Toxicology Letters 183 (2008) 112–117

Contents lists available at ScienceDirect

Toxicology Letters

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

DNA-repair-deficient *Rad54*/*Rad54B* mice are more sensitive to clastogens than wild-type mice

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ARTICLE INFO

Article history:
Received 3 July 2008
Received in revised form 6 October 2008
Accepted 10 October 2008
Available online 21 October 2008

Keywords:
DNA-repair
Double-strand breaks
Clastogens
LacZ mutant frequencies
Chromosomal rearrangements

ABSTRACT

The sensitivity of DNA-repair-deficient *Rad54*/*Rad54B* mice for clastogens was studied and compared to that of wild-type mice. *LacZ* mutant frequencies (MF) in *Rad54*/*Rad54B* mice, after treatment with mitomycin C (MMC), bleomycin (BLM) and γ-irradiation, were compared to those of the wild-type mice following the same treatments. While none of the clastogens showed an induction of the *lacZ* MF in the wild-type mice, there was a significant increase of the *lacZ* MF in the bone marrow of the *Rad54*/*Rad54B* mice after treatment with BLM and γ-irradiation and in the spleen after MMC treatment. As expected, the positive control ENU showed a significant increase in the *lacZ* MF in all tested organs in wild-type mice. Mutant colonies were hybridized with total mouse DNA in order to discriminate between small gene mutations and large DNA rearrangements and translocations (size-change mutations). The hybridization studies showed a significant increase in mouse DNA positive clones 4 days after treatment with MMC and BLM in the bone marrow of the wild-type mice, which is indicative for chromosomal rearrangements and translocations to occur. An even more pronounced increase was seen 28 days after treatment with the same compounds in the *Rad54*/*Rad54B* mice.

1. Introduction

Humans are exposed, on a daily basis, to numerous chemicals such as those present in food/feed additives, packing materials, drugs, cosmetics and pesticides. It is of vital importance that before marketing, chemicals present in those products are evaluated for their potential adverse health effects. Genotoxic agents are a major threat to the integritiy of chromosomes and the viability of cells, specially if the damage is not repaired, because it can lead to chromosome instability, cell cycle arrest, cell dysfunction, induction of apoptosis or carcinogenesis (Kirkland et al., 2005). For genotoxicity, two main endpoints are gene mutations and chromosome aberrations; the latter can either be structural (clastogenic) or numerical (aneugenic). The strategy for the assessment of the potential genotoxicity of chemicals is assessed in short-term *in vitro* and *in vivo* genotoxicity tests covering these endpoints.

For all genotoxic endpoints reliable and relatively validated *in vitro* tests exist. A positive *in vitro* test triggers *in vivo* testing for the same genotoxic endpoint. In contrast to chromosomal aberration inducers, when a chemical induces gene mutations *in vitro*, there is not a validated and reliable *in vivo* gene mutation assay. The *in vivo* gene mutation assay with transgenic animals may be a suitable and justified alternative *in vivo* test (Thybaud et al., 2003; Lambert et al., 2005). Lac operator-based transgenic reporter mice and rats contain many copies of a bacterial reporter gene. These transgenic, mainly the bacterial *lacI* or *lacZ* genes, are present in a shuttle vector and are transmitted by the germ cells, and thus present in multiple copies of every cell including the germ cells. The two commercially available mouse models, Muta™Mouse and Big Blue® are able to detect point mutations and small deletions or insertions. The main restriction of these models is that large deletions cannot be detected because in order for the bacteriophage to infect *Escherichia coli* for the recovery of a lengthy insert, flanking cos-sites are essential. The transgenic mouse model with the *lacZ* transgene on a plasmid vector, the pUR288 plasmid mouse model designed by Boerrigter et al. (1995) can surpass this problem and is capable of detecting large deletions in addition to point mutations (>500 base pairs; Dollé et

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doi:10.1016/j.toxlet.2008.10.005

al., 1999; Mirsalis et al., 1995; Vijg et al., 1997). Large deletions or translocations may lead to the *ad random* fusion of the *lacZ* transgene with mouse chromosomal DNA, such mutations are referred to as "size-change mutations" (Dollé et al., 1999). The majority of these size-change mutations detected by the *lacZ* plasmid mouse model are chromosomal rearrangements (Busuttil et al., 2006). These include translocations, resulting from breaks and incorrect repair or DNA fusion. Therefore, both genotoxic endpoints may be assessed in one instead of two separate tests reducing the number of laboratory animals.

Previous studies investigating the capacity of the pUR288 plasmid mouse model to detect clastogens demonstrated some sensitivity towards clastogens, although the response was not very robust (Mahabir et al., 2008). A possible reason for this maybe that these mice are repair-proficient and are able to counteract clastogenic actions of compounds. In the present study we investigated the effect of DNA-repair on the sensitivity of pUR288 mice towards clastogenic compounds. Wild-type (WT) and *Rad54*/*Rad54B* repair-deficient (*Rad54*/*Rad54B*) mice (Essers et al., 1997; Wesoly et al., 2006), both harboring the *lacZ* gene, were exposed to three clastogens, mitomycin C (MMC), bleomycin (BLM) or γ-irradiation. The *Rad54*/*Rad54B* mutations, introduced to increase the number of chromosomal rearrangements, were compared to WT mice. The *Rad54* and *Rad54B* genes are involved in homologous recombination (HR) repair. Deletion of *Rad54* and *Rad54B* results in defective HR, and might result in a shift towards other repair systems for chromosomal breaks like non-homologous end-joining (NHEJ) repair. As NHEJ is error-prone, it may repair the initial damage erroneously, leading to detectable chromosome rearrangements, as compared to the wild-type situation. The *in vivo* micronucleus test (MN test) was used as a classical control for detection of clastogenic properties of the compounds used.

2. Animals, materials and methods

2.1. Animals and treatments

Animal protocols were approved by the institutional animal ethics committee. Eight- to 12-week-old wild-type (WT) and *Rad54*/*Rad54B* repair-deficient (*Rad54*/*Rad54B*) mice both harboring pUR288 plasmids (*lacZ*) were bred and maintained under specific pathogen-free conditions at the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Mice were weighed before dosing and on the day of necropsy. Mice were *intraperitoneally* (*ip*) treated with 1.25 mg/kg body weight (bw) MMC (CAS no. 50-07-7), 2.5 mg/kg bw BLM (CAS no. 9041-93-4), 100 mg/kg bw ENU (only in WT mice, CAS no. 759-73-9), all in a volume of 100 μl phosphate buffered saline (PBS) or irradiated with 5 Gy γ-irradiation. Untreated control mice of both genotypes received (through *ip*) the vehicle PBS in a volume of 100 μl.

Both at 4 and 28 days after treatment, mice were sacrificed by cervical dislocation. Tissues (bone marrow, liver and spleen) were collected, snap frozen in liquid N_2 and stored at −80 °C until used for DNA isolation. At the same time points three drops of peripheral blood were collected by orbital punction in EDTA coated tubes for the micronucleus test. The remaining peripheral blood was collected in EDTA coated tubes for histological analysis.

2.2. LacZ gene mutation assay with transgenic animals

The mutant frequency (MF) in WT and *Rad54*/*Rad54B* mice was determined using a procedure described by Dollé et al. (1996). Briefly, pUR288 plasmids were rescued from total genomic DNA (20–50 μg) using magnetic beads coated with the *lacZ*/*lacI* fusion protein. After detachment from the beads, these plasmids were subsequently transfected into electrocompetent *E. coli* strain C (*lacZ*$^-$ *galE*$^-$). A fraction (2 μl of the 2 ml total) of the bacterial sample was plated on nonselective 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) plates to determine the rescue efficiency; the remainder onto selective phenyl-β-D-galactoside (P-gal) plates to select for mutants. The *lacZ* mutant frequency was calculated by dividing the number of mutants by the total number of rescued colonies × dilution factor (1000).

2.3. Hybridization of mutant colonies with total mouse DNA

LacZ mutants that originate from aberrant translocation events and that carrying "ad random" mouse DNA fragments were made visible through hybridization using total mouse DNA as a probe. Hereto total mouse DNA (250 ng in MilliQ-UF) was incubated with HindIII in digestion mix (100 mM Tris pH 7.6, 80 mM $MgCl_2$ and 10 mM DTT). After digestion, the DNA was stored at −20 °C. A Gene Images Random Prime Labeling kit was used containing nucleotide mix, primer and enzyme solution (Klenow). A maximum of 50 ng total mouse DNA was denaturized at 100 °C and put on ice. After centrifugation, the DNA was collected and a mixture of nucleotide mix, primer and enzyme solution, was added before incubation at 37 °C for 1 h. The reaction was stopped by adding 0.5 M EDTA pH 8.0 and the mixture was put on ice. This labeled DNA was used as a probe for hybridization of the mutant pUR288 clones.

LacZ mutant colonies were grown overnight on selective P-gal plates at 37 °C and the individual colonies were grafted on a Hybond-N$^+$ filter (Amershan). To obtain exclusively DNA bound to the filter, the cells were lysed and washed twice with a denaturation buffer and twice in a neutralization buffer to remove cell debris and unbound DNA. The filter was washed with 2× SSC (3 M sodium chloride and 0.3 M sodium citrate) and dried avoiding contact with other DNA sources. For the hybridization the pre-hybridization mix (1 M NaCl, 10% dextran sulphate and 1% SDS) was pre-warmed at 65 °C. Herring sperm DNA solution was denaturized at 100 °C for 5 min and added to the pre-hybridization mix (1/100, v/v) solution. The mixture was pre-hybridized at 65 °C for approximately 3 h. The probe was denaturized at 100 °C for 5 min. Before adding the probe to the hybridization mixture and the blot, it was allowed to cool on ice. After adding the probe to the hybridization mixture, the blot was hybridized overnight at 65 °C while shaking carefully. After washing once with 1× SSC (3 M sodium chloride and 0.3 M sodium citrate) and 0.1% SDS, and once with 0.5× SSC (3 M sodium chloride and 0.3 M sodium citrate) and 0.1% SDS, the blot was packed in wrap foil (Saran), put against a Phosphor imager (Storm 860, Molecular Dynamics, GE Healthcare Bio-Sciences) and illuminated. Mutants that hybridized with mouse DNA were counted.

2.4. The peripheral blood micronucleus test

The peripheral blood samples of treated and control WT and *Rad54*/*Rad54B* mice were analyzed using acridine orange staining (Hayashi et al., 1990). Slides were coated with acridine orange (10 μl, 1 mg/ml) on a pre-heated plate (65 °C). Eight microliters of FBS (fetal bovine serum) and 3 μl blood was pipetted on slides and covered with a cover slip. The slides were analyzed under a Zeiss Axioscope fluorescence microscope. Per animal, the frequency of MN was analyzed in 1000 polychromatic erythrocytes (PCE) in both WT mice and *Rad54*/*54B* mice. The percentage PCEs in 2000 normochromatic erythrocytes (NCE) was determined as an indicator of cytotoxicity in both mouse models.

2.5. Statistical analysis

The mean *lacZ* plasmid mutant frequency, the percentages of micronucleated PCEs and the size-change mutations (chromosomal rearrangements) for the different treatment groups were tested for significance with the Student's *t*-test.

3. Results

3.1. LacZ gene mutation assay with transgenic animals

WT and *Rad54*/*Rad54B* mice were treated *ip* once with PBS (negative control) and three different clastogens, MMC, BLM or γ-irradiation. Only WT mice were treated with the mutagen ENU, which served as a positive control for the *lacZ* plasmid rescue procedure. The *lacZ* mutant frequency was determined 4 and 28 days after treatment in bone marrow, liver and spleen (Figs. 1 and 2). In the WT mice, no consistent increase in the *lacZ* MF was found in all tissues tested after treatment with the clastogens compared to the untreated control. However, in *Rad54*/*Rad54B* mice treated with BLM and γ-irradiation a slight but statistically significant ($p < 0.05$) increase in the *lacZ* MF in the bone marrow compared to the untreated control was found (Fig. 2A). This increase was already observed 4 days after treatment with BLM. This result may indicate that the *Rad54*/*Rad54B* mice are more sensitive to clastogens compared to the WT mice. Although there was no consistent increase in the *lacZ* MF of the liver in the *Rad54*/*Rad54B* mice after treatment with all compounds, there was a rather high background *lacZ* MF in the untreated control compared to the untreated control in the liver of WT mice.

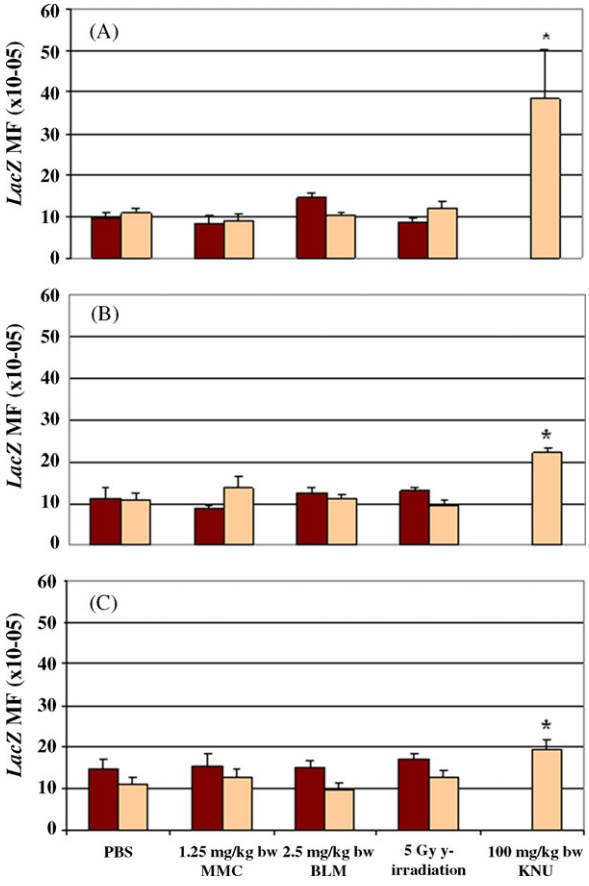

Fig. 1. The *lacZ* MF in WT mice 4 (black bars) and 28 days (grey bars) after treatment with PBS (negative control), ENU (positive control), clastogens MMC, BLM and γ-irradiation, were given at the indicated doses. (A) Bone marrow, (B) liver and (C) spleen.

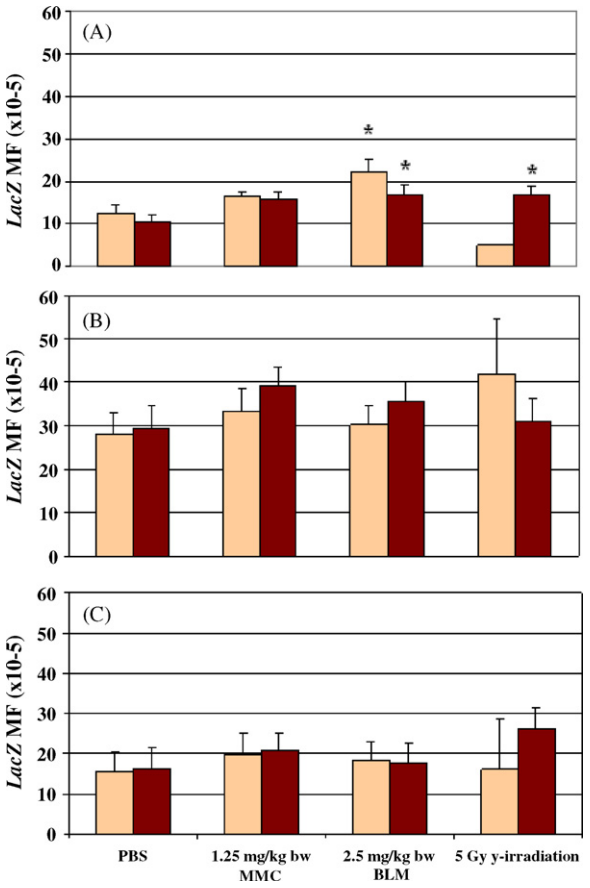

Fig. 2. The *lacZ* MF in *Rad54*/*Rad54B* mice 4 (black bars) and 28 days (grey bars) after treatment with PBS (negative control), MMC, BLM and γ-irradiation. (A) Bone marrow, (B) liver and (C) spleen.

As expected, the positive control compound ENU, tested in WT mice only, showed a statistically significant ($p < 0.05$) increase in *lacZ* MF in all tissues analyzed compared to the untreated control.

3.2. Hybridization of mutant colonies with total mouse DNA

The percentages of mutant colonies with size mutations and chromosomal rearrangements were determined by hybridization with total mouse DNA in the bone marrow of WT and *Rad54*/*Rad54B* mice after treatment with the different compounds (Fig. 3). In WT mice there was an increase in the percentages of mouse DNA positive colonies in the bone marrow only 4 days after treatment with MMC and BLM compared to the untreated control. Interestingly, an almost similar increase was seen 28 days after treatment with MMC and BLM in the bone marrow of the *Rad54*/*Rad54B* mice with an intermediate result 4 days after treatment with MMC only. In contrast, no significant increase in the percentages of mouse DNA positive clones were found 28 days after γ-irradiation in both mouse models nor, as expected, after ENU treatment in WT mice (data not shown).

3.3. Micronucleus test

The MN test was performed as a classical control for detection of clastogenic properties of the compounds used. The percentages of PCEs (Tables 1 and 2), which is an indication of cytotoxicity, was decreased statistically significant in the blood of all treated mice,

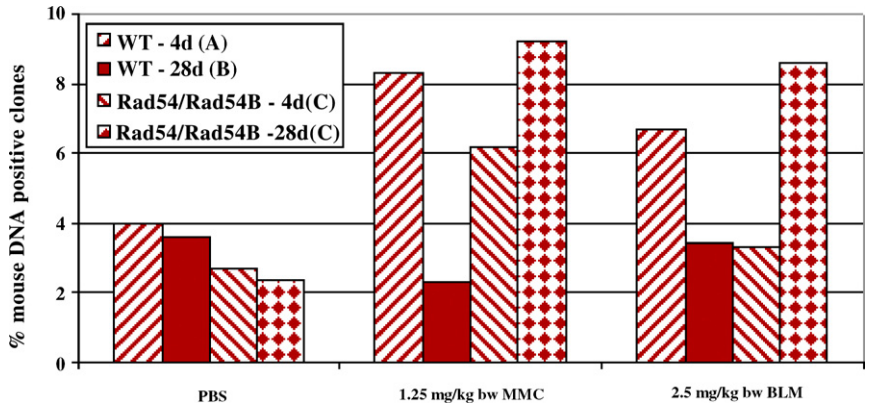

Fig. 3. Quantification of mouse DNA positive *lacZ* mutant clones from the bone marrow of WT and *Rad54*/*Rad54B* mice 4 and 28 days upon treatment with PBS (negative control), MMC and BLM. (A) WT mice 4 days after treatment, (B) WT mice 28 days after treatment, (C) *Rad54*/*Rad54B* mice 4 days after treatment and (D) *Rad54*/*Rad54B* mice 28 days after treatment.

except for the *Rad54*/*Rad54B* mice 4 days after treatment with BLM. This decrease is an indication of sufficient exposure of the target cells of both genotypes.

A statistically significant increase in the percentages of MN was seen after treatment with all clastogens 4 and 28 days after treatment in both genotypes. There were no significant differences in the induction of MN between the two genotypes.

Unexpectedly, ENU, considered as a compound which predominantly induces point mutations and thus expected to be negative in the MN test (Asita et al., 1992), showed a higher and statistically significant increase in cells with MN in WT mice.

4. Discussion

In the present paper, we investigated whether clastogenic properties of compounds can be detected in mice harboring the *lacZ* gene. This was performed by exposure of these mice to three clastogens: MMC, BLM or γ-irradiation. To increase the sensitivity of

Table 1
Micronucleus induction in peripheral blood of WT mice 4 and 28 days after treatment.

Compound	Dose (mg/kg bw)	Animal number	WT mice (4 days)		WT mice (28 days)	
			%MnPCE (mean ± S.E.M.)	%PCE (mean ± S.E.M.)	%MnRET (mean ± S.E.M.)	%PCE (mean ± S.E.M.)
PBS	–	1	2.60	3.50	2.20	5.00
		2	3.80	3.10	4.20	4.35
		3	2.20	2.60	3.10	3.70
		4	2.40	3.80	2.80	3.40
		5	2.40	3.35	3.00	3.00
		6	3.20	3.00	4.60	2.80
			2.77 ± 0.25	3.23 ± 0.17	3.23 ± 0.17	3.71 ± 0.34
ENU	100	1	ND[a]	ND	5.40	2.95
		2			3.90	2.95
		3			6.00	2.10
					5.10 ± 0.62*	2.67 ± 0.28*
MMC	1.25	1	6.40	1 30	7.00	1.90
		2	7.00	1.70	6.20	2.20
		3	6.20	1.55	5.50	3.00
		4	6.80	1.50	7.60	1.70
		5	4.20	1.60	7.40	2.00
		6	4.00	1.80	7.00	2.10
			5.77 ± 0.54**	1.58 ± 0.07**	6.78 ± 0.32**	2.15 ± 0.18**
BLM	2.5	1	5.00	1.80	6.00	2.10
		2	5.70	2.65	4.60	3.00
		3	4.80	2.20	5.50	3.20
		4	6.60	1.70	7.00	2.50
		5	3.70	4.75	5.90	2.75
		6	3.30	2.35	4.00	2.40
			4.85 ± 0.50**	2.58 ± 0.46*	5.50 ± 0.44*	2.66 ± 0.17*
γ-Irradiation	5 Gy	1	ND	ND	2.80	2.90
		2			7.40	3.15
		3			8.00	2.70
		4			5.10	4.15
		5			6.60	2.00
		6			4.30	4.00
					5.70 ± 0.81*	3.15 ± 0.33*

[a] Not determined.
* $p < 0.05$.
** $p < 0.001$.

Table 2
Micronucleus induction in peripheral blood of *Rad54*/*Rad54B* mice 4 and 28 days after treatment.

Compound	Dose (mg/kg bw)	Animal number	*Rad54*/*Rad54B* mice (4 days)		*Rad54*/*Rad54B* mice (28 days)	
			%MnPCE (mean ± S.E.M.)	%PCE (mean ± S.E.M.)	%MnRET (mean ± S.E.M.)	%PCE (mean ± S.E.M.)
PBS	–	1	2.30	3.40	2.70	2.85
		2	2.90	3.05	2.00	3.05
		3	2.20	2.60	2.40	3.25
		4	2.30	4.10	1.70	3.60
		5	2.10	3.15	8.80	2.20
		6	3.30	3.30	2.00	3.10
		7	2.52 ± 0.18	3.27 ± 0.20	2.20	2.50
					3.11 ± 0.96	2.94 ± 0.18
MMC	1.25	1	8.00	1.50	5.10	1.90
		2	7.50	1.60	6.60	1.55
		3	6.40	1.50	7.20	2.05
		4	8.40	1.70	8.50	1.90
		5	8.20	1.55	8.60	1.65
		6	6.40	1.70	4.70	2.10
		7	3.90	3.00	6.78 ± 0.67*	1.86 ± 0.09**
		8	7.20	1.70		
			7.00 ± 0.52**	1.78 ± 0.18**		
BLM	2.5	1	7.50	2.00	6.90	2.15
		2	5.80	2.15	6.10	2.10
		3	4.40	4.90	5.90	2.25
		4	5.60	2.10	7.40	1.70
		5	5.10	2.45	5.40	1.95
		6	8.20	2.25	5.40	1.50
			6.10 ± 0.59**	2.64 ± 0.46	6.18 ± 0.33*	1.94 ± 0.12**
γ-Irradiation	5 Gy	1	ND[a]	ND	7.50	1.40
		2			6.30	1.85
		3			5.00	2.25
		4			5.50	2.35
		5			4.50	2.85
		6			5.10	2.05
					5.65 ± 0.44*	2.13 ± 0.20*

[a] Not determined.
* $p < 0.05$.
** $p < 0.001$.

mice towards clastogens, we used mice (also harboring the *lacZ* gene) with a defect in the *Rad54* and *Rad54B* genes. These genes are involved in the HR repair. With a defect in the HR, there may be a potential shift in the repair of DNA double-strand breaks towards NHEJ repair. NHEJ is an error-prone mechanism causing an accumulation of chromosomal damage, which can lead to an increase in gene mutations.

None of the clastogens tested showed an absolute induction in the *lacZ* MF in the bone marrow, liver and spleen of the WT mice compared to the untreated control at 4 days and 28 days. The lack of *lacZ* MF induction in the WT mice could not be ascribed to the performance of the test because ENU, the positive control (Suzuki et al., 1997; Wang et al., 2004), showed a statistically significant *lacZ* MF induction in all organs tested. In contrast, exposure to BLM (both at 4 and 28 days after treatment) and γ-irradiation (only 28 days after treatment) showed a slight but statistically significant induction in the *lacZ* MF in the bone marrow of the *Rad54*/*Rad54B* mice. In the other tissues tested no statistically significant induction of *lacZ* MF was observed.

Surprisingly, there was a higher background *lacZ* MF found in the liver of the *Rad54*/*Rad54B* mice. This higher background is probably due to deficiencies in the *Rad54* and the *Rad54B* genes. *Rad54*/*54B* mutants cause an accumulation of mutations and a higher *lacZ* MF baseline frequency in some, but certainly not all, organs. We propose that in fast proliferating tissues, like the spleen and bone marrow, there is a selection in cell division on cells having no DNA damage, leading to the suppression of the *lacZ* MF, even in a DNA-repair-deficient background. This selection mechanism is absent in slow or non-proliferating (somatic) tissues like the liver.

The responses of *in vivo* assays are a reflection of different processes such as absorption, distribution, metabolism and elimination. The low *lacZ* MF induction upon clastogen exposure could be due to an inappropriate route of administration. This could result in insufficient exposure of the target cells to the different compounds, hence leading to low induction in *lacZ* MF. Lynch et al. (2008) showed that bioavailability is important for the effect of compounds on different tissues/organs. In their study, BLM was found both clastogenic and cytotoxic to the bone marrow when administered *ip*, but not when given orally. Thus bio-availability is an important factor in the genotoxicity testing of compounds *in vivo*. However, in our present study a decreased % of PCEs after treatment in the MN test indicated that the target organ (bone marrow) was clearly exposed to the various compounds.

Since we found only a slight induction of the *lacZ* MF in the *Rad54*/*Rad54B* mice, we wanted to investigate whether there was a difference in a "mutational fingerprint" between the two genotypes. This was performed by hybridizing the mutant colonies with totally labeled mouse DNA. A positive signal is indicative for large deletions (size-change mutations) or chromosomal rearrangements (Dollé et al., 1999).

An increase was seen both in WT and *Rad54*/*Rad54B* mice. Interestingly, the response in the bone marrow of WT mice was most overt 4 days after treatment, whereas the response in *Rad54*/*Rad54B* mice was optimal 28 days after treatment with both MMC and BLM (see Fig. 3). An explanation for this finding could be that WT mice, having active DNA-repair, are able to repair DNA damage caused by MMC and BLM. Apparently, 4 days after treatment this repair is not complete yet, leading to size-change mutations at this

point. In contrast, the *Rad54*/*Rad54B* mice have compromised HR repair, consequently, leading to a shift in the repair of DNA double strands through NHEJ repair, which is not error free. Apparently, size-change mutations accumulate under these conditions and are highest 28 days after treatment with the clastogens MMC and BLM. Based on these findings we propose that the pUR288 model is promising in detecting clastogens, but the optimal conditions are not established yet.

Unexpectedly, we observed that ENU induced MN in peripheral blood of WT mice (Table 1). However, it is known from the literature (Douglas et al., 1996) that ENU is capable to form various types of DNA adducts. One of these, N7-ethylguanine, may easily lead to apurinic sites, which on their turn may result in chromosomal damage causing this compound to be positive in the MN test.

In conclusion, the aim of this study was to investigate whether the *lacZ* plasmid mouse model is capable of detecting clastogenic effects of compounds. Although there was no clear induction in the *lacZ* MF after treatment with the clastogens in WT mice and only a subtle induction in the *lacZ* MF in the bone marrow of *Rad54*/*Rad54B* mice, the hybridization studies demonstrate an induction of rearranged *lacZ* variants carrying mouse chromosomal DNA after treatment with MMC and BLM. These results indicate that the pUR288 model still may be promising enough for the detection of clastogenic properties of compounds. Apparently, the correct conditions are not established yet. Next, the present approach is quite laborious and therefore, certainly is not suitable for high-throughput testing. It may be recommendable to develop an *in vitro* system using cells obtained form the mice used in this study to find out whether the plasmid system is not only promising but indeed capable of efficiently recognizing clastogens. The latter approach, if successful, will have an additional advantage, in that far less animals will be required for testing.

Conflict of interest

None declared.

Acknowledgements

The authors wish to thank the animal technicians of the animal facilities of the Netherlands Vaccine Institute (NVI, The Netherlands) and Mr. Jan de Wit of the Erasmus Medical Centre (Erasmus MC, Rotterdam, The Netherlands) for his help with the γ-irradiation studies. Dr. Lya Hernandez is acknowledged for critical reading of the manuscript.

The study was financially supported by ZonMW project number 3170.0068.

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