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REVIEW

Current status of biodosimetry based on standard cytogenetic methods

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Abstract Knowledge about dose levels in radiation protection is an important step for risk assessment. However, in most cases of real or suspected accidental exposures to ionizing radiation (IR), physical dosimetry cannot be performed for retrospective estimates. In such situations, biological dosimetry has been proposed as an alternative for investigation. Briefly, biodosimetry can be defined as individual dose evaluation based on biological endpoints induced by IR (so-called biomarkers). The relationship between biological endpoints and absorbed dose is not always straightforward: nausea, vomiting and diarrhoea, for example, are the most well-known biological effects of individual irradiation, but a precise correlation between those symptoms and absorbed dose is hardly achieved. The scoring of unstable chromosomal-type aberrations (such as dicentrics and rings) and micronuclei in mitogen-stimulated peripheral blood, up till today, has been the most extensively biodosimetry assay employed for such purposes. Dicentric assay is the gold standard in biodosimetry, since its presence is generally considered to be specific to radiation exposure; scoring of micronuclei (a kind of by-product of chromosomal damages) is easier and faster than that of dicentrics for dose assessment. In this context, the aim of this work is to present an overview on biodosimetry based on standard cytogenetic methods, highlighting its advantages and limitations as tool in monitoring of radiation workers' doses or investigation into accidental exposures. Recent advances and perspectives are also briefly presented.

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Introduction

Since its discovery, ionizing radiation (IR) has provided great benefit to mankind through its applications in agriculture, industry, medicine and research. The continuous improvement in radiation protection (radioprotection), the science in charge of protecting people (radiation workers and the general public), is based on knowledge about the interactions of IR with living tissues (UNSCEAR 2000).

The importance of radioprotection programmes has grown due to increasing of IR application and to the public interest on potential risks associated with the usage of radiation sources. Still today, radioprotection management is based on evaluation of specific dosimetric quantities. Among them, absorbed dose, defined as the amount of energy delivered by IR to matter per unit of mass, is the fundamental physical quantity to evaluate potential biological response resulting from exposure to radiation. In the international system of units, the unit of absorbed dose is J kg^{-1}, and its specific name is Gray (ICRP 1991).

To regulate the exposure of workers and the public at large, two derived dose quantities are suggested by the International Commission on Radiological Protection (ICRP): equivalent and effective doses, both expressed in Sievert (Sv) to distinguish them from absorbed dose in Gray (Gy) (ICRP 1991). The effective dose takes into account radiosensitivity of organs and tissues of the human body (Streffer 2007) and was introduced to represent the long-term risk of harm from radiation exposure, in particular radiation-induced cancer (ICRP 1991; Amaral 2005).

Usually, physical dosimeters (film badges, thermoluminescent crystals and semiconductors) have been used for monitoring of personnel absorbed dose in all facilities that use regulated radiation sources, in order to maintain the exposure as low as reasonably achievable (ALARA

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principle), following the recommendations of the major organizations of radioprotection, ICRP and International Atomic Energy Agency (IAEA).

Nevertheless, in the majority of incidents involving radiation exposure, the affected individual does not wear such dosimeters. In these situations, physical dosimetry is not an easy task. For example, in case of emergency responses to a large-scale accident, medical treatment of most seriously affected persons might be delayed or even prejudiced due to lack of sound dosimetric data (Lloyd 2005). In an attempt to overcome this drawback, alternative methods for dose assessment using biological endpoints (or biomarkers) have been purposed. Dosimetric evaluations based on biomarkers studies are referred to as biodosimetry (Prasanna et al. 2005).

This paper presents an overview on biodosimetry, pointing out advantages and limitations, based on standard cytogenetic methods, as important tool either in monitoring of radiation workers' doses or in the investigation into accidental exposures. Recent advances and perspectives are briefly presented as well.

Clinical signals and haematological alterations

The first method employed to correlate human biological parameters with absorbed dose was based on the observation of intensity, frequency and duration of some symptoms displayed after radiation overexposure (IAEA and WHO 2000). Those symptoms characterize the so-called prodromal phase of the acute radiation syndrome (ARS), which involve hematopoietic, gastrointestinal and neurovascular systems. Table 1 summarizes clinical symptoms with absorbed dose levels. From this table, health disturbances of ARS only appear after whole-body acute exposures greater than 1 Gy. Screening and more accurate classification of irradiated population with doses above 2 Gy are carried out by haematological counts of lymphocytes, polymorphonuclear leucocyte and platelets levels. These data are supported by a well-known relationship between a large exposure levels and ARS's hematopoietic disturbances, by which the production of one or more blood components is stopped or intensely reduced (so-called cytopenia) (Waselenko et al. 2004).

Lymphopenia occurs before the manifestation of other cytopenias (granulocytopenia and thrombocytopenia) within the first 6–24 h after a moderate- to high-dose exposure (Dainiak et al. 2003). Based on the level of lymphocyte count, the degrees of ARS have been described as shown in Table 2. This rapid and common laboratory test enables a rapid screening of injured victims in case of radiological emergency (Stepanova et al. 2008).

The application of those clinical and laboratory assays is limited principally by two reasons: First, severity of these manifestations also depends on dose rate, radiosensitivity of tissues involved, body area exposed and damage level suffered by organic systems. Other factors are adverse environmental conditions and pathologic agents (virus and bacterium), which can enhance ARS-related disturbs or promote health alterations similar to those induced by ionizing radiations (Dainiak et al. 2003); Second, the

Table 1 Early symptoms of the acute radiation syndrome (extracted from Table VIII–Prodromal phase of acute radiation syndrome (IAEA and WHO 1998); WBE: Whole-body exposure

Symptoms	ARS degree and approximate dose of acute WBE (Gy)				
	Mild (1–2 Gy)	Moderate (2–4 Gy)	Severe (4–6 Gy)	Very severe (6–8 Gy)	Lethal (>8 Gy)
Vomiting	Present	Present	Present	Present	Present
Onset	~2 h	1–2 h	Earlier than 1 h	Earlier than 30 min	Earlier than 10 min
Incidence	10–50%	70–90%	100%	100%	100%
Diarrhoea	–	–	Mild	Heavy	Heavy
Onset	None	None	3–8 h	1–3 h	Within minutes or 1 h
Incidence	–	–	<10%	>10%	~100%
Headache	Slight	Mild	Moderate	Severe	Severe
Onset	–	–	4–24 h	3–4 h	1–2 h
Incidence	–	–	50%	80%	80–90%
Consciousness	Unaffected	Unaffected	Unaffected	May be altered	Unconsciousness
Onset	–	–	–	–	Seconds/minutes
Incidence	–	–	–	–	100% (at >50 Gy)
Body temperature	Normal	Increased	Fever	High fever	High fever
Onset	–	1–3 h	1–2 h	<1 h	<1 h
Incidence	–	10–80%	80–100%	100%	100%

Table 2 Relationship between absolute lymphocyte count and absorbed dose after 6 days since first exposure (IAEA and WHO 1998)

Degrees of ARS	Dose (Gy)	Lymphocyte count (per L)
Pre-clinical phase	0.1–1.0	1.5–2.5 × 10^9
Mild	1.0–2.0	0.7–1.5 × 10^9
Moderate	2.0–4.0	0.5–0.8 × 10^9
Severe	4.0–6.0	0.3–0.5 × 10^9
Very severe	6.0–8.0	0.1–0.3 × 10^9
Lethal	>8.0	0.0–0.05 × 10^9

minimum radiation dose for observation of such events (~1 Gy) is many times higher than that usually involved in occupational exposures. Indeed, nuclear workers are generally under low-level radiation at low-dose rate (<20 mSv per year) that is considered an acceptable level of radiation exposure (Prise et al. 2001; Koenig et al. 2005). Thus, dose estimation by clinical and laboratory assays is not precise, but they can be used for initial screening of exposed individuals and fast deciding upon proper medical care in radiological accidents involving tens or hundreds of individuals (Berger et al. 2006). For a more precise evaluation, it has been proposed the use of cytogenetic dosimetry that is based on investigation into radiation-induced changes in chromatin structure.

Cytogenetic dosimetry

Genomic instability is directly related to rearrangements of chromatin structure, resulting in chromosome aberrations (CAs) that are classified in accordance with their mechanism of formation. Chromatid-type aberrations are usually related to chemical stress, whereas chromosome-type aberrations are more specific to IR (Bonassi and Au 2002).

The most commonly used cell models for the study of CAs are fibroblasts and lymphocytes. When IR interacts with these cells, which are in the quiescence phase, radiation-induced DNA lesions can remain latent and persist through the DNA replication process (Fringer and Grinnell 2003; Yusuf and Fruman 2003). The analysis of radiation-induced CAs requires artificial induction of cell proliferation, subsequent processing of DNA lesions by DNA synthesis occurring in S phase, and then arresting of cells in mitosis.

Lymphocyte cultures require a shorter culture time and involve a lower risk of contamination than fibroblast cultures. Lymphocytes also can be obtained by procedures that are simpler (venous punction) than those for fibroblasts (biopsy). Additionally, lymphocytes circulate throughout the body, being a better model for biodosimetric assays (Stankeová et al. 2003; Garcia-Sagredo 2008).

In vitro culture, a lymphocyte population is stimulated to divide by addition of a mitogen, generally phytohaemagglutinin (PHA) (Carloni et al. 2001). Highly condensed and better defined chromosomes are observed in the metaphase stage after blocking of mitosis with an arresting agent, usually colcemid. This prevents formation of the mitotic spindle, due to inhibition of microtubule polymerization during chromosome segregation at anaphase (Kanda et al. 1994).

After culture, it is possible to identify CAs in metaphase chromosomes. Depending on life-time/viability of bearing cells, CAs are classified as unstable or stable aberrations. Dicentrics, acentric rings and fragments are types of unstable aberrations, whereas insertions, reciprocal and non-reciprocal translocations are referred to as stable aberrations (Obe et al. 2002).

Unstable aberrations are usually associated with loss of genomic material that can occur in two pathways: (1) acentric fragments fail to wrap on the mitotic spindle during anaphase; (2) anaphase bridges are formed from dicentrics resulting in ruptures of affected chromosomes. Both events contribute to dose underestimation if IR exposure occurred thereafter 3 months (Lloyd 1998). On the other hand, stable aberrations are transmissible through successive divisions, providing important information to assess absorbed dose over long periods of time (Léonard et al. 2005; Rodrigues et al. 2005).

These different features are employed on distinct situations in personal monitoring, depending on the period of time between irradiation and development of the biodosimetric assays. More details are presented below.

Dicentric scoring: dose–effect curves and absorbed dose

The first studies proposing the employment of CAs to detect previous exposures to radiation and quantify absorbed dose were published by Bender and Gooch in 1962 (see Hoffmann and Schmitz-Feuerhake 1999). In those researches, the frequency of CAs was scored in 1,000 metaphases from eight individuals who were exposed to radiation in a nuclear accident in Idaho Falls, United States, in 1961. Quantitative dose–effect information could not be measured because the bioassay was performed several years after irradiation, but CAs were still present in most of the cases (Kawata et al. 2004). Later in the 1960s, the scoring of CAs was successfully tested in a number of cases of accidental exposure to IR, and good correlation between the number of dicentric chromosomes and absorbed dose predicted by physical models was obtained.

Today, as a general rule, it has been proposed that the analysis of CAs should be performed using 500 cells, if an incident involving low doses (≤1 Gy) might have happened. On the other hand, for an incident involving higher doses (>1 Gy), it is recommended to count 100 dicentrics (IAEA 2001). However, for a preliminary estimation of

dose in a radiological accident, only 50 metaphases or 30 dicentrics per subject need to be scored (Lloyd et al. 2000).

The dose–effect relationship obtained after in vitro irradiation of blood can be used as a calibration curve to estimate effects from an irradiation in vivo (Doloy et al. 1991; IAEA 2001). Generally, frequencies of dicentrics are related to known radiation doses, depending on radiation quality (or LET–Linear Energy Transfer) and relative biological effectiveness (RBE). This makes possible the estimation of absorbed dose in uniformly irradiated persons (accidental or occupational exposure), because in this case lymphocytes should have been also exposed (Amaral 2002; Voisin et al. 2002).

Dose–effect curves have shapes and slopes that vary as a function of the LET and RBE, as illustrated in Fig. 1.

For low-LET radiation (e.g. gamma and X rays), the dose–effect curve for dicentrics fits better as linear-quadratic model: $Y = A + \alpha D + \beta D^2$. Here, Y is the dicentrics' yield, A is the frequency of dicentrics at dose zero, α and β are fitted parameters and D is the dose. On the other hand, for high-LET radiations (e.g. neutrons and alpha particles), the shape of the calibration curve fits better to a linear dose response ($Y = A + \alpha D$). The reason for that is related to differences in the quantity of energy deposited per micrometre of an ionization track, leading to a different biological effectiveness as a function of different types of radiation (IAEA 2001). Thus, for an accurate assessment of absorbed dose involving individual exposure to mixed fields, such as gamma and fission neutrons, it is essential to estimate separately the contribution of each component (Ballarini et al. 2003; Voisin et al. 2004a).

It was not before the year 2004 when dicentrics scoring was recognized as an international standard by the International Organization for Standardization (ISO) that published a guideline for performing laboratory services (ISO 2004; Stricklin et al. 2007). Principal parameters of ISO 2004 regulations are cited below:

1. Incorporation of good laboratory practices;
2. Appropriate documentation of protocols;
3. Development of an own dose–response curve at each laboratory, excluding inter-laboratorial differences;
4. Set-up of dicentrics scoring only by trained technicians;
5. Demonstration of scoring experience by intra- and inter-laboratory comparisons, using standard scoring criteria (Roy et al. 2004; Yoshida et al. 2007; Wilkins et al. 2008).

The principal advantages of the dicentrics assay in biodosimetry are as follows:

- High specificity to IR–only a few genotoxic mutagens may produce a similar effect (bleomycin, neocarcinostatin and mitomycin C). Nevertheless, these radiomimetic agents are employed mainly to chemotherapy treatments, presenting high toxicity;
- Low background in non-exposed populations–about 1–2 in 1,000 cells;
- High occurrence among unstable aberrations–in the order of 60%;
- Considerable range of dose detection–lower detection limit is about 0.1 and 0.01 Gy for low- and high-LET radiation, respectively;
- Low cost method for chromosome staining–conventional staining with Giemsa (Fig. 2).

Nevertheless, dicentrics have a limited applicability in procedures that involve fractionated irradiation as well as

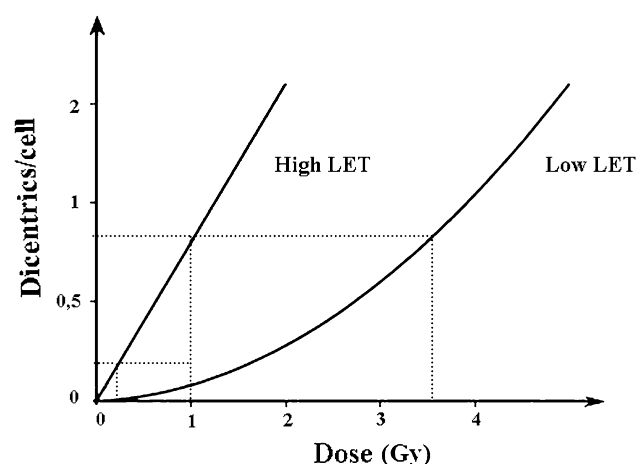

Fig. 1 Dose–effect curves for different quality radiations (IAEA 2001)

Fig. 2 Unstable aberrations in irradiated lymphocytes, visualized by conventional staining with Giemsa. The dicentric chromosome and its respective fragment are marked by an *arrow* and a *circle*, respectively

late exposures, which may result in a lower dicentrics yield than if the same dose is received acutely.

In fractionated exposures, primary breaks in adjacent chromosomes induced during the first irradiation can be corrected by repair mechanisms before induction of additional breaks by a subsequent dose fraction. If the time interval between two consecutive irradiations is greater than about 2 h (which corresponds to the mean lifetime of the breaks), then the mis-rejoining of damaged chromatin and dicentrics is minimized or even avoided (IAEA 2001).

In the case of late exposures, cells bearing dicentrics and other unstable aberrations are naturally eliminated (about 50% at each mitosis) from the circulating lymphocyte pool by a mechanism called apoptosis (programmed cell death), which may cause underestimation of the absorbed dose if the bioassay was performed more than 3 months after exposure. Probably, this selective removal is a strategy of the immune system to prevent or minimize late biological effects, as cancer and leukaemia (Pala et al. 2001; Belloni et al. 2008). Thus, cells bearing dicentrics have to be scored only after first post-irradiation division (M1), in order to maintain the quantitative response.

This is particularly important after partial-body irradiation, because a fraction of the affected cells may not have enough time to reach the M1 stage within 48-h cell culture (conventional time). These cells present a delayed cell cycle, especially at check points, in comparison with non-irradiated lymphocytes, as a result of their abnormalities (Suzuki et al. 2006). One experimental option to mathematical extrapolations (Dolphin and Qdr models) has been to extend cell culture time and add colcemid at the beginning of the culture, reaching better dose estimations (Fernandes et al. 2008b).

Other laboratorial factors that could influence cell progression rate and yield of radiation-induced CAs in culture must be fit appropriately, such as temperature, culture time and culture medium (Purrot et al. 1981; Gumrich et al. 1985; Virsik-Peuckert and Harder 1985; Hone et al. 2005).

Alternative staining methods, such as fluorescence plus Giemsa (FPG) using bromodeoxyuridine, have enabled to restrict the analyses to M1 cells, but some practical limitations have been detected. The main drawback of the FPG method is the final optical resolution of chromosomes, which is very poor and blurred (Hayata et al. 1992; Roy et al. 1996).

Dicentric assay is a time-consuming method, demanding skilled technicians for accurate identification of this type of CAs. For this reason, several studies have indicated that fluorescent in situ hybridization (FISH) and C-banding methods to highlight centromeric regions may allow faster scoring with same accuracy (Fernandes et al. 2006; Fernandes et al. 2008a; Mestres et al. 2008).

Stable aberrations: relationship with absorbed dose

The introduction of stable chromosome aberrations as IR biomarker has been essential to overcome some limitations of dicentrics scoring that can lead to an underestimation of dose after late exposure or long-term irradiation (Bauchinger 1998; Moquet et al. 2000).

Unlike cells bearing dicentrics and other types of unstable CAs, those with stable aberrations usually do not undergo negative selection during mitoses, given that the presence of stable CAs is not necessarily lethal for cells and the loss of genetic content is considerably limited during successive mitosis. For this reason, this biomarker has been more reliable for retrospective evaluations (Tucker 2001; Rodríguez et al. 2004).

Symmetrical (or reciprocal) translocations and insertions exhibit higher persistence than other stable CAs in the peripheral blood lymphocytes pool, as cell renewal occurs. Identification of such abnormalities requires the cell culture of irradiated lymphocytes, similarly as done to dicentrics (Edwards et al. 2005). However, staining methods other than Giemsa staining are carried out to detect translocations, as later described in this paper.

Dose–effect curves based on translocations are also obtained in a similar way as those for dicentrics, with the dose–effect relationship being a function of the same equations (linear-quadratic for low-LET radiation, and linear for high-LET radiation) (Bothwell et al. 2000; Darroudi 2000; Rodríguez et al. 2004; Hande et al. 2005). The yield of translocations shows distinct dose–effect responses depending on conditions of exposure (early or long-term irradiations). At low doses ($\leq$1 Gy), most translocations are of simple nature for both acute and chronic exposures, while at higher doses (>2 Gy) complex aberrations predominate. The latter arise from multiple double-strand breaks (DSBs) followed by a multi-way exchange involving more than three chromosomes (Anderson et al. 2003; Camparoto et al. 2003).

Up to today, many studies have been conducted to validate the accuracy of translocations as a retrospective biomarker. Some issues have been cleared appropriately, while others still remain ambiguous. The use of stable CAs in cytogenetic dosimetry has some limitations:

- High background frequency of translocations (2–10 per 1,000 cells);
- Significant inter-individual variations, especially with age (>40 years);
- Lower range of dose detection than with dicentrics assay (0.25–2 Gy);
- Influence of environmental and lifestyle factors;
- Lack of standardization in the choice of chromosome pairs to be studied, as well as of the staining method

and nomenclature system to analyse and classify translocations, respectively.

Staining methods and nomenclature systems of stable aberrations

Initially, cytogenetic identification of translocations was carried out by banding techniques either using proteolytic enzymes (trypsina) in G-banding or denaturation/renaturation of chromatin techniques (Moquet et al. 2000).

As for dicentric studies, these methodologies are time-consuming, and data analyses require skilled personnel for recognizing both normal and modified longitudinal patterns of each chromosome pair (Tawn and Whitehouse 2005). Besides, banding analyses also present limitations when rearrangements involving small amounts of material or multi-way exchanges appear. Such events can hardly be detected, especially when banding and spreading of chromosomes are not adequate. As a result, the G-banding and denaturation/renaturation of chromatin techniques are not suitable for large-scale screening of populations (Tucker 1998).

However, an improvement in translocation analyses for cytogenetic dosimetry has been reached with the introduction of molecular cytogenetic methodologies, particularly fluorescent in situ hybridization (FISH). This staining technique is based on the use of DNA-specific fluorescent probes that bind to target chromosomes by complementary base pairing. Scoring of highlighted translocations is straightforward, less subjective and faster than with G-banding (Sorokine-Durm et al. 2000; Müller et al. 2005).

Most common laboratory practice is painting three of the larger chromosomes, representing about 20% of the genome. The total genomic translocation frequencies can be estimated according to a formula proposed by Lucas et al. (1992), which considers simple pair-wise exchanges (see Lucas and Deng 2000), as follows:

$$F_G = F_p/2.05f_p\left(1 - f_p\right) \quad (1)$$

where F_G is the full genome aberration frequency, F_p is the translocation yield detected by FISH and f_p is the fraction of the genome hybridized, taking into account the gender of the investigated subject.

Equation 1 is based on the hypothesis that the frequency of translocations observed for each chromosome is proportional to its DNA content. Thus, larger chromosomes would show a higher probability to undergo interaction with IR. A number of studies have shown contradictory results, either confirming Lucas' hypothesis or suggesting that specific chromosomes are more radiation sensitive than others. In this context, a recent methodology, the so-called spectral karyotype (or SKY), appears as an additional operational tool, providing specific fluorescent painting of all chromosome pairs. SKY permits detection of minimum damages and multiple chromosomal rearrangements, which could be useful for understanding of early biological effects associated with irradiation. As a result, "hidden aberrations" produced after high-level exposure may be detected (Braselmann et al. 2005; Zeljezic and Garaj-Vrhovac 2006).

Using fluorescence techniques, two methods allow a better description of events involved in chromosome exchanges than conventional terminology of routine cytogenetic scoring, including some unexpected and anomalous patterns, namely: PAINT (Protocol for Aberration Identification and Nomenclature Terminology), created by Tucker et al. (1995a, b), and the S&S System, performed by Savage and Simpson (1994).

Table 3 contrasts these two methods. Actually, these two systems are not mutually exclusive but they are complementary. The option for one or the other system depends on research purpose. For dose estimation, both methods have shown similar results, so that the choice of one of them is not crucial to biodosimetric measurements (Tucker 2008).

On the other hand, although translocations and dicentrics are widely studied for radiation protection purposes, a kind of by-product of chromosome damage has been proposed as an alternative tool for such investigation, namely the micronucleus.

Cytokinesis-block micronucleus (CBMN) assay

Unrepaired (or misrepaired) DNA lesions and chromosome malsegregation produced from mitotic malfunction (damaged kinetochores and spindle fibre defects) lead to loss of entire chromosomes or to acentric fragments. At the end of cell division, this "lost" genomic material appears as small nucleus-like particles, so-called micronuclei (MN) (Fenech et al. 1999).

The preferred method for assessing MN in lymphocyte cultures is to block cytokinesis using cytochalasin B. In the cytokinesis-block micronucleus (CBMN) assay, cells that have completed one or more nuclear divisions are blocked on cytokinesis phase, being visualized as bi- or trinucleated units (M1 and M2 cells, respectively), as illustrated in Fig. 3 (Fenech 2000).

The addition of cytochalasin B guarantees the control of lymphocyte cell division, avoiding non-homogenous response of these cells to a mitogenic stimulus. This method is important for dose estimations because the scoring can focus on binucleated cells only, excluding cells that passed successive divisions (e.g. M2, M3, M4) (Müller et al. 1996).

In order to establish standard laboratory procedures in MN analyses, the HUMN Project (The International

Table 3 Comparison of PAINT and S&S systems (modified from Savage and Tucker 1996; Knher and Bauchinger 2000)

Paint	S&S
Provides a descriptive analysis of aberrant chromosome patterns	Allows critical quantitative work, by investigation into exchange origins and their mechanisms of formation
Considers and classifies, individually, each abnormal painted chromosome or fragment, independent of any relationship with other painted signals in the cell	Considers and classifies each exchange as a whole, taking into account all abnormal painted signals in the cell, given a unique designation
Appropriate to any number of chromosome-specific probes	Is only applicable to single probes
Applicable with chromosome cocktails of same colour or multiple colours (the latter providing a sound classification)	Applicable with chromosome cocktails multi-coloured, only
Despite importance of centromeric identification, it is not indispensable for quantification studies	Centromeric identification is essential to reach accurate classification

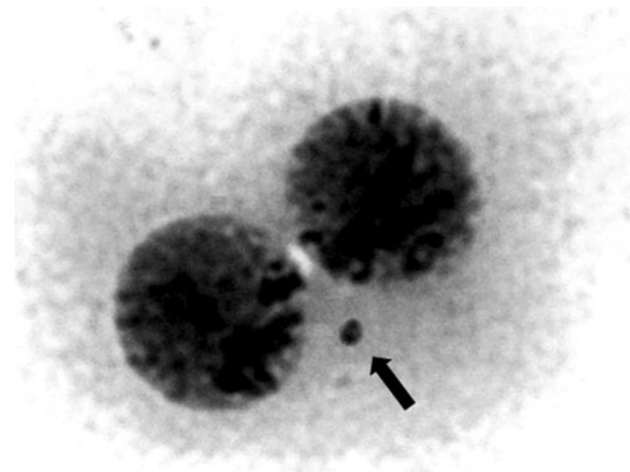

Fig. 3 Binucleated cell with a micronucleus marked by an *arrow*

Collaborative Project on Micronucleus Frequency in Human Populations) was initiated by Fenech et al. (1999). As part of this project, data were collected on:

- MN baseline frequencies in different populations and cell types;
- Influence of environmental and life-style factors on dose reconstruction;
- Suitability of MN as a biomarker of risk for cancer and other diseases.

In this project, a suggestive correlation between the genotoxicity of some agents, particularly IR, and the increase in MN frequencies in humans was found, which has been supported by further studies (Ramírez et al. 1999; Bonassi et al. 2007).

The standard criteria for selecting binucleated cells and for scoring micronuclei were described by Fenech (2000):

- Main nuclei should have intact nuclear membranes, be situated within the same cytoplasmic boundary and share similar features (e. g size, staining pattern and staining intensity);
- Nucleoplasmic bridges wider than one-fourth of the nuclear diameter are not acceptable;
- A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable;
- Cellular membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells;
- MN in human lymphocytes usually vary between one-sixteenth and one-third of the mean diameter of the main nuclei;
- MN can not be refractive or linked to the main nuclei;
- MN usually have the same staining intensity as the main nuclei, but occasionally staining may be more intense.

MN show considerable advantages compared to classical cytogenetic biomarkers (dicentrics and translocations) in terms of victims screening following high radiation exposure, because it is an easy and fast method of scoring of irradiated cells. However, unlike dicentrics, MN are not a radiation-specific biomarker (Silva-Barbosa et al. 2005; Fernandes et al. 2006) and can be induced, for example, by age and gender.

Still compared with dicentrics, the MN frequency is substantially lower, especially at high doses. As a consequence, it is necessary to increase the number of analysed cells in order to achieve a statistically significant dose–effect relationship. In this case, an experimental alternative to improve the mitotic index of first cycle metaphase was obtained by Paul et al. (1997) through synchronized culture method with addition of methotrexate. This approach induces a 2–3 times higher mitotic growth than obtained with conventional method.

Other critical points are the high spontaneous frequency of MN in non-irradiated population and the inter-individual variability, which results in a low specificity of this biomarker at low radiation doses (Thierens et al. 2000). As mentioned, the baseline frequency of MN in human lymphocytes (mean 7.8 MN per 1,000 cells) is strongly

influenced by factors as age (associated with loss of whole chromosomes), gender (women exhibit a higher MN frequency, due to excess micronucleation of sex chromosomes) and also by the effect of smoking habits and genotoxic agents (Norppa and Falck 2003; Joksic et al. 2004).

It is possible to distinguish spontaneous from radiation-induced MN using FISH with pancentromeric and pantelomeric probes. Predominantly, spontaneous MN contain whole chromosomes (centromeric-positive: C^{+}) with single chromatid, whereas radiation-induced MN contain acentric fragments (centromeric-negative: C^{-}) harbouring chromatid-type terminal fragments (Fig. 4) (Wojcik et al. 2000; Lindberg et al. 2008).

For high doses of radiation, the dose–response curve starts to level off at about 5–7 Gy and 3–4 Gy, for low- and high-LET radiation, respectively. This phenomenon is well known also for other cytogenetic endpoints, as for dicentrics, and it is interpreted as selection against heavily damaged cells which cannot enter mitosis, due to very severe damage resulting in apoptosis (Müller and Rode 2002).

Up to today, validation of MN assays as a triage tool in biological dosimetry has been quite successful at high doses (≥1 Gy) by scoring at least 200 binucleated cells (McNamee et al. 2009). However, it is not suited for assessing either partial-body exposure or whole-body exposures at low doses (<1 Gy), given its low sensitivity to IR (Wojcik et al. 2009).

In this context, the importance of scoring nucleoplasmic bridges (NPBs) should not be underestimated because it provides direct evidence of genome instability. Probably, NPBs result from non-disjunction of one or more dicentric chromosomes whose centromeres were pulled to opposite poles of the binucleated cells (Thomas et al. 2003; Fenech 2006).

A great advance of the CBMN assay as a “cytome” assay of chromosomal instability is the inclusion of cell parameters, such as viability, mitotic status and chromosomal instability, in evaluation exposure to cytotoxic and genotoxic agents. This new concept employs the use of centromeric probes and kinetochore antibodies for measuring chromosome breakage, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis. Furthermore, molecular tools also enable the cytological scoring of target cells in terms of their viability status, mitotic status and chromosomal instability or damage status (Fenech 2006; Duan et al. 2009).

Premature chromosome condensation (PCC) assay

Analyses of current biomarkers in cytogenetic dosimetry (dicentrics, translocations and micronuclei) are generally performed from metaphase chromosome preparations obtained through mitogenic stimuli in vitro. Traditionally, after lymphocyte cell culture (~48–72 h), the yield of chromosome-type aberrations or related by-products are scored for dose assessment (Prasanna et al. 1997). Those assays are characterized by a high detection limit of approximately 4 Gy. For higher doses, radiation-induced mitotic delay and cell death of irradiated cells overcome the yield of mitotic cells, causing underestimation of the deduced radiation dose (Prasanna et al. 1997).

By contrast, the PCC assay allows an accurate evaluation of damage at high doses after acute exposures. Analysis of radiation-induced lesions is performed on interphase cells, within a few hours (3–4 h) after blood sampling. This method also permits an accurate discrimination between total- and partial-body exposures to low- and high-LET radiation, and can be carried out in very low mitotic indices, such as in pathologic situations (IAEA 2001).

The induction of PCC occurs by fusion of isolated human lymphocytes with mitotic cells (e.g. Chinese hamster ovary–CHO) in the presence of a fusing agent (e.g.

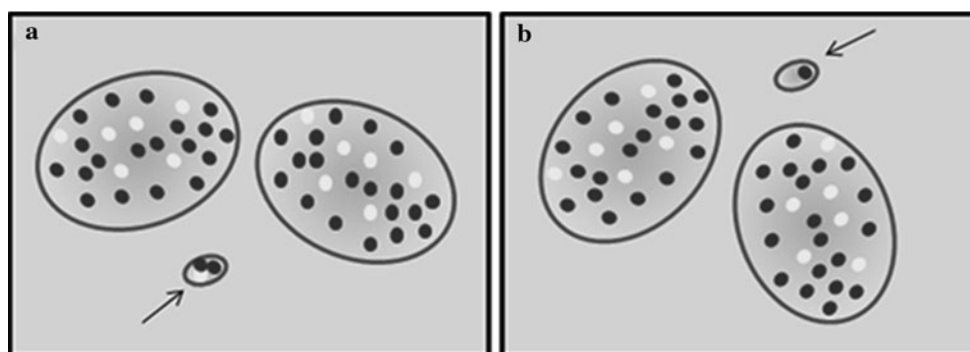

Fig. 4 Scheme of human lymphocytes painted with FISH using pancentromeric (*white*) and pantelomeric (*black*) probes. *Arrows* indicate labelled micronucleus: **a** radiation-induced MN (one centromere signal and two telomere signals) and **b** spontaneous MN (one telomere signal)

polyethylene glycol, okadaic acid, calyculin A, virus). Both fusion and chromosome condensation process depend on the chosen fusing agent: for example, in the presence of polyethylene glycol, mitotic CHO cells release a mitotic factor that acts on lymphocyte interphase cells promoting an early condensation (Gotoh and Durante 2006).

The morphology of chromosomes, in PCC assay, varies according to cell cycle position of the investigated lymphocyte at time of fusion: (1) G1-phase cells show a single chromatid; (2) S-phase cells include chromosomes with fragmented and pulverized appearance (3) G2-phase cells show double chromatids that are greatly extended and generally much longer than prometaphase elements (Ravi et al. 2007).

Similarly to dicentrics, choice of the staining chromosome technique applied for translocations and micronuclei depends on the purpose of research and, for the PCC assay, the biomarker to be analysed: radiation-induced chromosome breaks can be stained with the Giemsa or FPG techniques, while dicentrics can be visualized after pretreatment with C-banding.

A recent work performed by Lindholm et al. (2010) compared the dicentric (gold standard) with the PCC assay, in terms of cytogenetic dosimetry. This study showed that PCC rings are a better radiation-induced biomarker to evaluate high-dose individual exposure (>6 Gy), whereas dicentric assay is better suited for assessing whole-body low-dose estimates (0–6 Gy). Hence, the PCC assay is a suitable tool in cases of a massive radiological emergency when people may be exposed to high doses (5–50 Gy) and is crucial to identify those who will require medical care as early as possible (Emamchai et al. 2009; Wanga et al. 2009).

The principal features about biological dosimetry are summarized in the Table 4.

Molecular biomarkers: emerging tools for biodosimetry

With the advent of new technologies, a better comprehension of cellular mechanisms involved in the biological response to physico-chemical stresses has been achieved. As a result, several researches have been able to identify molecular markers that may reflect radiation-induced DNA damages, correlating them with inhibition, reduction and/or over-expression of an ever-expanding number of genes. Those early transcriptional responses may be useful to predict consequences for the cell that occur later after irradiation (surveillance, cell cycle arrest, apoptosis or necrosis) (Coleman et al. 2003; Dainiak et al. 2005). Below, some cell parameters suggested for estimating exposure to IR are briefly discussed, namely: glycophorin A (GPA), hypoxanthine guanine phosphoribosyltransferase (HPRT) and p53 protein.

Glycophorin A (GPA)

The autosomal *gpa* locus, mapped to chromosome 4q28-31, codes for the major abundant cell-surface sialoglycoprotein in human erythrocytes, named glycophorin A (GPA). This protein is present at about 5×10^5 copies per cell (Bigbee et al. 1998; Ha et al. 2002). The GPA gene occurs in two co-dominantly expressed allelic forms, gpa^M and gpa^N, which differ by two of 131 amino acid residues. The codified glycophorin A can be labelled with fluorescent monoclonal antibodies specific for the M and N allelic forms and analysed by flow cytometry (Bigbee et al. 1998). The GPA assay allows to identify the most common genotype, the $gpa^{M/N}$, which is present in 50% of all individuals, and to measure variant frequencies in cell types (N0, M0, NN and MM). Hemizygous N0 and M0 variants indicate the occurrence of point mutations and deletions, whereas the homozygous NN and MM variants indicate events such as chromosome mis-segregation, somatic recombination or gene conversion (Saenko et al. 2000; Ha et al. 2002).

The presence of a GPA mutant, especially of the N0 and NN variants, is a persistent indicator of past radiation exposure to high doses (≥1 Gy). Once these variants are released from damaged bone marrow stem cells to the circulating blood, gpa mutants are found after exposure even when the blood cells are replaced, unlike dicentrics and complex translocations (Ha et al. 2002).

Furthermore, the GPA assay exhibits several practical advantages: (1) it represents a cheap method, (2) only 1 ml of blood per subject is required, (3) the blood collected can be stored at refrigerator temperature (4°C) up to 1 week prior to analysis, (4) the employment of flow cytometry reduces time of analysis, allowing to study large populations and (5) it is not influenced by environmental parameters and life-style factors.

On the other hand, this biomarker shows a linear dose dependence, and a detailed mutation spectrum cannot be obtained, due to absence of nucleic acids in erythrocytes. Besides, GPA mutations have not been observed in low radiation exposures (Jones et al. 2001). Further limitations are as follows: (1) there is no in vitro system to calibrate GPA assays, (2) less than 50% of the population is M/N heterozygous and, therefore, is eligible for the assay, (3) it is not suitable to quantify low doses (<1 Gy) and (4) it shows a high inter-individual variation.

Considering the advantages and limitations of the GPA assay as a retrospective biomarker to radiation exposure, the International Commission on Radiation Units and Measurements (ICRU) concluded that the use of the GPA assay is more adequate in association with other biological indicators, to determine average doses in a large population exposed to a high radiation dose (Kleinerman et al. 2006).

Table 4 Principal features of biological dosimetry; ARS: acute radiation syndrome; IR: ionizing radiation

Procedure	Observed endpoint	Readout/Time of onset	Lower limit	Application	Advantages	Limitations
Clinical signals[a]	Vomiting Fever Headache	48 h	1–3 Gy	Triage of injured persons after radiological incident	Serve as a basis for sorting persons exposed to IR and deciding upon proper medical care	Manifestation of symptoms only arise at high doses Severity of injury depends on irradiation condition and radiosensitivity of involved tissues
Blood count[b]	Decreasing absolute lymphocyte count	24–72 h	0.5 Gy	Determination of radiation exposure in prodromal phase of ARS	Quickness of obtaining data Methodological simplicity	Strong influence of pathologic factors Significant inter-individual variability
Cytogenetic dosimetry	Dicentrics, rings and fragments[c]	48–51 h	0.1 Gy	Estimation of dose after weeks or few months	High specificity to IR Low background in non-exposed populations Low limit of dose in biodosimetry	Limited applicability in protracted and late exposures Selective removal of dicentrics along cell division Necessity of technician with good experience
	Translocations[d]	72–75 h	0.25 Gy	Estimation of dose after long period of time	Do not undergo negative selection during mitoses; can be used in cases of old or long-term irradiation	High background frequency Not recommended for persons older than 40 years Significant inter-individual variations Unclear dependence on lifestyle factors
	Micronucleus[e]	72 h	0.32 Gy	As a genetic toxicology testing and in biomonitoring of individuals exposed to genotoxic agents	Easy identification of micronucleus Does not require technician with long-lasting experience	High background frequency Dependence on genotoxic compounds and lifestyle factors Significant inter-individual variability
	Premature condensed chromosome[f]	3–4 h	4 Gy	Triage of injured persons after high level exposure to IR	Quickness of obtaining data Does not require mitogen stimuli in vitro Allows accurate discrimination between total- and partial-body exposures	Not suitable at low-dose range Cell analysis is more complicated than conventional lymphocyte cell culture

[a] IAEA and WHO 1998
[b] Waselenko et al. 2004
[c] Lloyd et al. 2006
[d] Tucker 2008
[e] Voisin et al. 2004b
[f] Lindholm et al. 2010

Hypoxanthine guanine phosphoribosyltransferase (HPRT)

The gene encoding HPRT is located on the Xq26 chromosome of mammalian cells and exhibits hemizygous features. This gene codes for a constitutive enzyme associated with purine metabolism that is not essential to survival of a cell (Bigbee et al. 1998). Mutant peripheral blood T lymphocytes do not express an active *hprt* gene product. These mutant clones can be scored and clonally expanded by selective growth (with or without 6-thioguanine) and have been used in studies of radiation-induced alterations, because genetic changes at this locus are tolerated (Bigbee et al. 1998).

For analyses of deletions and other molecular alterations, the multiplex polymerase chain reaction technique (multiplex-PCR) has been employed. In general, gene changes result in gene inactivation by mutations (~85%), while the remaining changes (15%) show larger structural modifications. By molecular analysis, the dose–response curves can be obtained in vitro for high- and low-dose rates and then used, for example, to estimate the radiation dose of persons exposed accidentally (Bigbee et al. 1998; Kumar et al. 2006).

Currently, it appears that the *hprt* locus frequently involves large-scale genomic rearrangements from rejoining of double-strand breaks (DSB). Considering that the presence of DSBs is associated with a negative selection of dicentric-bearing cells, this biomarker may be only indicated to evaluate an exposure that occurred a short time before analysis (Rothkamm et al. 2008).

In spite of the limited usefulness of the HPRT assay for detecting exposures involving high acute doses, the effectiveness of this biomarker is well documented for biodosimetric purposes (Jianlin et al. 2004; Rothkamm et al. 2008). Furthermore, the sensitivity of this assay can be improved by correcting for confounding factors that may influence the mutant frequency, as age and smoking habits (Kumar et al. 2006).

p53 protein expression level

The p53 gene is a tumour suppressor gene mapped to chromosome 17. The protein encoded by this gene binds to the DNA and acts as a transcription factor for several genes participating in the control of cell survival. After DNA damage, p53 activation can lead to cell cycle arrest and DNA repair or cell death. For this reason, this protein is essential for maintaining genome integrity (Lamb and Crawford 1986; Okorokov and Orlova 2009). In contrast, mutant p53 can no longer bind to the DNA in an effective way and, consequently, does not regulate suitably expression of its target genes. Cells bearing these changes cannot promote a correct repair or induce apoptosis, starting an anomalous process of cell division that may result in tumour formation (Bahl et al. 2000).

Under normal conditions, p53 is present in the cytoplasm in a low concentration and shows a short half-life (~6–20 min). By contrast, in response to stress signals produced by genotoxic agents, the p53 half-life increases from minutes to hours (~6 h). This immediately leads to a higher p53 concentration permitting its detection and correlation with biological and physico-chemical stress (Novellino et al. 2003; Rössner et al. 2004; Riley et al. 2008). Several studies have proposed the evaluation of changes in p53 levels as biomarker of individual exposure to IR (Lu-Hesselmann et al. 2006; Cavalcanti et al. 2008). As an example of the potential of this method in biodosimetry, Fig. 5 presents flow cytometry results of p53 protein expression levels of irradiated (a) and non-irradiated (b) blood samples from a healthy donor. The method of

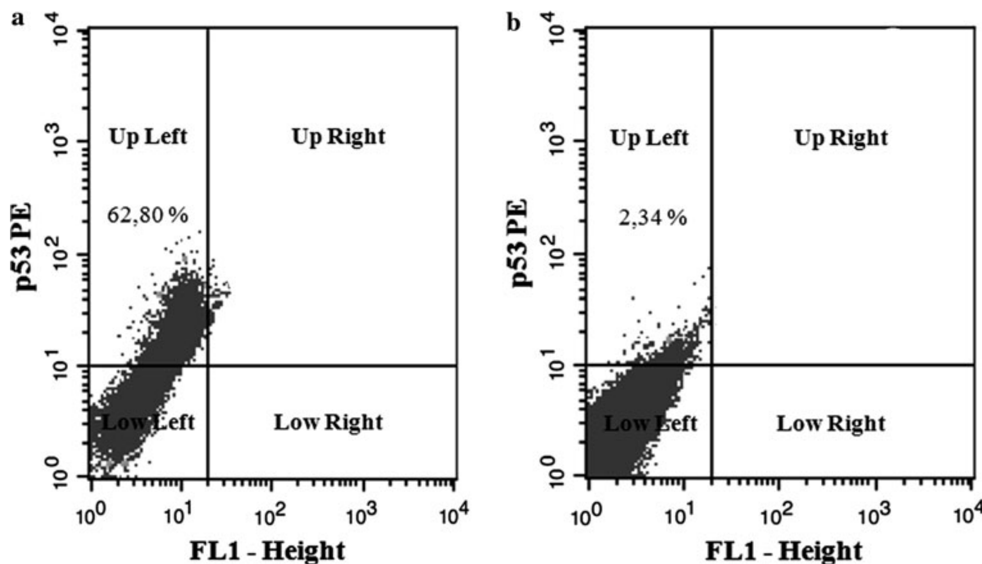

Fig. 5 Flow cytometry analysis of (**a**) samples irradiated with 4 Gy and (**b**) non-irradiated samples. Lymphocyte cells were marked with monoclonal anti-p53 antibody conjugated with phycoerythrin (PE), and the results are presented in a dot plot format. Cells positively marked for p53 are observed in the up left region. As dot plot is a graphic of bidimensional representation, FL1 channel was open but no fluorescence was read

analysis was performed according to Cavalcanti et al. (2008). The Figure 5 demonstrates that it is possible to verify the increase of this protein level with radiation dose, for a time period between blood sampling and data acquisition of about 6 h, for the data presented. Considering that, in general, cytogenetic dosimetry is time-consuming, requiring days or even weeks to provide accurate data, the methodology proposed by Cavalcanti and co-workers has high potential as a rapid screening method in case of a large-scale nuclear incident. However, further studies must be carried out to evaluate the usefulness of the method considering inter-individual differences (Amaral et al. 2008).

Final considerations

Cytogenetic dosimetry is a powerful tool for evaluating individual radiation-induced risks, complementary or alternative to physical dosimetry, having dicentric assays as the gold standard. Several emerging technologies, such as flow cytometry and multiplex-PCR, have motivated the investigation into new molecular biomarkers for rapid dose assessment. These new approaches may allow to better predicting health consequences after individual overexposure by taking into account individual radio-sensitivity and thus are very promising for biodosimetric purposes. Obviously, these new methods demand further studies including a large number of subjects in terms of in vivo response, inter-individual variability and radiation specificity. Besides, as those biological indicators belong to a recent branch in the biodosimetry field, it is essential to investigate their usefulness as biological signatures of radiation exposures in terms of radiation quality (low and high-LET), dose rate, irradiation conditions (whole- and partial-body) and environmental and individual life-style parameters.

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