclei after staining with Acridine orange was slightly higher than that of Giemsa staining following irradiation (Fig. 3).

After sorting out lymphocytes from the cultivated peripheral blood, the frequency of micronuclei was compared with a fixed slide of duplicated B-lymphocytes with a different staining process (Fig.4). The results obtained from this experiment on stain methods, and the trend of determination of micronuclei after staining with Acridine orange, were slightly higher than those of Giemsa stain.

Dose-response data obtained in our study by scoring micronuclei after staining with Giemsa and Acridine orange in B-lymphocytes is summarized in Figure 5. The micronuclei frequency in B-lymphocytes was significantly elevated from 10 to 30cGy ϒ-irradiation. The frequency of micronuclei in B-lymphocytes after staining with Acridine orange ($y=0.023+2.58\times10^{-2}D-2.83\times10^{-5}D^2$, $r^2=0.96$) was observed to be at least two times higher than those of T-lymphocytes in dose ranges of 10 to 50cGy ϒ-irradiation. However, there was no significant increase observed in the frequency of radiation-induced micronuclei in T-lymphocytes ($y=0.0143+2.9\times10^{-4}D+0.5\times10^{-6}D^2$, $r^2=0.54$).

DISCUSSION

The micronucleus test is a relatively simple method, and this test offers numerous possibilities for chromosomal breaking effects by chemical means [1,2]. All these properties render the micronucleus test highly suitable for routine radiological screening [7]. However, a large number of granules from various cells were scattered over the slides when smeared preparations were made from experimental animal and human tissue samples [5,8]. These granules, when stained with Giemsa, were occasionally incorrectly identified as micronuclei. Previous reports that the nucleic acid extraction showed the yellowish green fluorescence of the micronucleus in Acridine orange disappeared after treatment with DNAse but not with RNAse [10]. Another report shows that the Giemsa stained inter-chromosomal network was selectively removed by the RNAse treatment [20]. These reports suggest that the specificity of Acridine orange staining for micronuclei greatly increases the probability of true micronuclei being easily distinguished from misscored potential artifacts. In agreement with previous reports, the results of this study suggest that Acridine orange fluorescence can quite readily distinguish true micronuclei (Fig. 1). Additionally, the simplicity of this method, as well as the greater spectral separation of the DNA fraction containing cells, should make the Acridine orange staining technique exceedingly suitable for biological dosimetry.

Because of the advantages described above, we believe that the Acridine orange method is particularly well suited to scoring radiation induced micronuclei in peripheral lymphocytes. Since the frequency of micronuclei may be influenced by various conditions, for each subject duplicate slides were prepared and stained from each culture. The results obtained by the two staining methods are in close agreement with each other, even though the frequency of Giemsa stained micronuclei was shown to be a little higher than Acridine orange after irradiation (Fig. 2). It was interesting to observe that the frequency of micronuclei per cells with micronuclei was generally high after Acridine orange staining (Fig. 3). Moreover, the specificity of Acridine orange stain for micronuclei in B-lymphocytes reduces the possibility of misscoring potential artifacts, which have caused confusion with Giemsa (Fig. 4). These findings suggest that one might expect the frequency of micronucleated cells to be detected by Acridine orange more readily than that of Giemsa stain as the tendency for population of damaged cells [10]. It can therefore be argued that it would be informative to measure the frequency of Acridine orange stained micronuclei when scoring for micronucleation within irradiated cells. The over-dispersion of micronucleus frequency is in agreement with what has been observed previously for micronuclei in mouse erythrocytes after chemical treatment [6,9,10]. Therefore, this simplified micronucleus test method with human lymphocytes and Acridine orange seems to be at least as sensitive as the Giemsa, and shows great potential as an appropriate method for biological radiation dosimetry.

In our experiment, a clear difference in frequency of micronuclei between T-lymphocytes and B-lymphocytes in low-dose radiation was noticed. The results of this study show that the frequency of micronuclei in B-lymphocytes

was about two times higher when compared to those in T-lymphocytes in dose ranges of 10 to 50cGy (Fig. 5). Moreover, the micronuclei frequencies of B-lymphocytes found in the study of Vral *et al* [17] for the applied doses of 10 (0.57) and 25cGy (0.07) were similar to the values found in this study. This is further strengthened by an earlier report that described that micronuclei are observed to be higher in B-lymphocytes than T-lymphocytes for low-dose radiation under 1Gy [17,18]. The results also support our findings, which relate to range of dose estimation. However, they scored micronuclei after Giemsa staining, which has some disadvantages because it shows considerable variation in frequency of micronuclei, dependant on both observers and laboratory conditions. Therefore, the present investigation is unique as a study, as it was done using low-dose radiation induced micronuclei in B-lymphocytes with Acridine orange stain, thus minimizing the artifact. Although similar trends in the frequency of micronuclei have been reported previously, in this study more reliable data were obtained with the utilization of Acridine orange, and the experimental errors by misscored micronuclei could be minimized. However, these results are independent of the opinion that B-lymphocytes have a high probability to operate various mechanisms that relate to radiosensitivity. It has been reported that the high sensitivity to radiation might be related to DNA-PK [21]. The fact that the DNA repair has been delayed due to enzyme deficiency in the repair process of the damaged DNA has been considered. It has been reported that a mutual relationship for Ku86 exists between the mechanism of DNA repair and the sensitivity of B-lymphocytes to low-dose irradiation [22]. Therefore, the results of this study acknowledge the possibility of using B-lymphocytes as a biological parameter for estimating the low-dose radiation effects. It is a generally established theory that, when exposed to ionizing radiation during radiation therapy or accidents, the numbers of lymphocytes in peripheral blood decrease and change in the formation of lymphocytes that react to radiation [23]. Hence, the study of genetic phenomena between the frequency of micronuclei in B-lymphocytes and their repair mechanisms after low dose radiation needs deeper study. In summary, the results in this study suggest that the Acridine orange staining method gives more reliable results than the usual Giemsa staining method in micronucleus assay. The determination of radiation-induced micronuclei in T-lymphocytes as well as B-lymphocytes after staining with Acridine orange is likely to have the greatest potential in the estimation of low-dose radiation exposure.

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