Improvement Of Biological Dosimetry By Cytogenetics For Operational Purposes: Dicentrics Or Micronuclei?

P. Voisin, N. Paillole, M. Benderitter, M. Claraz, V. Chambrette, M. Delbos, V. Durand, A. Leroy and I. Sorokine-Durm.
Institut de Protection et Sûreté Nucléaire, Département de Protection de la santé de l'Homme et de Dosimétrie, IPSN, BP6, F-92265 Fontenay-aux-Roses Cedex, France.

1. Introduction

The biological dosimetry is an important part of the diagnostic and the pronostic in case of overexposure suspicion to ionizing radiations. It intervenes besides the clinical symptoms and the physical reconstruction, in order to help the medical team in charge of irradiated patients for adopting the best therapy strategy. Whatever for a radiological expertise or a nuclear accident, biological dosimetry currently is assessed by the scoring, in the lymphocytes of peripheral blood, of the unstable chromosome aberrations (dicentrics + centric rings), induced by ionizing radiations. The cells are observed at the stage of the mitosis. The aberrations frequency is then brought back to a whole body-equivalent dose. To ensure this cytogenetic dosimetry, a calibration curve is established from the chromosome aberrations scoring in lymphocytes coming from blood samples *in vitro* irradiated with known doses.

These chromosome aberrations, even in case of high irradiation, remain however rare events. For a homogeneous exposure, their distribution approaches to a statistical distribution known as Poisson Law. It is then possible to predict the error of estimate according to the number of chromosome aberrations scored and the number of metaphases observed. The adopted step is however different according to the goal to reach, accidental expertise or radiological urgency (1,2).

In the case of an irradiation suspicion implying few people and considering the known (or unknown!) circumstances of the radiological accident, the quality of the preparations and the precision of the estimate are privileged. So that the confidence interval is satisfactory, several hundreds of metaphases must be observed. It is a long and tiresome job, requiring several working days for skilled observers.

In the event of accident involving a large population exposed to irradiation, uncertainties of physical dosimetry or clinical symptoms increase in proportion of the number of potentially irradiated individuals, which justifies the interest of a biological dosimetry on a purely individual basis. Its utility fully appears only if the dose estimates can be returned in the hours following the real or supposed irradiation. The measuring accuracy and the quality of the preparations appear in proportion less important than the deadline. A particular procedure making it possible to combine these various factors was developed and checked during simulation training. The metaphase preparation and chromosome analysis techniques were simplified, and the number of observed metaphases reduced. We will see below the effectiveness of this process.

Nevertheless, the conventional cytogenetics remains a heavy technique limited to people qualified in cytogenetics. There is another easier and potentially faster cytogenetic technique, which, in the case of a triage, could usefully replace the conventional cytogenetics. The micronuclei scoring in the blood peripheral lymphocytes is a method increasingly used to evaluate DNA damage induced by many genotoxic factors (3). The micronuclei appear as small mass of DNA beside the main nuclei and arise from either fragments or whole chromosome excluded from the daughter nuclei during cell division. Micronuclei are scored in lymphocytes blocked in their second interphase. This is obtained by the addition of Cytochalasin B, which modifies, neither the DNA replication, nor the mitosis course, but blocks the cytokinesis step. Their binucleated aspect in which the micronuclei are scored using Giemsa or fluorescent staining identifies these cells (4).

We thus applied the same adaptation principle to the micronuclei assay that we tested during emergency trainings. The results obtained are also reported in this paper and the comparison with the conventional cytogenetics data.

2. Conventional cytogenetics

2.1 Standard preparative method:

Blood is taken by venous puncture on lithium heparinate in Vacutainer ®. The whole blood cultures are carried out according to a procedure similar to this recommended by the IAEA report (1) and published elsewhere (2). Briefly, a blood aliquot is cultured in a appropriate cell medium, added with a mitogenic agent, the phytohemaglutinin (Life Technologies), several antibiotics and 10 % fœtal calf serum (SVF, Life Technologies). The cultures are carried out in duplicate the same day. In addition, safety culture series are carried out the following day in case of contamination problems, bad handling or poor culture rates.

For conventional cytogenetics, it is important to examine only the metaphases of first mitosis, because 50% of the cells carrying "unstable" chromosome aberrations are expected to be eliminated during each mitosis. The observation of second mitosis or more would lead to an underestimation of the dose. For this purpose, a thymidin analogue, the 5-Bromodeoxyuridin (Sigma), is added to each culture (5).

After 46 hours of culture to 37C, a mitotic inhibitor, demecolcin (Life Technologies), is added in the tubes, and the culture prolonged for 2 to 3 hours. The lymphocytes are harvested by centrifugation, and a KCl hypotonic shock (0.075 M) is applied to lyse red blood cells and to inflate lymphocytes nuclei. After centrifugation, the cells are fixed by addition of methanol/acetic acid mixture (3/1, v/v). The metaphases are spread out by projection over clean and wet microscope slides and visualized by technique FPG (3).

2.2 Unstable chromosome aberrations scoring

After staining with Giemsa (Life Technologies), the metaphases are examined under bright field microscope and all unstable chromosome aberrations (dicentrics, centric rings and acentrics) scored. Only the dicentrics and centric rings, really specific of the ionizing radiations, are used for the estimation of a dose. Indeed, the spontaneous rate of dicentrics in the normal population, according to our experimental conditions, is lower than 1 per 1500 metaphases. It is ten times lower for the centric rings. So that the confidence interval is satisfactory (at least equal to 0,2 Gy), a minimal scoring of 100 dicentrics is required or, if this number cannot be reached, 500 metaphases at least are observed (figure 1). It is necessary to keep in mind that only the complete metaphases (with 46 centromeres) are kept, which means that more metaphases are really observed, 10 % or more if the irradiation dose is high.

2.3 Modifications of the conventional cytogenetic procedure for a triage

As remind in the introduction, the "standard" procedure was adapted, in order to resolve the problem of the deadline in case of a population triage (Table 1). The preparation and analysis techniques of the chromosome aberrations were simplified, and the number of observed metaphases reduced to 50.

STEPS OF PREPARATION AND ANALYSIS	**EXPERTISE** *(precision factor)*	**TRIAGE** *(time factor)*
Number of operators	**2**	**2 teams of 2**
First culture (number of tubes/samples)	**2 to 10**	**2**
Additional culture (number of tubes/samples)	**2 to 10**	**2**
Hypotonic Shock	**KCl**	**KCl**
Fixation procedures	**3**	**3**
Number of metaphases observed	**250 to 500**	**50 at least**

Table 1. Main methodological differences in the preparation of the blood samples for biological dosimetry by conventional cytogenetics, according to the finality of the examination.

The observation of 50 metaphases is enough to obtain a 95 % confidence interval of ±1 Gy for a dose estimate. This uncertainty level seems satisfactory to the hospital teams in charge of the overexposed patients, for a first triage (figure 1). In order to maintain the ability to simultaneously process several tens of blood samples, it is important to periodically organize emergency training. Based upon the scoring of 50 metaphases only, these exercises pointed out that it was possible with six people to process 100 blood samples in 7 days, preparation included. The figure 2 shows an example of such training. The precision of the dose estimate was in the range expected by the statistics.

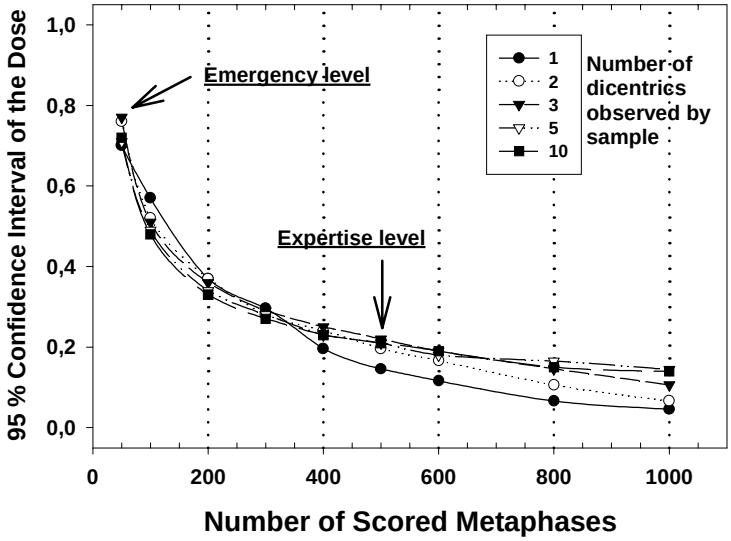

Figure 1 : Upper limit of the 95 % confidence interval of the dose estimate, according to the number of lymphocytes observed and calculated for γ-rays of ^{60}Co by using the dose-effect relationship Y=0.004+0.0374D+0.0549D2 (with Y=dicentrics yield and D=dose in Gy). The low influence of the dicentrics number must be noted.

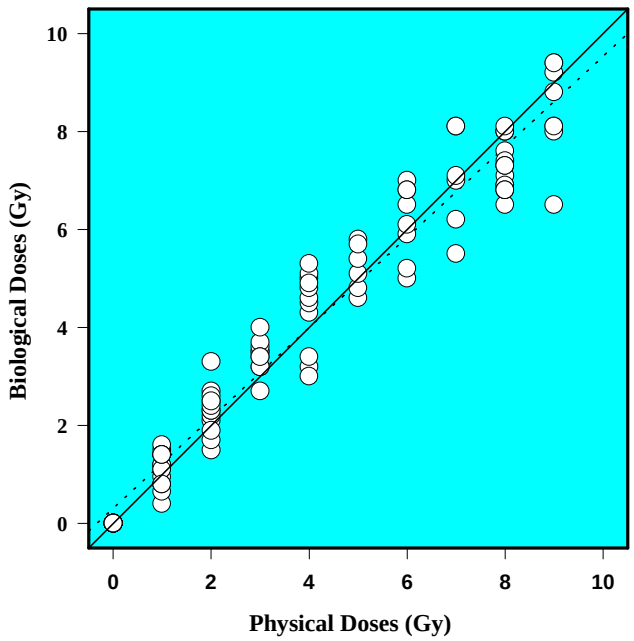

Figure 2**.** Experimental data of an emergency training during which more than 100 blood samples were simultaneously analysed by conventional cytogenetics, based on the 50 metaphases reading. The biological dose estimates (in ordinate) is expressed according to the physical dose (in abscissa). The dotted line represents the linear regression led through experimental points

In fact, the most probable situation to meet during a real accident should be intermediate, with a first population triage followed by a more precise dose estimate for the really overexposed people. A good solution is to process the blood samples for providing a large number of readable metaphases, but to score chromosome aberrations in only 50 metaphases for the first step. A second step consists in extending the scoring to 250

metaphases in order to increase the general precision of a factor x2. Last, the scoring could be supplemented to 500 for the most relevant cases.

2.4 Application to a real case: the second radiological accident of Georgia

At the end of July '98, three sources of ^{137}Cs (between 0.17 and 150 GBq) were found in the vicinity of the village of Matkhoji, located at 300 km in the West of Tbilissi. They would come from an old Russian military base, abandoned since 1992 and used now as pasture and playground for the children. The Georgian Ecological Department consequently required assistance to IAEA, which sent two missions to the site. It is come out from these investigations that a chronic exposure of a part of the population of the village was not to exclude. Thus, the IAEA asked for the technical assistance of the IPSN. A mission, with 4 members of IPSN and an IAEA representative, went to the site from 12 to 17 October 1998. This mission collected information on the circumstances of the exposure and selected a potentially involved cohort of 112 people, in three groups: children of more than five years and adults of less than 50 years attending the site, members of the family who had a source in their cattle shed. An analysis of hematologic parameters was performed on these people and 85 blood samples were taken for a biological dosimetry. The hematologic analysis carried out on the spot did not show any particular anomaly. The biological dosimetry by scoring of unstable chromosome aberrations (dicentric, rings centric, fragments) in blood peripheral lymphocytes was performed on blood samples after air transportation to the laboratory of the IPSN. Two successive procedures were initiated. The first one is a quick phase of triage not very precise but intended to check if some out of these people presented obvious signs of irradiation. Only 50 cells were observed for each individual. Seven days were needed to complete this phase. Dicentrics were found in three people only for a whole-body dose not exceeding 0.5 Gy on average. This triage was followed of a more complete but longer expertise, since the chromosome aberrations were searched in 250 cells. Two months and half were necessary to observe 22000 cells at the 85 people. Finally, 30 dicentrics were scored among only 17 Georgian patients. The strongest dose to the whole body estimated from the reference curve of the laboratory was 0.3 Gy. A majority of these dicentrics was found in the children having played on several occasions in the former military camp. Unfortunately, the information provided by the population was too fragmentary to allow an effective reconstitution of the received doses.

This intervention was particularly important, because it was located in the real framework of a reconstitution of dose by cytogenetic dosimetry in the event of radiological or nuclear accident. It confirms that in the case of a more complete expertise and especially more precise, the number of dicentrics possibly present in 50 cells is insufficient to determine a reliable dose. Other metaphases must thus be observed until a total number of 250 or 500, according to the cases and known circumstances of the irradiation.

3. The micronuclei assay

3.1 Methodology

The micronuclei assay can be performed from a blood aliquot of 150 µL, against 500 µL for the dicentrics test (4). The other culture conditions remain very close each other, even if the culture duration is lengthened at 64 hours and the foetal calf serum increased to 25%, for micronuclei assay. The cytochalasin B (Sigma) is added to the culture medium after 40 hours, to block a maximum of lymphocytes at the beginning of the second interphase. The lymphocytes are separated by centrifugation, and a KCl hypotonic shock (0.125 M) is also practised to lyse red blood cells and to inflate the lymphocytes. However, its higher concentration preserves the lymphocyte cytoplasm, essential to assess the micronuclei distribution. After centrifugation, the cells are fixed by addition of methanol/acetic acid (6/1, v/v). The cells are spread out by projection over clean microscope slides and observed in fluorescence, after addition of propidium iodide, a specific DNA dye. The micronuclei are counted only in binucleated lymphocytes. The observation of 1000 binucleates cells at least is necessary to obtain a statistical precision of the measurement of the same order of magnitude as that obtained by scoring the dicentrics in 500 metaphases. The speed of observation naturally depends on the richness of slides in binucleated cells and micronuclei, but the scoring can be achieved within 1 to 2 hours. This speed of observation, compared to the 2 to 3 days necessary for the manual dicentrics scoring in the 500 metaphases, is one of the major interests of this method.

3.2 Adaptation of micronuclei test to the emergency

As for the conventional cytogenetics, the micronuclei technique was adapted for the triage of a population potentially exposed to irradiation in a large accident (Table 2). A specific method (4) was developed by substantially decreasing the blood volume needed for cultures and by improving the treatment of the cells in order to preserve a sufficient ratio of binucleates, even for the highest radiation doses (4 Gy and above). To validate these changes, emergency training was also carried out by preparing slides and estimating doses from about sixty samples, with doses ranging between 0.5 and 4 Gy (γ rays of ^{60}Co, 0.5 $Gy.mn^{-1}$). The micronuclei

yield was observed in 500 binucleated cells, which corresponds, in our experimental conditions, to the mean time needed to observe dicentrics in 50 metaphases. The figure 3 shows data of such training. It can be noted that the higher scoring speed of micronuclei offsets the longer culture time necessary for producing them. The final result is that the total expertise duration (blood preparation + slide analysis) is half-day longer for the micronuclei than for dicentrics assays. In addition, the known variability of micronuclei data between individuals (4) is compensated by the higher level of measurements accuracy that gives the number of binucleates observed. In this respect, a ± 0.5 Gy confidence interval at 95 % statistical significance is expected from the observation of 500 binucleates for micronuclei data, compared to a ±1 Gy confidence interval expected from the observation of 50 metaphases for dicentrics scoring. The figure 3 shows that the results effectively obtained with micronuclei are quite similar to those obtained with dicentrics (figure 2), using the same experimental conditions. Last but not the least, the micronuclei test, easier and quicker than the dicentrics assay, does not require operators specialized in cytogenetics.

Methodology parameters	*Dicentrics*	*Micronuclei*
Blood volume/culture	0.8 ml	0.15 ml
Culture Time	48 hours	64 hours
Slide preparation time	4 hours	2 hours
Number of observed cells	50	500
Staining	Giemsa (bright field)	Propidium iodide (fluorescence)
Aberration scoring time/sample	1 to 2 hours	1 to 2 hours
Expected estimation accuracy	± 1 Gy	± 0.5 Gy
Lower statistical limit	1 Gy	0.5 Gy

Table 2. Key points of the lymphocyte culture conditions, slides preparation and number of cells observed, in case of dicentrics assay and micronuclei test. The experimental conditions shown here are those adapted for the simultaneous treatment of a great number of samples.

4. CONCLUSION

The dose assessment cannot be approached in the same way for expertise of overexposure suspicion or for triage of a large accident. For the former, quality and precision of assessment are needed and for the latter, speed and efficiency are the most important. To combine these different aims, improvement of the dosimetry by cytogenetics was developed in our laboratory using two different methods, dicentrics and micronuclei assays. Special procedures for the simultaneous preparation of several tens of blood samples and for the aberrations scoring with a reduced number of cells were designed and tested in simulated and real accidents. We show in this paper that the micronuclei have several advantages on dicentrics for a population triage, but a biological laboratory not specialised in cytogenetics can carry it out. Nevertheless, the micronuclei test has also some limitations which disserves this technique for an overexposure expertise:

- a lack of response for ionising radiation effect, at low dose (below 0.5 Gy) and when the exposure is heterogeneous or fractionated;
- a formation which seems take into account some aspects of an individual radiosensitivity;

A solution, which would consist to use micronuclei for triage and dicentrics for low doses or expertise, appears difficult to apply, for it would require doubling each analysis.

A more reasonable solution could be to supplement the classical micronuclei analysis by centromere and/or telomere staining by fluorescent in situ hybridization (6). It was shown recently that by this method, micronuclei of mutagen origin could be discriminated of those of clastogen origin. Practical aspects of this approach are in progress in our laboratory.

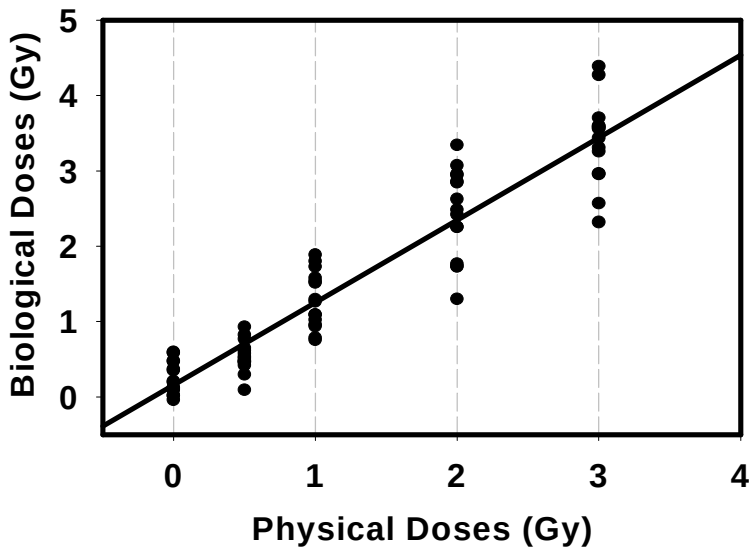

Figure 3. Experimental data obtained during an emergency training, by employing the micronuclei test. Fifty blood samples were treated according to the procedure described in table 2 and in the text, after *in vitro* irradiation up to 3 Gy (^{60}Co, 0.5 Gy.mn^{-1}) and a micronuclei scoring based on the observation of 500 binucleated lymphocytes. The solid line represents the linear regression led through experimental points.

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