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# Improvement of Accuracy of Chromosome Aberration Analysis for Biological Radiation Dosimetry

REIKO KANDA*

Division of Radiobiology and Biodosimetry, National Institute of Radiological Sciences, 9–1, Anagawa-4-chome, Inage-ku, Chiba 263–8555, Japan

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The frequency of chromosome aberrations in circulating lymphocytes is accepted as being the most reliable indicator of the absorbed dose of radiation. Researches done to improve the accuracy of cytogenetic analysis are described in this review. These include investigations of *in vitro* factors that affect the yield of radiation-induced aberrations and of *in vivo* factors that affect the chromosomal radiosensitivity of individuals. Improved chromosome-painting methods for accurate judgment of dicentrics and translocations are introduced. The practicality of these advanced cytogenetic techniques is shown by examinations of individuals exposed in the radiation accident at Tokaimura in 1999.

## INTRODUCTION

Biological dosimetry based on chromosomal damage to peripheral blood lymphocytes after accidental overexposure to radiation was first performed in 1962 on victims of the Recuplex criticality accident in Hanford[1]. It is now accepted as being the most reliable means of estimating the radiation dose[2]. In view of the growing importance of cytogenetic analysis, in 1986 the IAEA established a practical standard methodology[3]. This has served as a valuable laboratory manual and made clear that chromosome analysis is time-consuming work that requires expert skills. During the next decade, new techniques were introduced into cytogenetic analysis to improve its efficiency. The recent development of computer programs now permit semi- or fully-automated analysis of chromosome aberrations thereby saving physical labor of cytogeneticists. Another recent advance is chromosome painting that uses fluorescence *in situ* hybridization (FISH). This has facilitated rapid detection of stable aberrations, data on which is useful, in particular for dosimetry of old and long-term exposures. These researches have been promoted mainly because

*The author received the 1999 award for Young Investigators from the Japan Radiation Research Society. Phone; +81–43–251–2111, Fax; +81–43–251–9231, E-mail; kanda_r_@nirs.go.jp

of recent concerns about dose estimation and the biological meaning of low-dose exposures. Studies of low dose exposures in particular require analysis of an enormous number of cells to obtain statistically significant data[4].

Techniques for improving the accuracy of cytogenetic analysis have been steadily developed. Accurate scoring of aberrations requires specialized expertise in all the processes of blood culture, slide preparation and staining[4–6]. Hayata et al[7] improved these procedures thereby providing chromosome slide preparations of good quality which minimize error in scoring chromosome aberrations and producing a large number of analyzable metaphases per slide. Their improved procedures are currently optimal for automated systems and chromosome painting. Further investigations to improve each process in chromosome aberration analysis have contributed to the finding of *in vitro* and *in vivo* factors that influence the dose-response of chromosome aberrations[8,9] and to the development of new methods of chromosome painting[10,11].

## *IN VITRO* FACTORS INFLUENCING THE CYTOGENETIC DOSE-RESPONSE

When applying 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. Therefore, any factor influencing the cell progression rate in culture may affect the measurement of aberration frequencies. For example, the culture period, media, sera and temperature influence the frequency of first *in vitro* division[5,12,13]. To circumvent the problem of contamination of non-first mitosis, fluorescence plus Giemsa staining, which enables the distinguishing metaphases in the first division from those in later divisions, has been recommended in the IAEA technical report[3].

The improved procedures of Hayata et al[7] offer a more simple solution to this problem. Instead of the traditional 3-h treatment with colcemid[3], cultured lymphocytes are treated with colcemid throughout the culture period so as to arrest the cell cycle at metaphase more effectively. When purified lymphocytes were cultured in RPMI 1640 medium containing 20% fetal calf serum and PHA, the mitotic index, the metaphase rate in the first cell division and chromosome morphology together indicated that 0.025–0.05 $\mu$g/ml is appropriate for continuous treatment with colcemid[8], a much lower concentration than used traditionally (0.5 $\mu$g/ml)[3]. Further experiments showed that the yield of the dicentrics plus centric rings induced by radiation is influenced by the colcemid concentration within the optimal range[8]. These findings call attention to the importance of assuring the accuracy of *in vitro* factors, including the colcemid concentration, for cytogenetic radiation dosimetry.

## *IN VIVO* FACTORS INFLUENCING THE CYTOGENETIC DOSE-RESPONSE

A small number of *in vivo* factors clearly affect the dose-response of chromosome aberrations. Those that may influence the background frequency of chromosome aberrations, such as age, smoking habits and other mutagen exposures, have been well studied in low-dose exposed

and control populations. These studies revealed that age and smoking may more seriously confound dosimetry using stable aberrations than that using unstable aberrations[14,15].

The *in vivo* factors that influence radiosensitivity, however, have received little attention because differences in the inherent sensitivities of healthy individuals have been believed not to confound biodosimetry[5,16]. Several researchers agree that certain hypersensitive individuals exist in a community, the most numerous being persons with Down's Syndrome[16]. Other less frequently encountered syndromes, such as ataxia telangiectasia, retinoblastoma and Fanconi's anaemia, also may show enhanced yields of chromosome aberrations after irradiation[16,17].

Recently, Ricoul et al[18] provided the first evidence in humans that radiosensitivity may vary in relation to physiological conditions; pregnant women had increased chromosomal radiosensitivity during the second half of pregnancy, but this disappeared immediately after delivery. This phenomenon is at least partly due to the elevated estrogen concentration during pregnancy[9]. *In vitro* treatment with estradiol at 100 ng/ml (the estrogen level in the plasma of a woman during the last month of pregnancy) significantly increases the frequencies of dicentrics plus centric rings and the total chromosome breaks induced by irradiation, but this treatment has no effect on spontaneous aberration frequencies. A 20% increase in chromosome aberrations was seen in that *in vitro* study. Although this value is small for each individual, it may not be acceptable in terms of the human population. Therefore, hormonal conditions have been taken into account for biodosimetry so far.

## CHROMOSOME PAINTING: ITS PROBLEMS AND IMPROVEMENT

The accurate scoring of aberrations requires specialized expertise, the degree of skill of the observer having some affect on the aberration frequency observed in cells from a given blood culture[4–6]. The worldwide spread of FISH painting is partly due to its being considered to require no expert skill for the scoring of aberrations, selected chromosomes being stained entirely with fluorescent dye so that the interchange between the painted and unpainted chromosomes should be easily distinguishable. FISH painting has been frequently used for biodosimetry on the assumption that aberration breakpoints are randomly distributed, but this has not yet been proved. Recently, sex chromosomes, acrocentrics and chromosomes with an abundance of heterochromatin have been reported to be unsuitable for biodosimetry that uses chromosome painting because their aberration frequencies are not proportional to the DNA contents[19].

Furthermore, centromere positions in FISH painting preparations are difficult to identify, which may lead to high risk of the mis-scoring of dicentrics as translocations[10,20–21]. In fact, studies that used FISH painting reported a 1- to 12-fold induction of translocations than dicentrics by irradiation, even though their induction rates theoretically are even. A hybridization method that uses whole-chromosomal and pan-centromeric probes has been developed and is now commonly used, but it still produces inconsistent results regarding the yields of translocations and dicentrics[20,22]. Two improved methods of chromosome paintings now have been developed to locate centromeres under a bright-field microscope which has higher resolution than a fluorescence microscope: One is successive applications of Giemsa staining and FISH painting

on the same metaphase ("Giemsa-FISH"), the other non-fluorescent chromosome painting[10,11].

After the Giemsa-stained images of metaphases are photographed, the slides are destained, treated with RNase A and pepsin, postfixed and then given conventional FISH painting. Identification of translocations and dicentrics is made by the images of both Giemsa and the painting (Fig. 1). A successive application of painting and then Giemsa is not equivalent because the denaturing process of the painting tends to weaken the intensity and clarity of Giemsa staining thereby interfering with the analysis of chromosome aberrations. The Giemsa-FISH method does not give a statistical difference in the yields of translocations and dicentrics using irradiated lymphocytes[10]. When chromosome 4 is painted, the Giemsa-FISH method detects 10.8% of the unstable aberrations (dicentrics plus centric rings) produced in the total genome of irradiated lymphocytes, whereas conventional FISH detects 4–7%[23]. The value obtained by the Giemsa-FISH method is much closer to the value of 12%, which is predicted based on the chromosome 4 DNA contents in the total genome.

The Giemsa-FISH method is the most reliable means of identifying centromeres, but it is very laborious and time consuming. To solve this problem, non-fluorescent chromosome painting that uses a peroxidase/diaminobenzidine (DAB) reaction has been developed[11]. Before being hybridized with a biotinylated DNA probe specific for selected chromosomes and made visible by the peroxidase/DAB reaction, metaphase preparations are treated with the enzymes and denatured mildly, indispensable for obtaining uniformly painted chromosomes with good morphology (Fig. 2). DAB painting of chromosome 4 detects 11.5% of the unstable aberrations

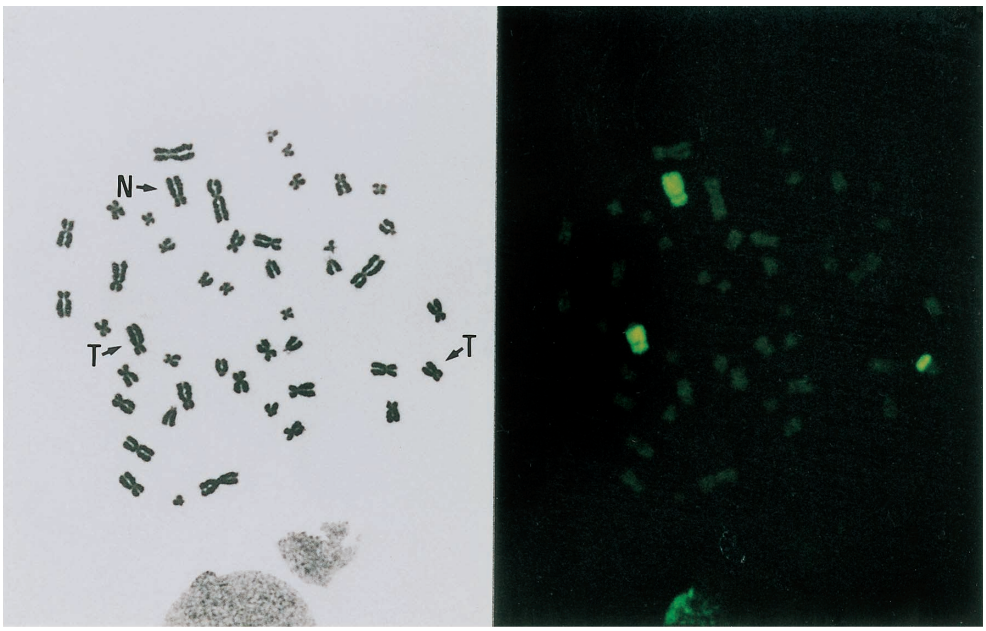


**Fig. 1.** Giemsa staining (left) and chromosome painting with a whole-chromosome probe for chromosome 4 (right) of the same human metaphase. One normal (N) and two translocated (T) chromosomes are present.

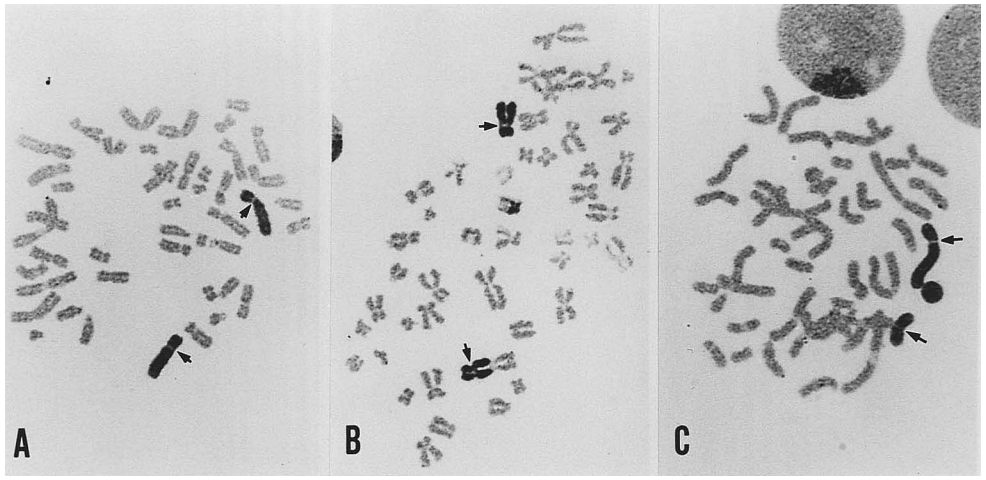


**Fig. 2.** Human metaphases painted with probes for chromosome 4 and made visible by the peroxidase/DAB reaction. Arrows indicate centromere positions on the painted regions. (A) a normal metaphase; (B) a metaphase with a dicentric and a fragment ; and (C) a metaphase with an acentric ring.

produced in the total genome, close to the predicted value cited above. Equal frequencies of radiation-induced translocations and dicentrics are obtained. DAB painting also has advantages over FISH painting: the preparation can be analyzed under a bright-field microscope to make the scoring more reliable, can be preserved permanently, and is suitable for an automated system.

## RECENT APPLICATIONS OF CYTOGENETIC DOSIMETRY

On September 30, 1999, a severe accident occurred in a uranium processing plant in Tokaimura, Ibaraki, Japan. As it involved exposure to neutrons, for which physical dosimetry is difficult, cytogenetic dosimetry has been expected to estimate doses necessarily. Of those who might have been exposed to low-doses, 43 persons agreed to a cytogenetic examination and have been analyzed for dicentrics plus centric rings. Dosimetry for the workers exposed to high doses was problematic as data on physical dosimetry and clinical symptoms indicated that the doses to the two most severely injured persons may have been above the upper limit measurable by conventional cytogenetic dosimetry.

After a high-dose exposure there are two practical problems: First rapid lymphopaenia is observed in the peripheral blood counts. This is not due primarily to cell killing, rather it is a physiological response whereby cells move away from the circulatory system into tissues and the lymphatic system[24], clearly reducing the numbers of cells per milliliter of blood available for cytogenetic analysis. This problem is solved by using a purified lymphocyte culture[7].

Second, few of the cells that remain available are able to enter mitosis when cultured and harvested at metaphase using a mitotic inhibitor such as colcemid. The technique of premature chromosome condensation (PCC) can be used to settle this second problem. Recently, chemical-

induced PCC has been reported to be applicable to dosimetry[25–27], thereby bypassing all the laborious work of the existing PCC method that uses mitotic cell fusion[28,29]. Of the chemical-induced PCC dosimetries, the most practical one is the scoring of rings in Giemsa-stained chromosomes that have been condensed by okadaic acid[27]. This biodosimetry enables estimation of up to 20 Gy X rays and minimizes time of slide preparation. This PCC method together with metaphase analysis allowed estimation of the doses to the three highly irradiated workers at 64 h after the accident.

## PERSPECTIVES

After a radiation accident biodosimetry is needed to supplement physical methods. In cases of high-dose exposures, information about the exposure greatly helps physicians decide on medical procedures, such as transplantation of marrow or stem cells and treatment with growth factors, that ideally should be required within the first 24 h[30]. On receipt of a blood specimen, the normal laboratory procedure is to culture the lymphocytes for 48 h and then fix and stain them. Doses, therefore, can be currently evaluated by cytogenetic analysis available from the third day. PCC cell induction by the mitotic cell fusion method can be performed on $G_0$ lymphocytes, which removes the requirement for a 2-day culture, thereby providing a quicker dose estimate. Therefore there is an incentive to extend the chemical-induction method to achieve PCC in $G_0$ lymphocytes but no one has succeeded yet. Moreover, for subjects exposed to low doses at an accident, early demonstration of very few chromosomal aberrations in the lymphocytes is valuable in terms of reassurance and psychological support[30]. The experience at the Tokaimura accident indicates that greater research efforts need to be directed to techniques that shorten the time for obtaining a dose estimate as well as to ways to improve accuracy.

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