Mutation Research - Genetic Toxicology and Environmental Mutagenesis 896 (2024) 503753

Contents lists available at ScienceDirect

Mutation Research - Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Chromosome-specific induction of micronuclei and chromosomal aberrations by mitomycin C: Involvement of human chromosomes 9, 1 and 16

Julia Catalán [a,b], Hilkka Järventaus [a], Ghita C.-M. Falck [a], Carlos Moreno [b], Hannu Norppa [a,*]

[a] Finnish Institute of Occupational Health, Box 40, Työterveyslaitos, Helsinki 00032, Finland
[b] Department of Anatomy Embryology and Genetics, University of Zaragoza, Zaragoza 50013, Spain

ARTICLE INFO

Keywords:
Mitomycin C
Chromosome 1
Chromosome 9
Chromosome 16
Micronucleus
Chromosomal aberrations

ABSTRACT

Cytogenetic studies have shown that human chromosomes 1, 9, and 16, with a large heterochromatic region of highly methylated classical satellite DNA, are prone to induction of chromatid breaks and interchanges by mitomycin C (MMC). A couple of studies have indicated that material from chromosome 9, and possibly also from chromosomes 1 and 16, are preferentially micronucleated by MMC. Here, we further examined the chromosome-specific induction of micronuclei (MN; with and without cytochalasin B) and chromosomal aberrations (CAs) by MMC. Cultures of isolated human lymphocytes from two male donors were treated (at 48 h of culture, for 24 h) with MMC (500 ng/ml), and the induced MN were examined by a pancentromeric DNA probe and paint probe for chromosome 9, and by paint probes for chromosomes 1 and 16. MMC increased the total frequency of MN by 6–8-fold but the frequency of chromosome 9 -positive (9^+) MN by 29–30-fold and the frequency of chromosome 1 -positive (1^+) MN and chromosome 16 -positive (16^+) MN by 12–16-fold and 10–17-fold, respectively. After treatment with MMC, 34–47 % of all MN were 9^+, 17–20 % 1^+, and 3–4 % 16^+. The majority (94–96 %) of the 9^+ MN contained no centromere and thus harboured acentric fragments. When MMC-induced CAs aberrations were characterized by using the pancentromeric DNA probe and probes for the classical satellite region and long- and short- arm telomeres of chromosome 9, a high proportion of chromosomal breaks (31 %) and interchanges (41 %) concerned chromosome 9. In 83 % of cases, the breakpoint in chromosome 9 was just below the region (9cen-q12) labelled by the classical satellite probe. Our results indicate that MMC specifically induces MN harbouring fragments of chromosome 9, 1, and 16. CAs of chromosome 9 are highly overrepresented in metaphases of MMC-treated lymphocytes. The preferential breakpoint is below the region 9q12.

1. Introduction

Although micronuclei (MN) are widely used as an indicator of genotoxic effects, the mechanisms of micronucleus formation are only partly understood [1]. Studies utilizing fluorescence *in situ* hybridization (FISH) with centromeric DNA probes have indicated that micronuclei consist of either whole chromosomes or chromosomal fragments [1,2]. However, little is known about the contribution to MN of fragments from specific chromosomes after mutagen treatment.

Mitomycin C (MMC), a natural antitumor antibiotic and alkylating cytostatic drug, is one of the few chemicals described to have a specific effect on the micronucleation of some human chromosomes [3–5]. MMC, inducing DNA interstrand cross-links (ICL) through an N-alkylation reaction [6], is genotoxic in various organisms and has been classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer [7] and as a genotoxic carcinogen (DEC95) [8]. MMC is one of the recommended positive controls in several Organisation for Economic Co-operation and Development genotoxicity test guidelines, e.g., OECD 487 (*In Vitro* Mammalian Cell Micronucleus Test) [9].

In cytogenetic studies, MMC primarily produces chromatid-type aberrations, especially chromatid interchanges, including exchanges between homologous chromosomes [10]. It has been known for a long time that the pericentromeric region of human chromosome 9 is

* Corresponding author.
E-mail address: hannu.norppa@ttl.fi (H. Norppa).

https://doi.org/10.1016/j.mrgentox.2024.503753
Received 26 January 2024; Received in revised form 20 March 2024; Accepted 26 March 2024
Available online 29 March 2024
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sensitive to chromosome aberration (CA) induction by MMC; in addition, chromosomes 1 and 16 have frequently been seen in MMC-induced CAs, with breakpoints in the C-bands of the chromosomes involved [4,10–18]. MMC has been observed to induce chromosomal breakage and under-condensation (also called attenuations or lateral extensions) preferentially in the pericentromeric heterochromatin blocks at 9q12, 1q12, and 16q12 [10,18]. Pericentromeric heterochromatin contains GC-rich repeats and harbours highly methylated DNA, which are more prone to induction of DNA lesions by MMC [10]. The MMC-induced chromatid-type aberrations of chromosome 9 may develop into chromosome-type rearrangements when the cells further divide; 70–118 h after a G_0 treatment with MMC of human lymphocytes, Kusakabe et al. [3] observed high rates of translocations (involving the exchange of the entire sidearm of chromosome 9 with another chromosome) and centric fragments (including the whole arm length of 9p). According to Abdel-Halim et al. [10], 97 % of the breakpoints in chromosomes 9 and 1 occurred in the paracentromeric heterochromatic bands 9q11–13 and 1q11–12. There are some data to suggest that, besides its clear clastogenicity, MMC can also have some aneugenic activity [10,18,19].

Only a few studies have examined the chromosomal content of MMC-induced MN in humans [3–5,20,22]. Kusakabe et al. [3] and Hovhannisyan et al. [5] reported that in human lymphocytes MMC preferentially induced MN positive for chromosome 9 but not for chromosome 9 α-satellite or centromere, indicating these MN contained acentric fragments. The latter study [5] also reported an over-representation of material from chromosome 16 in MMC-induced MN, but the observed number of MN did not statistically significantly differ from the number expected on the basis of chromosome length. Fauth et al. [4] noticed that MMC-induced MN predominantly contained material from chromosome 9; in one of the two donors, also material from chromosome 1 was twice as common in MMC-induced MN as in the control, but the finding was not statistically significant. However, it was not possible to elucidate, whether the observed MN contained an entire chromosome or chromosomal fragments [4].

In the present study, we have further investigated the involvement of human chromosomes 9, 1 and 16 in MMC-induced MN by FISH, using a paint probe for chromosome 9 together with a pancentromeric probe, and paint probes for chromosomes 1 and 16, in cultured lymphocytes. We have also characterized MMC-induced CAs by using the pancentromeric DNA probe and probes for the classical satellite region and long- and short-arm telomeres of chromosome 9, to better define the MMC-specific breakpoint in chromosome 9.

2. Materials and methods

2.1. Cell cultures and slide preparation

The blood samples used in the studies were donated by two healthy male donors (42 and 50 years old) who have earlier served as blood donors in several earlier in vitro MN and CA studies carried out in our laboratory and shown good lymphocyte growth and low baseline frequencies of MN and CAs. A written informed consent was obtained from both subject before the sampling. The study was conducted in accordance with the Declaration of Helsinki and approved by the Coordinating ethics committee of the Hospital District of Helsinki and Uusimaa (HUS) Joint Authority (Helsinki, Finland). Mononuclear leukocytes were isolated from the blood samples as previously described [23] and incubated at 37C in RPMI 1640 medium (Gibco, Paisley, Scotland, UK) containing 1 % L-glutamine (Gibco), 15 % fetal bovine serum (Gibco), 1 % penicillin-streptomycin (Gibco) and 1 % phytohemagglutinin (Remel, Kent, UK). Cultures were established at an initial cell density of 0.3×10^6 cells/ml, treated with mitomycin C (500 ng/ml; Sigma, St Louis, MO, US) for the last 24 h of incubation, and harvested at 72 h. Parallel control cultures without mitomycin C treatment were also established from the same individuals. All cultures were done in duplicate.

For the analyses of micronuclei, cells were cultured with and without 6 μg/ml of cytochalasin-B (Cyt-B; Sigma, St Louis, MO, US), because earlier studies have indicated that the use of Cyt-B reduces the micronucleation of acentric fragments [2]. When used, Cyt-B was added for the last 24 h of culture. The cells were harvested by a 2-min hypotonic treatment (RPMI:water = 1:1), followed by two fixations in methanol: acetic acid (3:1) and one final fixation in methanol:acetic acid (9:1). For the analyses of CAs, Colcemid (0.15 μg/ml; Gibco) was added for the last 2.5 h. After that, the cells were treated with 0.56 % KCl and fixed 3 times with methanol:acetic acid (3:1). After the fixation, the cells were dropped onto microscopic slides, which were air-dried and stored at −20 ℃.

2.2. FISH and chromosome-painting

The slides were pretreated with a mild pepsin solution (Sigma; 10 μg/ml in 0.01 N HCl, pH 3.0) at 37 °C for 15 min and washed briefly in distilled water and phosphate-buffered saline (PBS; Cambrex, Verviers, Belgium). Then, they were postfixed in 1 % (v/v) formaldehyde at 4 °C for 5 min, washed with PBS, and dehydrated in an increasing series of ethanol. The DNA of the cells was denatured in 70 % formamide/2xSSC (20xSSC: 173.3 g NaCl, 88.2 g sodium citrate and 1 l distilled water, pH 7.0) at 70 ℃ for 2 min, and the slides were dehydrated and allowed to dry. Directly conjugated DNA probes were prewarmed at 37 ℃ for 5 min, mixed according to the target analysis, denatured for 10 min at 85 °C and chilled on ice. Each slide received 10 μl of the probe mixture and was then coated with a glass coverslip, sealed and hybridized overnight at 37 ℃ in a moist chamber. On the following day, the slides were washed in 0.5xSSC at 72 ℃ for 5 min, in 0.25xSSC at 72 ℃ for 5 min (only for the micronuclei analyses), and in 1xPBD (Qbiogene RIST1370, Ohio, US) at RT for 5 min. The slides were then stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, washed in tap water, air dried, and mounted in antifade solution (Vectashield; Vector, Burlingame, CA, US). The techniques used in FISH and chromosome painting were partly based on our earlier studies [23] and on the instructions of the probe manufacturers.

For the analyses of micronuclei, parallel slides were separately hybridized either with a fluorescein isothiocyanate (FITC) -direct conjugated human pancentromeric probe (Star*Fish 1141-F; Cambio, Cambridge, UK) plus the chromosome 9 paint probe, direct red (Appligene-Oncor CP5609-R, Illkirch, France), or with the chromosome 1 paint probe, direct red (Appligene-Oncor CP5601-R, Illkirch, France) plus the chromosome 16 paint probe, direct green (Star*Fish 1083–16 F; Cambio, Cambridge, UK). In the first case, the pancentromeric probe was denatured at 85 ℃ for 10 min, whereas the denaturation of the chromosome 9 paint probe was done at 72 ℃ for 5 min and held at 37 ℃ for 30–60 min. Then, the probes were combined before use. In the second case, the chromosome 1 and 16 paint probes were mixed, and the probe mixture was denatured at 72 ℃ for 10 min and held at 37 ℃ for 30–60 min

For the analyses of CAs, the following probe mixture was used per slide: 7 μl of the FITC-direct conjugated human pan-centromeric probe (Star*Fish 1141-F; Cambio, Cambridge, UK), 1 μl of chromosome 9 classical satellite DNA probe, D9Z1, direct red (Appligene-Oncor D5016-R, Illkirch, France), 1 μl of chromosome 9p telomeric probe, direct red (Appligene-Oncor CP5417-R, Illkirch, France) and 1 μl of chromosome 9q telomeric probe, direct green (Appligene-Oncor CP5418-G, Illkirch, France). Then, the probe mixture was denatured at 90℃ for 10 min and hybridization was performed as described above.

2.3. Microscopy

The slides were coded and were scored by one microscopist under a Zeiss Axioplan 2E Universal fluorescence microscope (Zeiss, Jena, Germany) equipped with epifluorescence and filter blocks Set 49, Set 44 and Set 20 suited for the observation of DAPI, FITC and TRITC, respectively. Furthermore, a triple filter for the simultaneous observation of the three

fluorochromes was applied.

The analysis of the frequency of MN was scored on slides simultaneously used for the analysis of all centromeres and chromosome 9. For each replicate culture, the scoring was continued until (i) the contents of 100 MN, identified by DAPI fluorescence, had been checked for the presence of green (all centromeres) and red (chromosomes 9) signals, or (ii) until 2000 cells had been accumulated. In the latter case, the presence of all centromeres and chromosome 9 was still continued until 100 MN had been checked. On parallel slides, further 100 MN per replicate culture was analysed for the presence of chromosomes 1 (red signal) and 16 (green signal), separately with and without Cyt-B.

The frequency of MN in the MMC treatment was based on 10,959 cells (8365 and 2594 cells per donor) without Cyt-B and on 9490 binucleate cells (4980 and 4510 binucleate cells per donor) with Cyt-B.

Eventually, we had data on 400 MN (200 per donor) for each of the eight experimental points: controls with and without Cyt-B and MMC treatment with and without Cyt-B both in the study of centromeres and chromosome 9 and in the study of chromosomes 1 and 16. Altogether, 3200 MN were inspected for the presence of pancentromeric signals and chromosomes 9 or chromosomes 1 and 16.

CAs were analysed in 100 well-spread metaphases per subject and treatment. Therefore, 400 metaphases were analysed in total. Whenever chromosome 9 was involved, it was checked whether the aberration breakpoints were located in the band of the painted chromosome or in the pancentromeric region common to every human chromosome.

2.4. Statistical evaluation

An analysis of variance (ANOVA) was used to analyse associations between the percentages and frequencies of MN and the explanatory variables: MN method (cultures with and without Cyt-B), treatment (control *vs* MMC), individual (two subjects), and interactions among them. On the other hand, ANOVA was also used for analysing the number and type of CAs, excluding the method variable, as Cyt-B was not used in this assay.

To check whether chromosomes were broken or micronucleated more or less often than randomly expected, a Chi-square test was used to compare the observed values with the expected ones. For any whole autosome, the expected random probability of being micronucleated would be 2/46, whereas the expected random probability of being broken (giving rise to fragment-containing MN as well as chromosomal breaks and exchanges) would be equal to the length of such chromosome divided by the length of the total human genome. To do the corresponding calculations, the physical lengths of human chromosomes (in megabases) were taken from Morton [24].

Chromosome 9 signals were identified simultaneously with the pancentromeric signals, which allowed distinguishing MN harbouring whole chromosome 9 from MN harbouring an acentric fragment of chromosome 9. As such differentiation was, however, not possible for chromosomes 1 and 16, MN containing positive signals for these chromosomes could have represented acentric fragments or whole chromosomes. Thus, the corresponding expected random value of being produced was calculated in the following way:

[%C^- MN x (length of the chromosome/length of the total genome)] + (%C^+ MN x 2/46),

where C^- and C^+MN mean centromeric signal negative- and centromeric signal positive MN, respectively.

All the analyses were performed by the Harvey program (Harvey W*R*, 1987; LSMLMW Computing procedures and applications. Columbus, Ohio 43210).

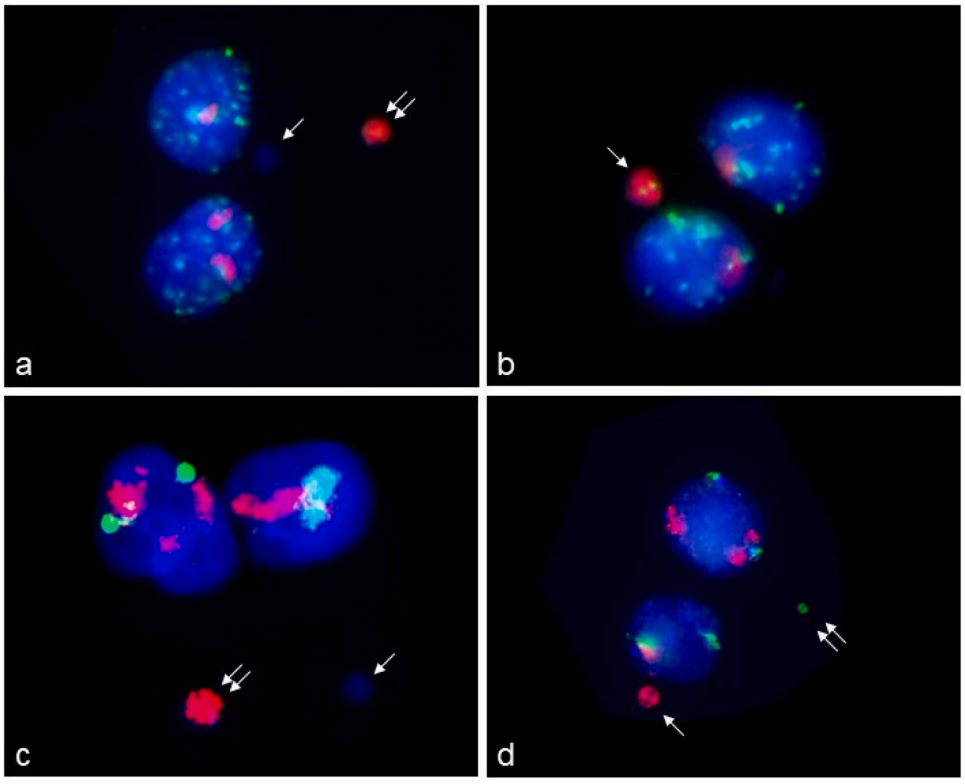

Figure 1. Micronucleated binucleate lymphocytes hybridized either with a pancentromeric DNA probe (green) and a chromosome 9 paint probe (red), simultaneously (a and b), or with chromosome 1 (red) and chromosome 16 (green) paint probes, simultaneously (c and d). a) A binucleate cell with two micronuclei, one without a signal (arrow) and one containing a chromosome 9 acentric fragment (double arrow). b) A binucleate cell with a micronucleus (arrow) containing chromosome 9 signal. c) A binucleate cell with two micronuclei, one without a signal (arrow) and one containing the chromosome 1 signal (red; double arrow). d) A binucleate cell with two micronuclei containing chromosome 1 signal (red; arrow) and chromosome 16 signal (green; double arrow).

3. Results

3.1. Micronucleus analysis

Figure 1 shows examples of micronucleated binucleate lymphocytes hybridized with the pancentromeric DNA probe (which recognizes the centromere of every human centromere) and the chromosome 9 paint probe, simultaneously (Figure 1, a and b); and with the chromosome 1 and chromosome 16 paint probes, simultaneously (Figure 1, c and d).

Table 1 shows the frequencies of total MN and the various categories of MN, as characterized by the pancentromeric probe, in the control and MMC-treated cultures with and without Cyt-B. As expected, MMC statistically significantly increased the frequency of total MN (8-fold without Cyt-B, 5.5-fold with Cyt-B) and MN containing acentric fragments (C^- MN; 8-fold without Cyt-B, 7-fold with Cyt-B). MN harbouring whole chromosomes (C^+ MN) showed a statistically significant elevation (6-fold) without Cyt-B but not when Cyt-B was used (1.5-fold); for this reason, ANOVA indicated a statistically significant interaction of MMC treatment and Cyt-B use in the frequency of C^+ MN. The difference between the donors was statistically significant for the frequency of all three MN categories, reflecting the fact that, in general, the older donor showed a higher MN response to MMC than the younger one. This difference was especially marked for the MMC-treated lymphocytes cultured without Cyt-B, which also explains the other interactions observed.

As binucleate cells have two nuclei, we also divided the MN frequencies in the Cyt-B series by two, to obtain MN frequencies per 1000 nuclei. As shown in Table S1 (Supplementary Material), this approach showed a statistically significant effect of Cyt-B use for all MN categories, with lower frequencies of total MN and C^- MN in the presence of Cyt-B, in both MMC-treated and control cultures. For C^+ MN, the Cyt-B series had a lower frequency only in the MMC-treated cultures.

The percentages (of all MN) of various types of MN, characterized for the presence of pancentromeric signal or DNA from chromosome 9, 1, and 16, in the control and MMC-treated cultures with and without Cyt-B, are shown in Table 2. MMC statistically significantly increased ($p<0.03$) the proportion of C^- MN (with a respective reduction in the contribution of C^+ MN), although this effect (1.25-fold) was only observed when Cyt-B was used (reflected as an interaction between MMC and Cyt-B use in ANOVA). The percentages of MN containing DNA from chromosomes 9, 1, and 16 were increased by the MMC treatment regardless of Cyt-B use. The clearest increase (7.8-fold) was observed in the contribution of 9^+C^- MN in the presence of Cyt-B; without Cyt-B, the effect was lower (3.7-fold), which was also seen as an interaction between MMC and Cyt-B use. In addition, MMC significantly elevated the percentage of MN containing chromosome 1 (1.8-fold without Cyt-B and 2.1-fold with Cyt-B) and chromosome 16 (1.7-fold and 1.9-fold, respectively).

When the percentages of various classes of MN in Table 2 were compared with the expected random values by the Chi-square test (Table 2), statistically significantly higher values than randomly expected were observed in the MMC treatment for 9^+C^- MN and 1^+ MN both with and without Cyt-B. The prevalence of 9^+C^- MN was also elevated in the control cultures without Cyt-B. For 9^+C^+ MN, lower than expected contribution was recorded in MMC-treated cultures with Cyt-B and control cultures without Cyt-B. No significant differences between the observed and expected percentages were observed for chromosome 16.

We then calculated the frequency (per 1000 cells) of the various categories of MN based on their contribution to all MN examined and the total frequency of MN (Table 3). The most pronounced effects of MMC were obtained for MN containing DNA from chromosome 9 (9^+ MN total; 30-fold without Cyt-B, 29-fold with Cyt-B) or an acentric fragment of chromosome 9 (9^+C^- MN; 32-fold without Cyt-B, 42-fold with Cyt-B) (Table 3), when the frequencies of all MN (8-fold without Cyt-B, 5.5-fold with Cyt-B) and all C^- MN (8-fold without Cyt-B, 7-fold with Cyt-B) showed clearly lower differences to control (Table 1). An increase was also seen in the frequency of 9^+C^+ MN, especially without Cyt-B (20-fold, 3-fold with Cyt-B), the effect again being higher than for all C^+ MN (6-fold without Cyt-B, 1.5-fold with Cyt-B) (Tables 1 and 3). In addition, the micronucleation of DNA from chromosome 1 (16-fold without Cyt-B, 11-fold with Cyt-B) and from chromosome 16 (17-fold without Cyt-B, 10-fold with Cyt-B) was clearly increased. There were also differences between the two donors, the older donor generally showing higher MN frequencies and a higher response to MMC than the younger donor, as reflected by the interactions observed.

Table 1
Mean (SD) frequency (per 1000 cells) of total micronuclei (MN) and MN labelled (C^+) or unlabelled (C^-) with pancentromeric DNA probe in control and mitomycin C (MMC) -treated cultures of isolated human lymphocytes (from two male donors) cultured with and without cytochalasin B (Cyt-B).[a]

Treatment	Frequency of MN[b,c,e,f]	Frequency of C^+ MN[b,c,d,f]	Frequency of C^- MN[b,c,e,f]
Cultures without Cyt-B			
Control	6.31 (0.75)	0.92 (0.31)	5.39 (0.62)
MMC (500 ng/ml)	50.64 (30.83)	5.56 (3.39)	45.07 (27.52)
Cultures with Cyt-B			
Control	7.69 (0.52)	2.02 (0.58)	5.67 (0.95)
MMC (500 ng/ml)	42.28 (2.70)	3.17 (0.24)	39.12 (2.62)

[a]The frequency of MN was calculated from 9490-16000 cells per treatment (4510–8000 cells per donor, 2600–8000 cells per replicate culture). The frequencies of C^- MN and C^+ MN were calculated from the total frequency of MN by using the percentage of C^- MN and C^+ MN (based on scoring 400 MN scored per treatment, 200 MN per donor, 100 MN per replicate culture).
Statistically significant effects (analysis of variance):
[b]MMC treatment ($p<0.02$).
[c]Difference between donors ($p<0.02$).
[d]Interaction between MMC treatment and Cyt-B use ($p=0.01$).
[e]Interaction between Cyt-B use and donor ($p<0.03$).
[f]Interaction between MMC treatment and donor ($p<0.04$).

Table 2
Percentage of micronuclei (MN) labelled (C^+) or not labelled (C^-) with pancentromeric DNA probe and paint probes for chromosomes 9 (9^+), 1 (1^+), and 16 (16^+) in control and mitomycin C (MMC) -treated cultures of isolated human lymphocytes (from two male donors) cultured with and without cytochalasin B (Cyt-B).[a]

Treatment	C^+ MN[b,d]	C^- MN[b,d]	9^+ MN			1^+ MN[b]	16^+ MN[b]
			Total[b,c,d]	C^- [b,c,d]	C^+		
Cultures without Cyt-B							
Control	14.5	85.5	9.5	8.25[e]	1.25[f]	9.5	2.0
MMC (500 ng/ml)	11.0	89.0	33.8	30.5[e]	3.25	17.3[e]	3.3
Cultures with Cyt-B							
Control	26.5	73.5	8.8	5.75	3.0	9.3	2.0
MMC (500 ng/ml)	7.5	92.5	46.5	44.75[e]	1.75[f]	19.5[e]	3.8

[a]The centromeric signals were analysed together with chromosome 9 paint, which facilitated distinction between MN harbouring whole chromosome 9 (C^+ 9^+) and acentric fragment from chromosome 9 (C^- 9^+). 400 MN scored per treatment (200 per donor, 100 per replicate culture) separately for the analysis of centromere and chromosome 9 and for the analysis of chromosomes 1 and 16.
Statistically significant effects:
[b]MMC treatment ($p<0.03$; analysis of variance).
[c]Cyt-B use ($p<0.008$; analysis of variance).
[d]Interaction between MMC treatment and Cyt-B use ($p<0.01$; analysis of variance).
[e]Higher than expected by random occurrence ($p<0.01$; Chi-square test).
[f]Lower than expected by random occurrence ($p<0.05$; Chi-square test).

Table 3
Mean (SD) frequency (per 1000 cells) of micronuclei (MN) positive for chromosome 9 (9^+) with (C^+) and without (C^-) centromeric label, and MN positive for chromosome 1 (1^+) and chromosome 16 (16^+) in control and mitomycin C (MMC) -treated cultures of human lymphocytes (of two male donors), with and without cytochalasin B (Cyt-B).[a]

Treatment	9^+ MN			1^+ MN[b,c,d,e,f]	16^+ MN[b,c,f]
	Total[b,c,d,e]	C^- MN[b,c,d,e]	C^+ MN[b]		
Cultures without Cyt-B					
Control	0.60 (0.18)	0.52 (0.17)	0.08 (0.13)	0.60 (0.15)	0.12 (0.05)
MMC (500 ng/ml)	18.13 (12.59)	16.52 (11.69)	1.61 (2.32)	9.36 (6.98)	1.99 (1.79)
Cultures with Cyt-B					
Control	0.67 (0.11)	0.45 (0.14)	0.46 (0.12)	0.72 (0.20)	0.16 (0.07)
MMC (500 ng/ml)	19.72 (2.96)	19.00 (3.39)	1.44 (0.62)	8.27 (1.18)	1.61 (1.17)

[a]The frequencies were calculated on the basis of the total frequencies of MN (scored from 9490-16000 cells per treatment, 4510–8000 cells per donor, 2600–8000 cells per replicate culture) and the percentage of each type of MN in 400 MN per treatment (200 per donor, 100 per replicate culture), separately for the analysis of centromere and chromosome 9 and for the analysis of chromosomes 1 and 16.
Statistically signicant effects (analysis of variance):
[b]MMC treatment ($p<0.02$).
[c]Difference between donors ($p<0.02$).
[d]Interaction between Cyt-B use and donor ($p<0.03$).
[e]Interaction between MMC treatment and donor ($p<0.04$).
[f]Interaction among MMC treatment, Cyt-B use and donor ($p=0.007$).

3.2. Chromosomal aberration analysis

The number of different types of CAs found in the metaphases of the control and MMC-treated cultures are shown in Table 4. As expected, MMC induced a statistically significant increase in the number of chromatid-type aberrations (both with and without gaps), as well as in the number of total CAs (with or without gaps), but not in the number of chromosome-type aberrations. From the total number of MMC-induced aberrations (including gaps), 94.4 % were chromatid-type. Most of the chromatid-type aberrations were breaks (1.85 per cell), whereas exchanges and gaps appeared at frequencies of 0.45 and 0.05 per cell, respectively. The number of multi-aberrant cells in the MMC-treated cultures was statistically significantly higher than in the control cultures. There was no statistically significant difference between the two donors in the CA analysis.

To investigate the involvement of chromosome 9 in the MMC-induced CAs, human metaphases were simultaneously hybridized with a pancentromeric probe (green), a chromosome 9 centromeric probe (red), a 9p telomeric probe (red) and a 9q telomeric probe (green), as shown in Figure 2. Chromosome 9, labelled with the four probes simultaneously, could easily be distinguish from the rest of the chromosomes which only showed the pancentromeric label. Figure 2 shows a chromatid break (a), exchanges between the two homologues of chromosome 9 (b and c) and among the chromosome 9 homologues and a third chromosome (d).

A more detailed description of breaks and exchanges involving chromosome 9 is shown in Table 5. MMC statistically significantly increased the frequency of breaks in chromosome 9 and in other chromosomes, as well as the frequency of interchanges involving chromosome 9 or other chromosomes.

To examine whether the increased level of breaks and exchanges in chromosome 9 was due to a higher susceptibility of this chromosome toward MMC, the observed values were compared with those expected assuming an equal probability for all chromosomes (Table 5). Chromosome 9 had a statistically significantly higher ($p<0.01$) number of MMC-induced breaks and exchanges than randomly expected. Breaks affecting chromosome 9 constituted 30.7 % of all MMC-induced breaks, whereas 40.7 % of all exchanges involved chromosome 9. Of the exchanges, 76 % concerned one chromosome 9 and other chromosome(s) and 24 % both homologues of chromosome 9, either alone or with other chromosomes. As reported in Table 5, and also illustrated in Figure 2, most of the MMC-induced breaks (82.6 %) and exchanges of chromosome 9 occurred exactly below the label of the chromosome 9 classical satellite probe (D9Z1) which covers the region 9ceb-q12. Therefore, the preferential breakpoint in chromosome 9 is immediately below band 9q12.

4. Discussion

In accordance with its well-known clastogenicity [3–5,10–19], MMC was able to strongly induce MN and CAs. In the MN assay, the effect particularly concerned MN harbouring acentric fragments (C^- MN), although also micronuclei containing a centromere (C^+ MN) were affected.

Previously, several studies have reported a clear induction of C^- MN or kinetochore-negative (K^-) MN by MMC, but the results have been conflicting for C^+ and kinetochore-positive (K^+) MN, [19,24–30]. Rosefort et al. [19], using a pancentromeric α consensus probe, observed almost similar increases in the frequencies of C^+ MN (4.6-fold) and C^- MN (4.9-fold) in binucleate human lymphocytes, and also other studies have presented evidence for an aneugenic effect of MMC [25–28]. Besides entire chromosomes, the C^+ and K^+ MN produced by MMC could originate from chromosomal breaks occurring near the centromeric region, resulting in the micronucleation of centric fragments [2]. However, Surrallès et al. [29], who found that MMC exclusively induced C^- and K^- MN, concluded that chromosome breakage by MMC at the area labelled by the SO-α-AllCen oligomer probe they used for centromeric FISH did not appear to be a problem. Yet, a similar argument was used

Table 4
Number of chromosomal aberrations and multi-aberrant cells in 200 metaphases (100 per donor, 50 per replicate culture) of control and mitomycin C (MMC; 500 ng/ml) -treated cultures of human lymphocytes (from two male donors).

Treatment	Chromatid-type					Chromosome-type			Total		No. multi-aberrant cells
	Gaps	Breaks	Exchanges	Total without gaps	Total with gaps	Breaks	Exchanges	Total	Without gaps	With gaps	
Control	3	8	0	8	11	5	0	5	13	16	2
MMC	11	370	90	460[a]	471[a]	27	1	28	488[a]	499[a]	100[a]

[a]Statistically significant effect of MMC treatment ($p<0.003$; analysis of variance).

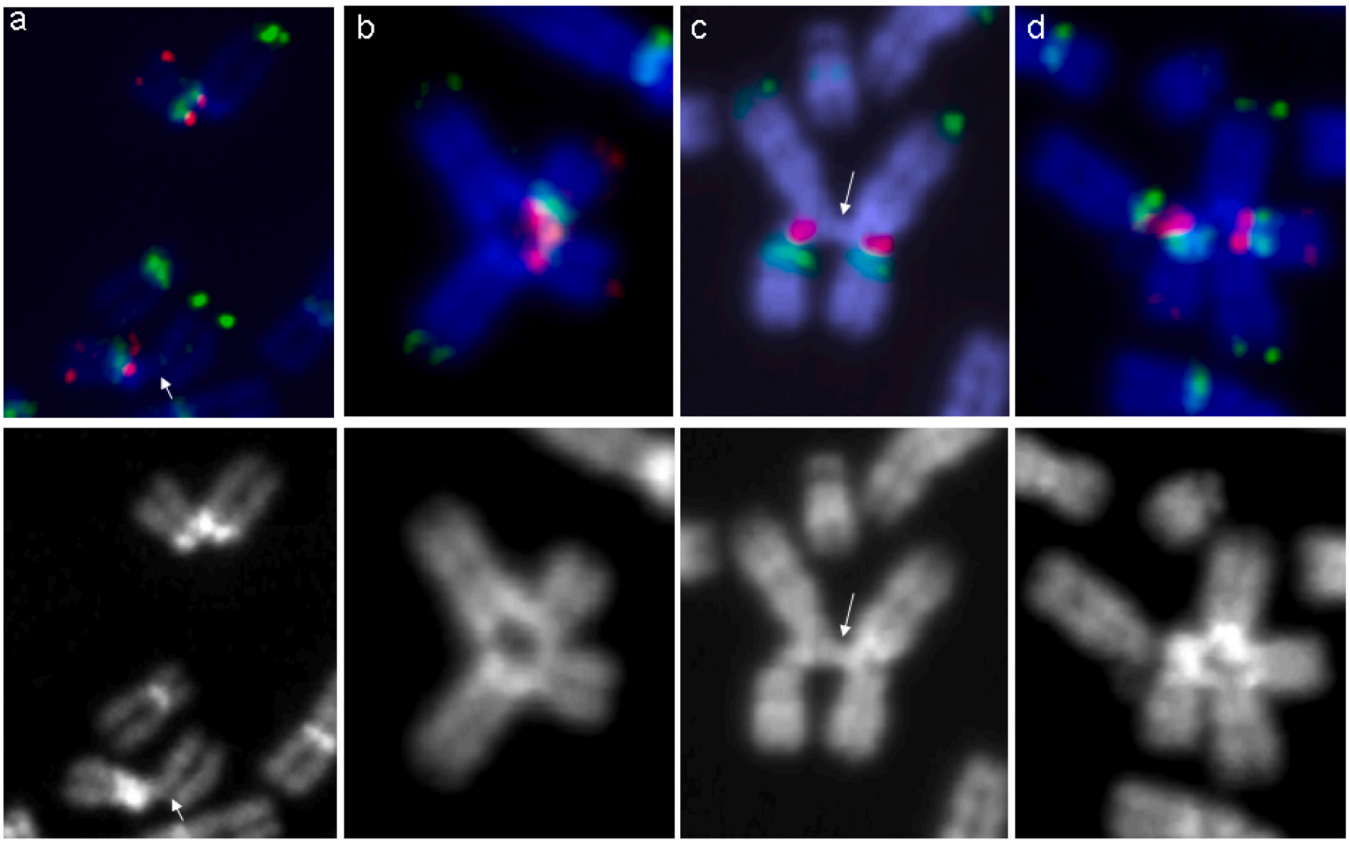

Figure 2. Examples of mitomycin C -induced chromosomal aberrations in human lymphocytes, studied by fluorescent *in situ* hybridization (upper row) combining human pancentromeric probe (green), a chromosome 9 classical satellite DNA probe (red), a chromosome 9p telomeric probe (red) and a chromosome 9q telomeric probe (green). Black and white images of the DAPI counterstained chromosomes are included (lower row) to visualize the aberrations at a higher contrast. a) Both chromosomes 9 are stained with the four probes; the chromosome 9 at the bottom of the image has a chromatid break (arrow). b) An interchange between the homologues of chromosome 9, with both exchange breakpoints below the chromosome 9 classical satellite DNA label (red). c) An interchange between chromosome 9 homologues, with the exchange breakpoint (arrow) below the chromosome 9 classical satellite DNA label (red). d) An interchange between the homologues of chromosome 9 and a third chromosome.

Table 5
Number of chromosomal breaks and exchanges involving chromosome 9 and other chromosomes in 200 metaphases (100 per donor; 50 per replicate culture) of control and mitomycin C (MMC; 500 ng/ml) -treated cultures of human lymphocytes (from two male donors).

Treatment	Chromosome 9											Other chromosomes			No. metaphases with other chromosomes normal
	Normal	Breaks				Exchanges						Breaks			Exchanges[b]
		CT-SAT[a]	CT-OT	CS-SAT	Total[b]	9–9	9–9-one OC	9–9-two OCs	9-one OC	9-two OCs	Total[b]	CT	CS	Total[b]	
Control	395	1	0	0	1	0	0	0	0	0	0	6	0	6	194
MMC	258	46	15	25	86[c]	9	2	1	31	7	50[c]	186	8	194	0 86 73

[a]Abbreviations: CS, chromosome break; CS-SAT, chromosome break exactly below chromosome 9 satellite probe; CT, chromatid break; CT-OT, chromatid break elsewhere in chromosome 9 than below the satellite probe; CT-SAT, chromatid break exactly below chromosome 9 satellite probe; 9–9, exchange between chromosome 9 homologues; 9–9-one OC, exchange of chromosome 9 homologues and one other chromosome; 9–9-two OCs, exchange of chromosome 9 homologues and two other chromosomes; 9-one OC, exchange between chromosome 9 and one other chromosome; 9-two OCs, exchange of chromosome 9 and two other chromosomes.
[b]Statistically significant effect of MMC treatment ($p<0.03$; analysis of variance).
[c]Value significantly higher than expected by random occurrence ($p<0.01$; Chi-square test).

by Rosefort et al. [19] about the consensus sequence they used in concluding that MMC has real aneugenic potential. One mechanism for an aneugenic effect could be disruption or detachment of kinetochores, reported in MMC-treated cycling murine splenocytes [28].

As Cyt-B has previously been observed to reduce the micronucleation of acentric fragments [see 2], we also looked at MN frequencies per 1000 nuclei. In this approach, the use of Cyt-B was associated with a decrease in MN harbouring acentric fragments, in both MMC-treated and control cultures. In the MMC treatment, Cyt-B also reduced the frequency (per 1000 nuclei) of C^+ MN. The lowered micronucleation of acentric

fragments in the presence of Cyt-B has been suggested to be due to shortened distance between the mitotic poles and lack of a contractile ring in cytokinesis-blocked cells [2].

Although many studies have reported the capacity of MMC to induce MN, only a few of them have investigated the chromosomes involved [3–5,20–21]. In the current study, painting probes were used for chromosomes 9, 1, and 16 as they have been suggested to be sensitive to MMC action. The simultaneous use of a pancentromeric probe together with the chromosome 9 painting probe allowed us to distinguish 9^+ MN containing whole chromosome 9 from those containing an acentric fragment of chromosome 9. Based on the results of ANOVA, we found that chromosomes 9, 1, and 16 were preferentially micronucleated by MMC. For chromosome 9, most of the MN induced were 9^+C^- and thus harboured acentric fragments. However, there was also an increase in 9^+C^+ MN, containing a whole chromosome 9 or a centric fragment of chromosome 9. When we evaluated, if the micronucleation of the three chromosomes by MMC was random, chromosomes 9 and (when Cyt-B was not used) chromosome 1 showed a statistically significant increase (Chi-square test). Furthermore, 9^+C^- MN were found more often than randomly expected in the controls (when Cyt-B was not used), indicating that acentric fragments of chromosome 9 are preferentially included also in spontaneous MN, as earlier suggested in a couple of studies [4,30,31].

The micronucleation of human chromosome 9 after MMC treatment has previously been investigated in three studies [3–5], all showing a high over-representation. The chromosome-specific micronucleus induction by MMC has earlier been examined for chromosome 16 in two papers [4,5] and for chromosome 1 in one paper [4].

Kusakabe et al. [3] analysed the contents of 57 MN induced by MMC (2 mg/ml for 2 h, at G_0) in 120-h lymphocyte cultures of a male donor and found a high induction of MN labelled with chromosome 9 paints (39.7 %, 25 MN out of 63 MN examined) but not with a chromosome 9 -specific α satellite probe, indicating that the MMC-induced breaks resulting in 9^+ MN were not induced within the α satellite repeats in the centromere of chromosome 9 but nearby, at the site of the α satellite spot on the 9q. This result is in agreement with the present study. The authors assumed that most of the MN with the chromosome 9 paint originated from an acentric 9q, based on their metaphase analyses showing that translocation of 9p or 9q to another chromosome and a centric fragment representing the entire length of 9p were characteristically generated from chromatid-type aberrations involving the centromeric region of chromosome 9. They proposed that nondisjunction of chromosome 9 may play a role in generating the unbalanced karyotypes of chromosome 9 they found, suggesting that MMC could also have aneugenic potential.

Fauth et al. [4] found a high occurrence (5–6 times the control) of chromosome 9 material in MN after MMC treatment (500 ng/ml for 23 h before harvest) in cultured lymphocytes from two female donors (donor one 62 %, 50 MN examined; donor two 69 %, 100 MN examined). These findings agree with our results with a 4–5-fold difference between the MMC treatment and the control in the prevalence of 9^+ MN. As 85 % of the MN showing chromosome 9 FISH signals also contained a DAPI-bright heterochromatin signal, Fauth et al. [4] suggested that chromosome 9 heterochromatin was enriched in MN, either because a complete chromosome 9 was lost in MN, or because a break within or near the heterochromatin block led to its preferential exclusion from the main nucleus. In addition, Fauth et al. [4] saw, in one of the two donors of the study, a two-fold increase over the control level in the percentage of 1^+ MN (not statistically significant), which is exactly the same result as we obtained, except that our finding was statistically significant. However, the other donor Fauth et al. [4] examined did not show any effect for chromosome 1. Chromosome 16 was not affected by MMC [4], whereas we could show an over-representation in MMC-induced MN also for chromosome 16. Also in our hands, the percentage of 1^+ and 16^+ MN among all MMC-induced MN were clearly lower than that of 9^+ MN, but our analysis was based on scoring 400 MN without Cyt-B and 400 with Cyt B. It may have been difficult to show such low prevalences with the fewer number of MN characterized by Fauth et al [4].

Hovhannisyan et al. [5] examined the involvement of nine human chromosomes, including chromosomes 9 and 16, in MMC-induced (100 ng/ml, for 50 h at hour 22 of culture) MN of cytokinesis-blocked human lymphocytes. They used sequential FISH with chromosome-specific centromeric probes and whole chromosome painting probes and observed that MMC primarily induced MN labelled with the whole chromosome probes rather than the centromeric probes. MN positive for whole chromosome 9 represented 32.6 % of the 994 MN induced by MMC in cultures of a male donor. MN positive for whole chromosome 16 comprised 3.0 % of the 1416 MMC-induced MN examined (another male donor) [11]. We used a 5 times higher dose of MMC than Hovhannisyan et al. [5] and obtained slightly higher figures: 46.6 % (33.8 % without Cyt-B) for chromosome 9 and 3.8 % (3.3 % without Cyt-B) for chromosome 16, the findings being statistically significant for both chromosomes. Hovhannisyan et al. [5] reported that, based on chromosome length (Chi-square test), chromosome 9 was statistically significantly over-represented (314 observed, 45 expected) in MMC-induced MN, while chromosome 16 was not (43 observed, 41 expected). Similarly, in our Chi-square tests on random occurrence of chromosomes in MN, we got a positive result for chromosome 9 (and chromosome 1) but not for chromosome 16, despite all three chromosomes we analysed showed a significant over-representation in ANOVA. However, as the other seven chromosomes examined by Hovhannisyan et al. [5] were all under-represented in MMC-induced MN - five of them (chromosomes 3, 4, 6, 7 and 17) statistically significantly - it can be concluded that the data are consistent with an over-representation of chromosome 16 in MMC-induced MN.

We studied the involvement of chromosome 9 in MMC-induced CAs by utilizing DNA probes for all centromeres, chromosome 9 classical satellite DNA, chromosome 9p telomere and chromosome 9q telomere. The rate of chromosome 9 breaks and exchanges was significantly higher than expected by chance, in agreement with previous classical cytogenetic papers [11–16] and the two FISH studies [3,10] that found an over-representation of human chromosome 9 in MMC-induced CAs. Most of the chromatid and chromosome breaks induced by MMC occurred exactly below the region covered by the classical satellite probe which hybridizes to pericentric heterochromatin more distant from the centromere than the region labelled by the pancentromeric probe.

Abdel-Halim et al. [10], using band-specific probes for 9q12–13 and 1q11–12 together with reverse colour painting for whole chromosome 9 and 1, found that about 50 % of all interchanges induced by MMC (1.3 mg/ml for 2 h, at G_0) concerned chromosomes 1 or 9. Interchanges between the chromosome 9 homologues comprised about 70 % of the interchanges involving chromosome 9. In our data, chromosome 9 was involved in 40.7 % of all exchanges, and 76 % of interchanges involving chromosome 9 occurred between the two homologues, which compares well with the results of Abdel-Halim et al. [10]. The same authors [10] also observed that 97 % of the breakpoints in chromosomes 9 and 1 occurred in the heterochromatic bands 9q11–13 and 1q11–12. As our data showed that the preferential breakpoint is immediately below 9cen-q12 (the region labelled by chromosome 9 classical satellite probe), it can be concluded that the preferential breakpoint in chromosome 9 is right below band 9q12.

Although the sensitivity of the pericentromeric region of chromosome 9, and of chromosomes 1 and 16, to MMC-induced CAs has been known for a long time [3,4,11–18], the mechanisms involved are not entirely clear. Pericentromeric heterochromatic c-bands contain GC-rich repeats with highly methylated DNA [10]. MMC preferentially cross-links CpG DNA sequences, as it reacts via N-alkylation with 7-N-guanine nucleotide residues of 5'-CG-3' sequences through the minor groove of DNA [34]. The yield of lesions increases with the number of successive CpG sequences [35] and by the 5-methylation of cytosines in these sequences [36]. ICLs impede DNA replication causing replication stress, which triggers different DNA repair pathways.

Heterochromatin is thought to be more susceptible to replication stress, resulting in DNA breakage or rearrangements, owing to the preponderance of repetitive DNA sequences, which makes them extremely vulnerable to improper DNA damage repair [37]. MMC-induced ICLs are preferentially repaired by the homologous recombination pathway, which uses homologous DNA sequences as templates for initiating the repair process [38]. In single-copy sequences, there is a unique homologous sequence (present in the sister chromatid or in the homologous chromosome) and the repair is mainly error-free. However, in heterochromatin, the existence of multiple homologous sequences in different chromosomes can initiate unequal sister chromatid exchange, or intra/inter-chromatid recombination, resulting in different chromosome aberrations [39].

Abdel-Halim et al. [10] observed that the pericentromeric heterochromatic regions of chromosome 9 homologues associated and colocalized in G_0-G_1-phase nuclei of human fibroblasts immediately after MMC treatment. They suggested that the pairing of homologous heterochromatin reflects the initiation of recombination-dependent ICL repair, ultimately leading to chromatid interchanges between homologous chromosomes in case of misrepair. Incomplete ICL repair would then lead to chromosomal breaks as intermediate repair products [10]. In a subsequent paper, Abdel-Halim et al. [40] proposed that the pairing of heterochromatin is a response to cellular stress. Considering micronucleus formation, fragments leading to C^- MN are also formed, for instance, from the type of interchanges shown in Fig. 2 c and d. After chromosome segregation, MN then originate from lagging fragments in early anaphase, as shown by live cell imaging in MMC-treated human lymphoblastoid cells [32]. The interchanges in Fig 2 also involve a dicentric chromatid that can form an anaphase bridge capable of breaking and thereby micronucleation later in anaphase/telophase. Whether the well-known under-condensation, observable in metaphase at the heterochromatic bands of chromosomes 9, 1, and 16 [10,11,16,17,24] in MMC treatment, has any role in micronucleus formation, remains to be seen. Several other possibilities for micronucleus formation exist [1,33]. As spontaneous chromatid interchanges are very rare, the preferential inclusion of chromosome 9 fragments in MN of untreated human cells, as reported here and in earlier studies [4,30,31], is probably based on partly different mechanisms than the induction of 9^+ MN by MMC.

5. Conclusions

In summary, our findings confirm the overrepresentation of acentric fragments from chromosome 9 in MMC-induced MN of human lymphocytes. Our results also support the preferential micronucleation by MMC of material from chromosomes 1 and 16. Furthermore, our data suggest that the prevalence of MN with acentric fragments of chromosome 9 is elevated in MN of untreated lymphocytes. In accordance with earlier studies, we show that chromosome 9 is highly involved in MMC-induced chromosomal breaks and interchanges. We can assign the preferential breakpoint immediately below 9q12. Future investigations should aim at elucidating further the mechanisms underlying the high sensitivity of chromosomes 9, 1 and 16 to chromosomal damage and micronucleation.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Julia Catalán: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Hilkka Järventaus:** Visualization, Methodology, Investigation, Formal analysis. **Ghita C.-M. Falck:** Methodology, Investigation. **Carlos Moreno:** Data curation. **Hannu Norppa:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

The authors would like to thank the two donors for their contribution to this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrgentox.2024.503753.

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