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Mutation Research 440 (1999) 147–156

Genetic Toxicology and Environmental Mutagenesis

Comparison of spontaneous and idoxuridine-induced micronuclei by chromosome painting

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Received 17 November 1998; received in revised form 2 February 1999; accepted 4 February 1999

Abstract

Fluorescence in situ hybridisation (FISH) technique with chromosome specific library (CSL) DNA probes for all human chromosomes were used to study about 9000 micronuclei (MN) in normal and idoxuridine (IUdR)-treated lymphocyte cultures of female and male donors. In addition, MN rates and structural chromosome aberrations were scored in Giemsa-stained chromosome spreads of these cultures. IUdR treatment (40 μg/ml) induced on the average a 12-fold increase of the MN rate. Metaphase analysis revealed no distinct increase of chromosome breaks but a preferential decondensation at chromosome 9q12 (28–79%) and to a lower extend at 1q12 (8–21%). Application of FISH technique with CSL probes to one male and one female untreated proband showed that all human chromosomes except chromosome 12 (and to a striking high frequency chromosomes 9, X and Y) occurred in spontaneous MN. In cultures containing IUdR, the chromosomal spectrum found in MN was reduced to 10 chromosomes in the male and 13 in the female proband. Eight chromosomes (2, 6, 12, 13, 14, 15, 17 and 18) did not occur in MN of both probands. On the contrary chromosomes 1 and especially 9 were found much more frequently in the MN of IUdR-treated cultures than in MN of control cultures. DAPI-staining revealed heterochromatin signals in most of the IUdR-induced MN. In an additional study, spontaneous and IUdR-induced MN were investigated in lymphocytes of another female donor using CSL probes only for chromosomes 1, 6, 9, 15, 16 and X. The results confirmed the previous finding that chromosomes 1 and 9 occur very often in MN after IUdR-treatment. The results indicate that decondensation of heterochromatic regions on chromosomes 1 and 9 caused by IUdR treatment strongly correlates with MN formation by these chromosomes.

Keywords: Chromosomal content of human micronucleus; Idoxuridine; Flourescence in situ hybridisation (FISH)

1. Introduction

Numerous genotoxic chemicals have been shown to increase the occurrence of micronuclei (MN) in cultured cells. MN may contain whole chromosomes or acentric fragments either as the consequence of spindle failure induced by aneugenic chemical compounds, or due to induction of chromatid breakage caused by clastogenic mutagens. For review, see Refs. [1,2].

The chromosomal content of MN can be determined by Fluorescence In Situ Hybridisation (FISH)

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1383-5718/99/$ - see front matter
PII: S1383-5718(99)00021-2

with chromosome specific DNA probes, which detect specific repeats in pericentromeric heterochromatin [3] or by chromosome painting probes, which label euchromatic parts of a given chromosome [4–7].

To date, the effects of mutagenic substances on the inclusion of specific chromosomes in MN have been investigated only with respect to the impact of vanadium compounds [8,9], colchicine [6,10], and 5-azacytidine (5-aza-C) [3,7]. Chromosome-painting (for review, see Ref. [11]) has furthermore been used to study the chromosomal content of radiation-induced MN [4–6]. However very little is known until now about the identity of chromosomes in spontaneous MN. Only Fauth et al. [7] presented data for one female proband by screening more than 60,000 lymphocytes. This high number indicates that such studies are very time consuming.

In the present paper, we extended these studies by investigating spontaneous MN of a male donor with chromosome specific library (CSL) DNA probes. In addition to the studies on spontaneous MN we analysed the chromosomal inclusion spectrum of MN induced by IUdR in lymphocytes of one male and one female proband. Moreover, the occurrence of chromosomes 1, 6, 9, 15, 16 and X was also investigated in another female proband. Chromosomes 1, 9, 15 and 16 were chosen because they have large heterochromatic regions near to the centromere. The chromosomes 6 and X served as heterochromatin-negative controls.

IUdR is a thymidine analogue, which has been shown to be an effective radiosensitiser of tumour cells when combined with irradiation or radiolabelled anti-tumour antibody radiotherapy [12–14].

IUdR is also known to induce sister chromatid exchanges (SCEs), chromosomal aberrations [15–18], and decondensation of heterochromatic blocks in metaphase chromosomes. Furthermore, IUdR induced the preferential occurrence of heterochromatin carrying chromosomes in MN. This effect was strongly related to decondensation and fragility of the heterochromatic region of chromosome 9 (9qh) as revealed by specific staining of 9qh in MN and application of the premature chromosome condensation (PCC) technique [19]. Because the study of Tommerup was restricted to chromosome 9, we studied the occurrence of all human chromosomes in IUdR-induced MN by use of CSL probes. These results were supplemented by staining with the heterochromatin-specific dye DAPI.

2. Materials and methods

2.1. Cell culture and slide preparation

Peripheral blood samples (10 ml) were obtained from one male and two female donors (age: 22–32 years) with normal karyotypes. Lymphocyte cultures were set up by adding 0.8 ml peripheral blood to 8 ml RPMI 1640 (readily supplemented with glutamine [300 μg/ml], Bio Whittaker, 15% foetal calf serum, Life Technologies, 1% penicillin–streptomycin, Bio Whittaker), and 22 μg/ml phytohemagglutinin (Life Technologies). Lymphocytes were cultured for 71 h at 37°C. For metaphase and MN preparations, 0.2 ml colcemid solution (0.001%, Serva) was added 2 h prior to harvesting. Cells were hypotonically treated (20 min 0.0375 M KCl at 37°C), fixed three times in methanol/acetic acid (3/1) and spread on clean glass slides.

IUdR (CAS # 54-42-2; final concentration, 40 μg/ml culture; Sigma) was added to the cells 23 h prior to harvest. Since IUdR is dissolved in dimethylsulfoxide (DMSO), addition of this drug results in a final concentration of 0.2% DMSO in the cultures. Therefore, DMSO (0.2%) supplemented control cultures were also investigated.

For the analysis of chromosomes and MN rates, preparations were Giemsa-stained according to standard methods.

2.2. DNA probes and labelling

Plasmid library DNA probes for all human chromosomes [20] were kindly provided by J.W. Gray, University of California (San Francisco). Plasmid DNA was obtained by midi-scale preparation according to Kaul and Scherthan [21]. Probe DNA was labelled with biotin-16-dUTP and digoxigenin-11-dUTP using Bio and Dig nick translation mixtures according to the manufacturer's protocol (Boehringer, Mannheim).

2.3. Fluorescence in situ hybridisation and detection

FISH of MN was performed according to Fauth et al. [7]. CSL DNA probes [20] were applied in pairwise, differentially labelled combinations. A hybridisation mixture (20 μl), containing 1 μg of biotinylated and 1 μg of digoxigenated CSL DNA probe, 1 μg herring sperm DNA, 10 μg human C_0t-1 DNA (Life Technologies), 50% formamide, 10% dextransulphate, 2 × SSC were applied per slide, covered with a 22 × 60 mm cover slip and sealed with rubber cement. Preparations were denatured for 3.5 min at 78°C on a hot plate. Subsequently, the slides were hybridised for two days at 37°C, then washed three times for 5 min in 0.05 × SSC at 42°C [22]. Biotinylated hybrid molecules were detected with fluorescein–conjugated avidin and digoxigenin-labelled hybrid molecules were detected with rhodamine anti-dig Fab fragments [7]. Finally slides were mounted in Vectashield (Vector) containing 1 μg/ml 4′6-diamidino-2-phenylindole (DAPI) as chromosomal counterstain.

2.4. Microscopic evaluation

Preparations were examined with a Leitz Orthoplan research microscope equipped with a Ploem opak fluorescence epiilumination system and filter blocks A and I_2 for excitation of green and red fluorescence and a green/red double band pass filter for simultaneous excitation of both emissions (Chroma). In each experiment at least 50 (average 88) micronuclei with associated interphase nuclei were scored for their chromosome content. The total number of screened MN was about 9000. Cells were regarded as micronucleated when fulfilling the criteria of Countryman and Heddle [23]. The only modified criteria was that the distance of a MN separated from the corresponding nucleus was not allowed to be more than the diameter of the main nucleus.

To avoid misinterpretations due to weak signals, we investigated only preparations with strong interphase signals in neighbouring nuclei and metaphases. Micronuclei (MN) rates were derived from the analysis of 2000–5000 cells per culture (Table 1).

2.5. Metaphase analysis

In parallel to all FISH experiments, chromosomes of 100 GTG-banded metaphase spreads from all cultures were analysed for the presence of breaks, gaps, acentric fragments, exchanges and decondensations. Chromosomes were classified as 'undercondensed' when the pericentromeric heterochromatin of chromosomes was outstretched for more than a chromatid width [24].

Table 1
MN rates in IUdR-treated lymphocyte cultures of three probands and control cultures with 0.2% DMSO

Proband (experiment no.)	*n*	MN rate (%) control	MN rate (%) IUdR
fem1-(1)	2000	0.15	2.6
fem1-(2)	2000	0.45	3.4
fem1-(3)	2000	0.35	2.9
fem1-(4)	5000	0.3	2.4
fem1-(5)	3000	0.2	3.5
male-(1)	2000	0.45	5.1
male-(2)	5000	0.2	2.7
fem2-(1)	5000	0.3	1.9
fem2-(2)	5000	0.3	5.4
Average (rounded)	3500	**0.3**	**3.3**

n, Number of cells investigated; fem, female; (), number of culture.

3. Results

3.1. MN rates

Spontaneous MN rates in cultures without and with 0.2% DMSO ranged from 0.15% to 0.45% (Table 1). The addition of DMSO (0.2%) had no effect on the spontaneous MN rate.

Nine cultures of the three different probands (fem1, fem2 and male) treated with IUdR (40 μg/ml) displayed MN rates ranging from 1.9% to 5.4% (Table 1). This represents an average increase by factor 12 as compared to spontaneous MN rates. About 95% of micronucleated cells showed one micronucleus, while a minor fraction (5%) contained two MN.

3.2. Metaphase analysis for structural aberrations

In untreated and DMSO-incubated cultures only few aberrations (mostly decondensations on C-group chromosomes) could be found in all the three probands (Table 2).

Table 2
Percentage of metaphases with decondensed heterochromatic regions of chromosomes 1, 9, 16, Y and other chomosomes in lymphocytes treated with IUdR and in control cultures

Proband	fem1			male			fem2		
Culture	a	b	c	a	b	c	a	b	c
Total[a]	0	2	37	3	5	58	3	8	79
1[b]	1	0	8	0	1	21	0	0	10
9[b]	0	0	28	1	1	38	0	0	79
16[b]	0	0	10	0	0	6	0	0	0
Y[b]				0	0	7			
Others[b]	0	1	4	0	2	4	3	7	0
Gaps[c]	0	0	1	0	1	2	0	1	5
Breaks[c]	0	0	0	0	2	2	0	0	0

Frequencies of metaphases with gaps or breaks are also shown. Number of metaphases investigated: 100 for each study.
a, Control culture; b, culture with 0.2% DMSO; c, culture with 40 μg/ml IUdR + 0.2% DMSO.
[a] Percentage metaphases with undercondensations (total).
[b] Percentage metaphases with undercondensations on chromosome #.
[c] Percentage metaphases with gaps/breaks.

IUdR treatment impaired condensation of the heterochromatic regions at 9q12, and to a lesser extend at 1q12, 16q12 and Yq12 in many metaphases of all three probands. The undercondensation involved one or both chromosomes 1 in 8–21%, chromosomes 9 in 28–79% and chromosomes 16 in 0–10% of metaphases. In 7% of metaphases the distal heterochromatic segment of the Y chromosome was undercondensed. Chromosome gaps and breaks were rarely found (in 1–5% of metaphases, Table 2).

3.3. Chromosomal content of spontaneous MN as revealed by chromosome painting

Human CSL DNA probes were hybridised pairwise to preparations obtained from normal lymphocyte cultures of a 32-year-old male. With exception of chromosomes 12 and 18, which were not detected in spontaneous MN of this proband, all other chromosomes were seen in spontaneous MN at frequencies ranging from 0.8 to 13% (Table 3). Most often the chromosomes 9, X and Y were included in MN (10–13%). The data of a normal female proband (fem1) were taken from an earlier study [7]. Nearly all chromosomes (except for 12 and 19) were present in spontaneous MN at about the same range as in the male proband (1–11.5%). Also most frequently chromosomes 9 and X were found in MN (9.8–11.5%).

Analysis of spontaneous MN of another female proband (fem2) by painting probes for chromosomes 1, 6, 9, 15, 16 and X revealed also a strikingly frequent occurrence of chromosomes 1, 9 and X at frequencies of 9.2, 16 and 8% of spontaneous MN, respectively. Chromosome 15 material was detected in 2% of spontaneous MN, while chromosomes 6 and 16 did not occur (Table 3).

3.4. Chromosomal content of IUdR-induced MN as revealed by chromosome painting

Chromosome painting analysis of male and female IUdR-induced MN revealed that the vast majority of MN of all three probands contained chromo-

Table 3
Frequency of individual chromosomes in MN (%) of normal and IUdR-treated lymphocyte cultures of one male and two female donors

Chromosome	Untreated			IUdR		
	fem1[a]	male	fem2	fem1	male	fem2
1	5.7	1	9.2	14.65	6	22
2	2.2	6.7		0	0	
3	7.7	3.8		0.7	2	
4	1.4	3.3		2.3	2	
5	3.9	5.1		5.7	3	
6	2.5	2.7	0	0	0	0
7	6.3	4.8		1.2	0	
8	2.8	1		2	2	
9	9.8	13	16	82	78.7	80.8
10	7	2		3.2	0	
11	1.3	5.9		0.9	0	
12	0	0		0	0	
13	2	3.3		0	0	
14	1.5	6		0	0	
15	5	2	2	0	0	0
16	2	8	0	4	5	0
17	2	4.8		0	0	
18	2.9	0		0	0	
19	0	0.8		0	1.7	
20	3.8	1.1		0.5	0	
21	1	1.3		0	1.2	
22	2	2		1.4	2	
X	11.5	10	8	3.1	0	0
Y		10			0	

[a] Values for fem1 were taken from our earlier study [7].

some 9 material (fem1: 82%; male: 78.7%; fem2: 80.8%; Table 3). These rates exceeded by far those from control cultures and represent 5- to 8-fold increases. Furthermore, occurrence of chromosome 1 material in MN was 2.5- to 6-fold enhanced by IUdR (Table 3). Frequencies of other chromosomes in IUdR-induced MN ranged from 1.2 to 5% in the male proband and 0.5–5.7% in the female (fem1) proband (Table 3; Fig. 1). Fig. 1 shows the frequencies of all human chromosomes in spontaneous and IUdR-induced MN by combining the values of fem1 and male.

Many chromosomes (2, 6, 12–15, 17, 18) did not occur at all in IUdR-induced MN: chromosomes 2, 6, 7, 10–15, 17, 18, 20, X and Y were not detected in IUdR-induced MN of the male proband and chromosomes 2, 6, 12–15, 17–19 and 21 were not detected in IUdR-induced MN of the female (fem1) proband (Table 3, Fig. 1). The IUdR-induced MN of fem2 which were examined only with selected CSL probes did not contain chromosomes 6, 15, 16 and X (Table 3).

3.5. Heterochromatic content of MN as revealed by DAPI staining

DAPI stains heterochromatin as bright blue clusters in interphase nuclei and metaphase chromosomes, preferentially the heterochromatic blocks at 1q12, 9q12, 16q12 and Yq12 [25,26]. These prominent DAPI-bright 'spots' can also be identified in MN and indicate the presence of pericentromeric heterochromatin of either chromosomes 1, 9, 16 or Y [7,27]. Such DAPI-positive signals were detected in 24% of spontaneous male MN ($n = 50$, Table 4) and 12% of spontaneous MN of fem1 [7].

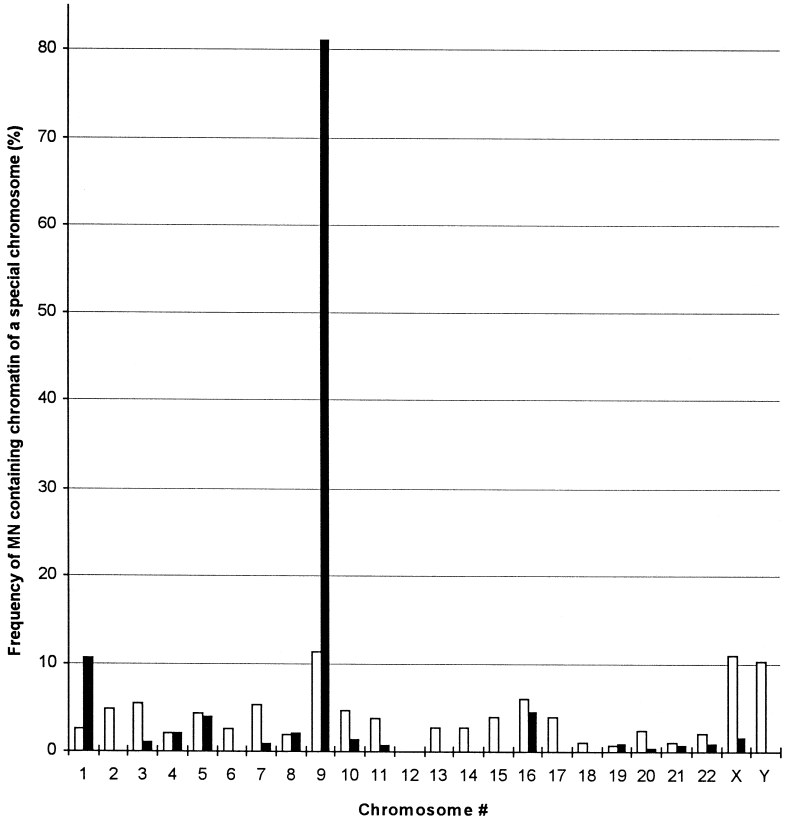

Fig. 1. Frequency of individual chromosomes in spontaneous (open bars) and IUdR-induced (solid bars) MN. For this diagram, the values of the male and the female (fem1) MN-study (see Table 3) were combined.

Table 4
Frequencies of MN (%) being positive for heterochromatic spots by DAPI staining, n = number of MN investigated

Proband	Spontaneous MN		IUdR-induced MN	
	DAPI+(%)	n	DAPI+(%)	n
fem1	12[a]	50	84	50
male	24	50	77.5	200

[a]This value is taken from our earlier study [7].

IUdR induced MN of fem1 contained DAPI-bright signals in 84%, the male proband showed DAPI-bright signals in 77.5% of IUdR-induced MN (Table 4).

4. Discussion

4.1. Structural metaphase analysis of control cultures

Control cultures showed only few (0–8%) structural chromosome abnormalities (Table 2). There were slight increases of aberrations in control cultures and with DMSO. Our frequency of spontaneous aberrations are corroborated by data in the literature, e.g., Refs. [28–31].

4.2. Spontaneous MN rates

Spontaneous MN rates of normal and DMSO treated cultures were in the range of 0.15–0.45% and are thus in agreement with previous studies [27,32–38]. Our results indicate that DMSO in the used concentration of 0.2% has no MN-inducing effect.

We performed the micronucleus test in a conventional way without using the cytochalasin b (CB)-method of Fenech and Morley [39]. The CB-treatment blocks cytokinesis but not karyokinesis resulting in double nucleated cells after the first cell division. Therefore, the occurring MN can be strictly assigned to cells which have finished the first division.

Although this method has several advantages (for reviews, see Refs. [2,40,41]) it was not suitable for our purposes, because the preserved cytoplasm interferes strongly with the FISH-technique. The DNA probes cannot penetrate efficiently and therefore we got only weak signals.

4.3. Frequency of human chromosomes in spontaneous MN

In the present study, the frequency of human chromosomes in spontaneous MN was not dependent on the size of chromosomes. These results are consistent with previous FISH investigations on interphase nuclei which showed that chromosomes 1 and 17 have similar loss rates [39]. The FISH results in MN are contrary to studies on metaphases which suggested that chromosome loss is correlated negatively with chromosome length [42,43]. This variance could be due to the different techniques applied. It seems also possible that cells tolerate the loss of small chromosomes better than that of large ones and therefore will enter metaphase more often.

Chromosome painting analysis of male MN (this investigation) and female MN [7] revealed that material of chromosomes 6, 8, 12, 18 and 19–22 is rarely included in spontaneous MN. While chromosome 12 was not found in MN of both probands, chromosome 19 missed only in MN of the female and chromosome 18 in MN of the male proband.

The increased occurrence of chromosome 9 in spontaneous MN of all three probands suggests a mechanistic link between heterochromatin decondensation and appearance of special chromosomes in MN and could be related to the presence of a large DAPI-positive heterochromatic block. However a female with an extremely large 9qh block did not show increased rates of chromosome 9 in MN [7]. Therefore the total amount of heterochromatin in a chromosome seems not to be a decisive factor. This disagreement could be explained by the fact that heterochromatic regions contain different types of satellite DNA. Especially in 9qh there is a complicated combination of all four types of satellite DNA [44], resulting in a multitude of polymorphism variants [45]. The composition and the amount of these different satellite DNA families within the 9qh block might be much more important than the size of the heterochromatic region.

In our study besides chromosome 9, only the X and Y chromosomes were frequently involved in spontaneous MN formation. There are several inves-

tigations pointing out a preferential loss of the X-chromosome in females and of the Y-chromosome in males, for review see Refs. [43,46].

In case of the Y-chromosome it is suggested that the elevated loss rates of this chromosome are related to accelerated shortening of the telomeres with increased cell division. This shortening of the telomeres influences chromosome-stabilising protein interactions with the adjacent heterochromatin, for review see [46].

Especially in older females the X-chromosome is preferentially lost. Recent studies could relate the loss to the inactivated X chromosome (X_i) [47–51]. Interestingly, MN-rates increase also with age and are associated with higher loss rates of X_i in older women [27,43,47,52–55]. This may be explained by the fact that the X_i is transcriptionally silenced [49].

The frequently observed occurrence of sex chromosomes in spontaneous MN of our probands is consistent with previous studies [27,52]. In our study the X chromosome occurred in quite similar frequencies in MN of young female and male probands. Therefore it seems unlikely that the inactivated female X-chromosome is more often included in MN as the active one. However this might change in older female.

4.4. Metaphase analysis after incubation with IUdR

IUdR caused few chromosomal gaps and breaks but led to undercondensations in metaphase chromosomes, preferentially to the decondensation of 9qh. The heterochromatin blocks of chromosomes 16 and Y were more rarely undercondensed as compared to chromosome 9, which is in agreement with a previous study [19]. In this study, the frequency of metaphases displaying decondensations of chromosome 9qh was higher than in our investigation. This could relate to interindividual variations of the sensitivity to drug treatment, because we observed distinct differences between the heterochromatin decondensation frequencies of the three probands tested.

With respect to heterochromatin undercondensations it is interesting that alpha and beta satellite DNA are not susceptible to decondensation inducing effects of 5-aza-C, while classical satellite DNA is undercondensed by 5-aza-C [56,57]. This might be related to a failure of proper heterochromatin formation since acetylation tends to maintain an open chromatin conformation [58].

4.5. Micronuclei analysis after incubation with IUdR

Treatment of lymphocytes with IUdR increased the MN rate approximately 12-fold with most micronucleated cells being associated with only one MN. These results corroborate those of Tommerup [19], who observed a quite similar increase of MN rates in IUdR treated lymphocytes.

In the present study, we could show that undercondensation of the centromeric heterochromatic regions in chromosome 9 led to its preferential occurrence in MN. The same effect was seen to a smaller extend for chromosome 1. This correlation is supported by the presence of DAPI-bright signals, which indicate heterochromatic material in about 80% of IUdR-induced MN (Table 4). The DAPI-positive MN can embody not only whole chromosomes but also chromosomal fragments containing parts of the heterochromatic block. This is consistent with the results of Tommerup [19] who demonstrated a 9h body in most MN containing a deleted long arm of chromosome 9. The author suggested that the presence of a 9h body can serve as a marker for the presence of a deleted chromosome 9q arm in a MN. Frequencies of 9p or q arms have to await painting analysis with arm specific probes which are now available [59]. To evaluate the ratio of whole chromosomes and chromosome fragments of chromosome 9 in IUdR-induced MN combined application of centromere and painting probes as described by Hindkjaer et al. [60] could also be informative.

Our results in chemically induced MN are somewhat contradictory to a chromosome paint analysis of MN induced by radiation [5]. The authors found a correlation between chromosome size and involvement in MN formation. This might be explained by a mostly random distribution of radiation damage in the genome. Chemical mutagens, on the other hand, target specific sub-genomic sites and thereby might induce a preferential loss of particular chromosomes or chromosomal fragments.

While IUdR treatment induced the preferential inclusion of chromosomes 1 and 9 in MN, other

chromosomes occurred very rarely or not at all (Table 3, Fig. 1). However, this effect probably does not reflect a real reduction of these chromosomes in MN. It seems more likely that we observed only a relative underrepresentation of other chromosomes caused by the extremely frequent occurrence of chromosome 9. This effect is obvious for the X- and Y-chromosomes which occur frequently in spontaneous MN but were found very rarely in IUdR-induced MN. On the other hand, the results from spontaneous MN indicate that the chromosomes are involved in MN formation not only by chance. As already mentioned, some chromosomes (especially 9, X, and Y) are included unexpected often. Others seem to be underrepresented. This might be true for chromosomes 4, 6, 8, 12, 13 and 18–22 which occur in less than 3% of spontaneous MN (combined results of present study and Fauth et al. [7]). It is of interest that most of these chromosomes (6, 12, 13, 18–21) are also very rarely seen (less than 1%) in IUdR-induced MN. Additionally, the chromosomes 12–14 and 21 were found very seldom in 5-aza-C-induced MN [7].

We conclude from these results that some chromosomes which are rarely included in spontaneous MN are also underrepresented in induced MN. However, this hypothesis must be confirmed by further studies.

Acknowledgements

We would like to thank Dr. Habil H. Scherthan for his methodical advice in the initial phases of the work and helpful discussions.

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