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

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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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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.