Mutation Research 741–742 (2013) 11–17

Contents lists available at SciVerse ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

No evidence for transgenerational genomic instability in the F1 or F2 descendants of Muta™Mouse males exposed to N-ethyl-N-nitrosourea

Jason M. O'Brien, Andrew Williams, John Gingerich, George R. Douglas, Francesco Marchetti, Carole L. Yauk*

Environmental Health Science and Research Bureau, Health Canada, Ottawa, ON K1A 0K9, Canada

ARTICLE INFO

Article history:
Received 11 December 2012
Received in revised form 27 February 2013
Accepted 28 February 2013
Available online 7 March 2013

Keywords:
Mouse
Germline
Mutation
Micronuclei

ABSTRACT

Exposure of male mice to genotoxic agents can increase mutation frequencies in their unexposed descendants. This phenomenon, known as transgenerational genomic instability (TGI), can persist for several generations. However, little is known about the underlying mechanisms. Chemically-induced TGI has been demonstrated in non-coding unstable tandem repeat DNA regions, but it is unclear whether it extends to other genetic endpoints. We investigated whether exposure of Muta™Mouse males to a single dose of 75 mg/kg N-ethyl-N-nitrosourea (ENU) increased the spontaneous frequency of gene mutations or chromosome damage in their offspring. Treated males were mated with untreated females 3 days, 6 weeks or 10 weeks post-exposure to produce the F1 generation. Offspring were thus conceived from germ cells exposed to ENU as mature spermatozoa, dividing spermatogonia, or spermatogonial stem cells, respectively. F2 mice were generated by mating F1 descendants with untreated partners. Mutations in the *lacZ* transgene were quantified in bone marrow and micronucleus frequencies were evaluated in red blood cells by flow-cytometry for all F0 and their descendants. *LacZ* mutant frequencies were also determined in sperm for all exposed males and their male descendants. In F0 males, *lacZ* mutant frequencies were significantly increased in bone marrow at least 10-fold at all three time points investigated. In sperm, *lacZ* mutant frequency was significantly increased 7–11-fold after exposure of dividing and stem cell spermatogonia, but not in replication-deficient haploid sperm. Micronucleus frequencies assessed two days after ENU treatment were increased 5-fold in F0 males, but returned to control levels after 10 weeks. Despite the strong mutagenic response in F0 males, pre- and post-meiotic ENU exposure did not significantly increase *lacZ* mutant or micronucleus frequencies in F1 or F2 offspring. These findings suggest that TGI may not extend to all genetic endpoints and that further investigation of this phenomenon and its health relevance will require multiple measures of genomic damage.

1. Introduction

There is ample evidence in rodents and mounting evidence in humans that exposure to mutagens can lead to germ cell DNA damage and germline mutations that can be inherited [1]. Heritable germ cell mutations and chromosome aberrations can cause numerous potential health disadvantages including an increased risk for genetic disease and a higher predisposition to cancer. However, a possibility that is of greater concern than the issue of heritable mutations, is the emerging evidence that the ability of some mutagens to induce new mutations is not constrained solely to the directly exposed individual, but can also manifest in their unexposed descendants [2–4]. This phenomenon, which has been dubbed transgenerational genomic instability (TGI), may have profound cross-generation health implications. TGI is characterized by an increased baseline mutation frequency in all descendants of the exposed individual (not only those that inherited a germline mutation) that is propagated for several generations. Because TGI is expressed in all offspring it cannot be due to an inherited gene-specific mutation, which would occur at a much lower frequency (e.g., mutation in DNA-repair gene in sperm leading to a mutator phenotype in the offspring). Additionally, because the effect is persistent for several generations, it cannot be the result of a delayed destabilization resulting from the inheritance of damaged paternal DNA (e.g., sperm DNA containing pre-mutational lesions). TGI is, therefore, most likely inherited through epigenetic means. Although TGI is typically transmitted through the germline of exposed males, it appears to be a non-targeted effect, given the

Abbreviations: ENU, N-ethyl-N-nitrosourea; ESTR, expanded simple tandem repeat; MN, micronuclei; TGI, transgenerational genomic instability.

* Corresponding author at: Environmental Health Centre, 50 Colombine Driveway, Tunney's Pasture, 0803A, Ottawa, ON K1A 0K9, Canada. Tel.: +1 613 941 7376; fax: +1 613 941 8530.
E-mail address: carole.yauk@hc-sc.gc.ca (C.L. Yauk).

0027-5107/$ – see front matter.
http://dx.doi.org/10.1016/j.mrfmmm.2013.02.004

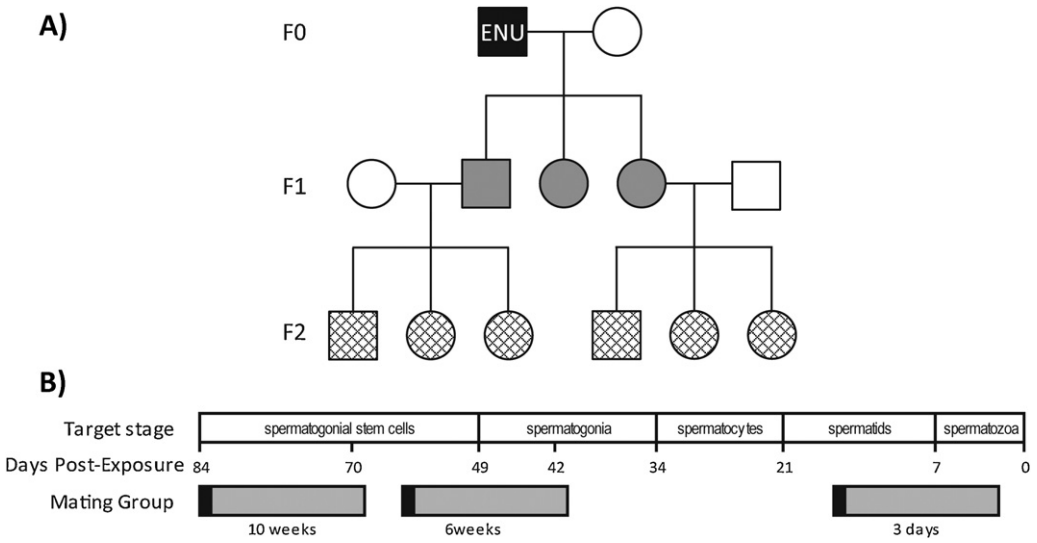

Fig. 1. Pedigree and mating regime. (A) Pedigree diagram. Males and females are represented by squares and circles, respectively. The ENU exposed F0 male is shaded black; F1 and F2 descendants of the F0 male are grey and hatched shapes, respectively. Unexposed breeding mates are represented by white shapes. (B) Stages of spermatogenesis targeted by delayed matings and tissue collection following exposure to ENU. Grey boxes represent stages targeted by the 2-week mating periods. Black boxes represent the stages targeted by the *lacZ* mutation assays (time of epididymis collection).

evidence that maternally inherited alleles are equally vulnerable to destabilization in the offspring of mutagen-exposed male mice [5]. The consequences of TGI may have very significant health ramifications, yet little is known about the biological mechanisms involved or its broad effects across the genome.

TGI occurs in rodents exposed to ionizing radiation [2,4,6–8] and recent evidence suggests that it is also induced by chemical mutagens [9,10]. The majority of studies that investigated TGI at the molecular level have focused on non-coding, highly unstable, expanded simple tandem repeat (ESTR) DNA regions. A few studies have demonstrated TGI induction across a spectrum of genetic endpoints in mice [2,3], but these have focused on the effects induced by relatively high doses of radiation [3]. Thus, the extent to which the genome is subject to TGI following exposure to physical and chemical mutagens is unclear. To address this issue, we employed the transgenic Muta^{TM}Mouse model to measure the mutant frequency of a non-ESTR protein-encoding *lacZ* transgene (transcriptionally silent in the mouse) in somatic and germ cells of male mice exposed to the model chemical mutagen N-ethyl-N-nitrosourea (ENU) and in their first- and second-generation descendants. We also examined the frequency of micronuclei (MN) in red blood cells, which is a traditional measure of chromosome damage and is a frequently measured endpoint in risk assessment test batteries [11]. Based on the broad TGI response observed in irradiated mice [3], we hypothesized that ENU-induced TGI impacts multiple genetic endpoints, and that descendants of exposed males should thus exhibit increased mutation frequencies at the *lacZ* transgene and an increase in MN frequency relative to descendants of unexposed males. Additional objectives of this study were to compare the responses between *lacZ* mutant and MN frequencies, and determine which, if any, male germ cell stages are most sensitive to induction of TGI.

2. Materials and methods

2.1. Mouse strain, dosing, and mating

A colony of the transgenic mouse strain 40.6 (Muta^{TM}Mouse) is maintained at Health Canada facilities. This strain harbors ~29 tandem copies of a recombinant λgt10 phage vector containing an *Escherichia coli lacZ* gene on both copies of chromosome 3 [12]. Adult male mice (14–15 weeks old) were administered a single dose of 75 mg/kg N-ethyl-N-nitrosourea (ENU) dissolved in phosphate buffered solution (PBS) via intraperitoneal (i.p.) injection (0.5 μl/g injection volume). Control males were given a single injection of PBS solution. Food and water were available to all animals ad libitum for the duration of the experiment. To produce the F1 generation, treated and control males were mated with unexposed adult females at three different time-points: 3 days, 6 weeks, or 10 weeks following ENU exposure. The F2 generation was produced by mating 10-week old F1 male and female offspring to unexposed adult partners from the Muta^{TM}Mouse colony (Fig. 1; the number of male and female mice from each group are indicated in Supplementary Table 1). Male F0 and F1 mice were euthanized after a 2-week breeding period. Female F1 mice were euthanized after F2 offspring were weaned (~6–7 weeks after breeding was initiated). Male and female F2 mice were euthanized at ~10–11 weeks of age. Bone marrow and peripheral blood were collected from all mice immediately following euthanasia by cervical dislocation and caudal epididymides were collected from all males. Bone marrow was collected by flushing femurs with PBS, centrifuging the suspension and freezing the resulting pellet in liquid nitrogen. Bone marrow pellets and cauda were stored at −80 °C until DNA isolation. Blood was stored briefly (<6 h) in anti-coagulant (Litron Laboratories, Rochester, NY, USA), and then processed for MN determination. All animal procedures were conducted according to protocols approved by Health Canada's Animal Care Committee.

Because of on-going breeding we did not collect blood for MN analysis for the F0 males of the 3-day post-exposure mating group. To fill this data gap, we substituted data from a separate ENU exposure study that employed a similar experimental design. For these samples, blood for MN determination was collected from 14-week old Muta^{TM}Mouse males 2 days following a single dose of 50 mg/kg ENU by oral gavage. This additional exposure was performed >2 years later than all other exposures described in the present study. The data is presented here to demonstrate that the dose utilized to investigate TGI was sufficient to induce a strong clastogenic response in the bone marrow of exposed animals.

2.2. DNA isolation

DNA from bone marrow was isolated as previously described [13]. Briefly, frozen bone marrow pellets were defrosted and incubated at 37 °C overnight with rotation in 5 ml of lysis buffer (10 mM Tris, pH 7.6, 10 mM EDTA, 0.1 M NaCl, 1% SDS, 1 mg/ml proteinase K), followed by a phenol/chloroform and chloroform/isoamyl alcohol extraction. DNA was precipitated in ethanol, and dissolved in 100 μl TE buffer (10 mM Tris pH 7.6, 1 mM EDTA). Sperm DNA was isolated as described in [14]. Briefly, caudal epididymides were minced in PBS and filtered through mesh filters. Filtrates were spun down and the pellet resuspended in cold 1× SSC (0.15 M NaCl, 15 mM trisodium citrate, pH 7.0). Somatic cells were lysed by the addition of 0.15% SDS. Sperm were pelleted by centrifugation, resuspended in 0.2× SSC, digested overnight at 37 °C with rotation after the addition of 1 M 2-mercaptoethanol, 10 mM EDTA, 1% SDS, and 1 mg/ml proteinase K. DNA was extracted 4× with phenol/chloroform, once with chloroform/isoamyl alcohol, precipitated by ethanol and dissolved in 40 μl TE buffer.

2.3. LacZ mutation assay

The mutant frequency of the *lacZ* gene in the λgt10 phage vector was determined by a P-Gal positive selection assay as previously described [15,16]. Briefly, single copies of the phage vector were isolated from bone marrow or sperm DNA