The micronucleus assay in radiation accidents

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Summary. The cytokinesis-block micronucleus assay in peripheral blood lymphocytes is a standardised and validated technique for biodosimetry. Automated scoring of micronuclei allows large scale applications as in population triage in case of radiation accidents or malevolent use of radioactive sources. The dose detection limit (95% confidence) of the micronucleus assay for individual dose assessment is restricted to 0.2 Gy but can be decreased to 0.1 Gy by scoring centromeres in micronuclei using fluorescence in situ hybridization (FISH). In the past the micronucleus assay was applied for a number of large scale biomonitoring studies of nuclear power plant workers and hospital workers. Baseline micronucleus frequencies depend strongly on age and gender. The assay was also already used for biodosimetry of radiation accidents. In a multiple endpoint biodosimetry study for dose assessment of a worker exposed accidentally in 2003 to X-rays, a good agreement was obtained between dose estimates resulting from the micronucleus assay, the scoring of dicentrics and translocations. Automated scoring of micronuclei in combination with centromere signals, allowing systematic biodosimetry of exposed populations, remains a challenge for the future.

Key words: micronucleus assay, biodosimetry, radiation accidents.

Riassunto *(Il test dei micronuclei negli incidenti radiologici)*. Il test dei micronuclei, basato sul blocco della citochinesi, effettuato su linfociti da sangue periferico, è una tecnica standardizzata e validata per la biodosimetria. Lo *scoring* automatico dei micronuclei permette applicazioni su larga scala come ad esempio il triage in caso di incidenti radiologici o di uso malevolo di sorgenti radioattive. Il limite di rivelazione (livello di confidenza 95%) del test dei micronuclei per la valutazione della dose individuale è limitato a 0.2 Gy, ma può essere significativamente ridotto (0.1 Gy) con lo *scoring* dei centromeri nei micronuclei usando la *fluorescence in situ hybridization* (FISH). Il test dei micronuclei è stato applicato in passato a un gran numero di studi di monitoraggio biologico di lavoratori di impianti di produzione di energia nucleare e di operatori sanitari. Le frequenze di fondo di micronuclei dipendono fortemente dall'età e dal genere. Questo saggio è già stato usato per la biodosimetria di incidenti radiologici. In uno studio di biodosimetria su endpoint multipli per la valutazione della dose di un lavoratore esposto nel 2003 in modo accidentale a raggi X è stato raggiunto un buon accordo tra le stime di dose ottenute dal test dei micronuclei, lo *scoring* con dicentrici e le traslocazioni. Lo *scoring* automatizzato di micronuclei in combinazione con i segnali dei centromeri, che permettono una biodosimetria sistematica delle popolazioni esposte, rimane una sfida per il futuro.

Parole chiave: test dei micronuclei, biodosimetria, incidenti radiologici.

THE MICRONUCLEUS ASSAY AS BIOLOGICAL DOSIMETRY TOOL

The *in vitro* cytokinesis-block micronucleus (CBMN) assay, developed by Fenech and Morley in 1985, is a reliable method to quantify chromosome breakage and loss in nucleated cells [1, 2]. Micronuclei (MNi) can be the result of small acentric chromosome fragments that are not incorporated into the daughter nuclei during cell division. They are enveloped by a nuclear membrane and appear as small nuclei – micronuclei – in the cytoplasm outside the main daughter nuclei. They arise during exposure to various clastogenic agents and are the result of non- or misrepaired DNA double strand breaks. MNi can also contain whole chromosomes that lag behind at anaphase during nuclear division and by consequence are not incorporated in the main nuclei. These MNi arise during exposure to aneugenic agents and represent also the main fraction of spontaneously occurring MNi. In the CBMN assay the scoring of MNi is restricted to once-divided cells, which are recognised by their binucleate (BN) appearance after inhibition of cytokinesis by cytochalasin B. This restriction prevents confounding effects caused by variable cell division kinetics [1]. In *Figure 1* an example is given of binucleate cells with and without a micronucleus.

Because of its good reliability and reproducibility, the CBMN assay has become one of the standard cytogenetic techniques for genetic toxicology testing in human and mammalian cells in general [3]. In the field of radiation protection, the CBMN assay for

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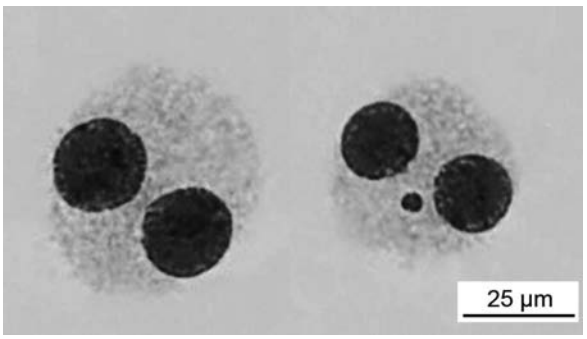

Fig. 1 | *Example of binucleated cells without and with one micronucleus.*

peripheral blood lymphocytes (PBL) is an appropriate biological dosimetry tool to evaluate *in vivo* radiation exposure of occupational, medical and accidentally exposed individuals and to assess *in vitro* radiosensitivity and cancer susceptibility [4-19]. Many studies showed that the number of radiation induced MNi is strongly correlated with radiation dose and radiation quality [20-25].

AUTOMATED SCORING OF MNi

Compared to the dicentric assay, which is the standard technique for biological dosimetry [26], the easy and rapid scoring of MNi makes this method very attractive for 1) large scale assessment of genetic damage in radiation workers receiving a high radiation burden and, 2) for population triage in case of large scale radiation accidents. Automation of the MN scoring further results in a more reproducible analysis of MN frequencies due to withdrawal of the subjective MN identification by technical staff. Several algorithms for automated image analysis of the CBMN assay were already developed in the nineties [27, 28]. These systems however showed limitations such as a relative high inaccuracy in classification of the BN cells. More recently, new and better automated image analysis systems for the CBMN assay have been developed. The MN software module for the Metafer 4, developed and commercialised by Metasystems, identifies a BN cell by the occurrence of two similar nuclei, close to each other but completely separated. In a second step, MNi are counted in a circular area defined around the two nuclei of the BN cell [29, 30]. The system developed by Decordier *et al.* (2008) [31] for use in biomonitoring of *in vivo* exposure to mutagenic agents, uses a PathFinder™Cellscan™ capture station and two MN analysis workstations. This system identifies firstly the cytoplasm of cells, subsequently detects the number of nuclei in the cell thus allowing identification of BN cells and in a third step it scores the MNi. However, this system still requires visual validation and processes only 12 slides a day. The study performed recently by our research group [32], using the Metasystems software, demonstrates the suitability and advantage of automated MN scoring for population triage in case of large scale radiation accidents, where it is important to distinguish severely exposed individuals (≥ 1 Gy), who require early medical follow up and treatment, from those less exposed. In our study, the MN frequencies scored automatically were highly correlated with the manual MN scores ($r^2 = 0.917$) and a visual validation was not needed [32]. The reference dose response curve obtained for automated MN scoring, based on MN data of 10 individuals, showed that the uncertainty on a dose determination of 1 Gy amounts to 0.25 Gy. The 95% confidence intervals of the 0 Gy and 1 Gy doses do not overlap. Accurate dose estimations were also achieved at the higher doses (2 and 3 Gy) investigated.

In the study it was estimated that 2 technicians can process at least 60 blood samples (120 slides) in a 12 hour shift [32]. The restriction here is the number of slides that can be scored automatically with a Metafer 4 system equipped with a slide feeder in one day. Automation of the MN scoring further offers the advantage that, when different laboratories, equipped with a Metafer 4 platform, use the same fixation protocol and MN classifiers, very comparable results can be obtained. Before the automated MN assay can be implemented as a triage tool, it should be further validated using data obtained in an international network setting.

THE MICRONUCLEUS-CENTROMERE ASSAY

The sensitivity of the micronucleus assay is limited to 0.2 Gy. This is due to the relatively high and variable spontaneous MN yield that is an inherent shortcoming of the MN assay for low dose estimation. It is well known that most of the radiation induced MNi originate primarily from acentric fragments while spontaneous MNi contain especially whole chromosomes. By using a pancentromeric probe and applying fluorescence in situ hybridization (FISH) one can distinguish between MNi containing acentric fragments and those containing whole chromosomes [6, 33-36]. By applying this method, the sensitivity of the MN assay can be substantially increased in the low dose range [6, 35]. In both studies, using the pancentromeric p82H probe, the majority of spontaneous MNi were centromere positive (MNCM+) (resp. 73 and 71%). Most radiation induced MNi were centromere negative (MNCM-) while the number of MNCM+ only showed a very small increase with dose (resp. 3.7 and 5.3 MNCM+ per Gy per 1000 BN cells). By manual scoring of MNCM- in 2000 BN cells a detection limit, at the 95% confidence limit, of 0.1 Gy can be achieved. According to the results of Algunes *et al.* [36], this detection limit is even lower: 0.05Gy. The CBMN-centromere assay requires more time (2000 BN cells in half a day) and effort compared to the conventional CBMN assay. The development of an au-

tomated "CBMN-centromere" scoring procedure, which is under progress, will reduce scoring time. Such an automated CBMN-centromere assay will allow systematic biomonitoring of radiation workers exposed to low doses.

APPLICATION OF THE MICRONUCLEUS ASSAY FOR BIOMONITORING

The micronucleus assay was validated as *in vivo* radiation biomonitor by our research group in a study of patients treated with large field radiotherapy for cervical cancer or Hodgkin's disease [4]. After this validation stage the micronucleus assay was applied for large scale biomonitoring of nuclear power plant (NPP) workers and hospital workers [5-9]. For the first study (1996) the CBMN assay with manual scoring of 1000 BN cells was used to assess genetic damage in 269 nuclear power plant workers exposed occupationally to accumulated doses ranging from 10 to 248 mSv [5]. In the second study (1999) the MN-centromere assay was used to evaluate the clastogenic and aneuploidogenic actions of radiation in 111 radiation workers exposed to doses ranging from 10 to 104 mSv over the last ten years versus a matched control group of 104 members of administrative staff [7]. The third population under study (2000) comprised 71 hospital workers, exposed to ionising radiation as doctors, technicians or nurses of Departments of Radiology, Radiotherapy, Nuclear Medicine. Cytogenetic monitoring with the MN-centromere assay was performed for this population versus a matched control group of nurses and doctors working in the Department of Pediatrics [8]. In the study of the year 2002 the application of the micronucleus assay as radiation susceptibility biomarker was investigated in a population of 99 nuclear power plant workers [9].

These large scale studies led to a number of general conclusions. The spontaneous micronucleus yield is increasing systematically with age. For a male population values of 0.35 MNi/year and 0.44 MNi/year were obtained respectively in the NPP and hospital workers studies [5, 8]. These values are in agreement with the large scale study of Fenech of variables influencing baseline micronucleus frequencies: 0.31 MNi/year [37]. For the female population of the hospital worker study [8] an increase of 0.58 MNi/year was found, again in agreement with Fenech: 0.52 MNi/year [37]. The increase is thus more prominent for a female population. Detection of centromeres showed that the age increase of baseline MN frequencies can be attributed almost totally to centromere-positive MN reflecting an increased chromosome loss with age. The X-chromosome is almost completely responsible for this spontaneously occurring chromosome loss [38, 39]. This explains also the gender difference in spontaneous MN frequencies observed in the performed studies: for a population with mean age 41.4 and 41.8 years the mean spontaneous MN frequency amounts to 16.4 for males and 23.5 for females respectively. On the contrary the difference in centromere-negative MNi is not significant: 6.7 *vs* 7.7. In the performed studies a restricted number of individuals (about 1-2%) showed very high MN yields (40-70 MNi/1000 BN cells). Repeated sampling confirmed these high values. Centromere staining showed that the high MN yields are almost completely centromere-positive MNi. The studies performed by our research group could not demonstrate a clear smoking effect on the MN frequency. Literature data regarding this point are contradictory [40].

The monitoring studies of radiation workers allowed to investigate the dependence of MNi of the accumulated dose over the 10 years preceding the venepuncture. In the first study (1996) a linear regression of the individual micronucleus frequencies, corrected for the age effect, showed an increase of 0.0175 MNi/mSv with Pearson correlation coefficient value of 0.10. Application of the micronucleus-centromere assay in the second study of radiation workers resulted in almost the same increase of MNi with dose, 0.025 MNi/mSv and demonstrated that this dose dependence is completely due to MNCM-pointing to the clastogenic action of ionising radiation. Dose dependence of MNi in an occupational exposure setting was also studied by Vaglenov *et al.* [41]. They reported an increase of 0.03 MNi/mSv. Large scale biomonitoring studies show that the micronucleus assay is able to demonstrate genetic damage at population level for accumulated doses received occupationally exceeding 50 mSv.

APPLICATION OF THE MICRONUCLEUS ASSAY IN RADIATION ACCIDENTS

In Belgium the research group applies systematically the conventional micronucleus assay for workers exceeding the 20 mSv dose limits, imposed legally for occupationally exposed workers. The prescription for this examination is given by the occupational medicine service of the worker, a blood sample is taken and sent to our department. The procedures followed for biological dosimetry in case of overexposures are described in detail in [10]. For the determination of the dose corresponding to the scored micronucleus frequency, Y_{MN}, the laboratory's dose response curve with age dependent background level MN_{BG} is used ($Y_{MN} = MN_{BG} + 49.946\ 10^{-3}D + 41.402\ 10^{-3}D^2$ with Y_{MN} the micronucleus frequency and D the dose). The α- and β-coefficients of this relation are derived from the dose response of the *in vitro* X-ray induced MNi averaged over 10 donors [42]. For a 40 year old worker MNBG is adopted to be $1.58\ 10^{-2}$ for males and $2.25\ 10^{-2}$ for females [8]. For the age dependence of MN_{BG} values of 0.44 MNi/y and 0.58 MNi/y are adopted for males and females respectively [8]. The determination of the dose range corresponding to the 95% confidence interval is based on the interindividual differences among 10 donors in dose response of the *in vitro*

Fig. 2 | *Dose range (Y-axis) according to the 95% confidence level corresponding to a dose estimate derived from MNi scored as described in present work (X-axis).*

radiation induced MNi, published earlier [42]. For the uncertainty on MN_{BG} at the 95% confidence level (1.96 SD) for the reference age of 40 years a value of 1.19 10^{-2} is deduced from the data published earlier by adjusting the micronucleus data for the age dependence [8]. Combining the uncertainties on dose response and MN_{BG} allows the determination of the minimal and maximal dose levels (95% confidence limits) corresponding to the dose estimate derived from Y_{MN} using the mean dose response, given above. The result of this analysis is depicted graphically in *Figure 2*.

In 2003 this biological dosimetry protocol was applied for retrospective assessment of the dose received by a hospital worker exposed accidentally by a 50 kV contact radiotherapy X-ray device during maintenance. A dose estimate of 0.73 Gy was obtained with 95% confidence limits 0.54-0.96 Gy. Scoring of dicentrics resulted in a dose estimate of 0.62 Gy (range 0.45-0.90 Gy), in very good agreement with the micronucleus assay dose. The skin injury at the back of the worker indicated that the worker accidental overexposure was a partial body irradiation. From the overdispersion of the dicentrics data we could deduce that a fraction of 49% of the body was irradiated. It was not possible to apply this type of analysis to the micronucleus assay data to determine the fraction of the body exposed as those data always display an overdispersion even in the case of a total body irradiation. To study the decrease of micronucleus frequency with time post-exposure a second blood sampling was performed one year after the first one. MNi disappear with a half-time of 342 days, very close to the value of 377 days, obtained for dicentrics. This result is in agreement with the decline in the micronucleus frequency with post-irradiation time down to about 60% after 1 year post-treatment, observed in radiotherapy patients [43].

CONCLUSIONS

The CBMN assay is a valuable technique of biological dosimetry, thoroughly validated and standardized. In view of the reliable automated scoring, worked out recently, the assay is of special interest for large scale applications as population triage. Scoring of centromeres by FISH increases the sensitivity significantly. Automated scoring of MNi in combination with centromere signals remains a challenge for the future.

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