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RELATIONSHIP BETWEEN UNSCHEDULED DNA SYNTHESIS AND MUTATION
INDUCTION IN MALE MICE*

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Abbreviations: dThd, thymidine; EMS, ethyl methanesulfonate; LSC, liquid scintillation counting; 7 MeG, 7-methylguanine; MMS, methyl methanesulfonate.

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SUMMARY

Unscheduled DNA synthesis (UDS) induced in the germ cells of male mice by chemical and physical agents can be studied in vivo by making use of the timing of spermatogenesis and spermiogenesis. In meiotic and post-meiotic germ-cell stages UDS occurs from leptotene through mid-spermatid stages but is not detected in later stages. No consistent correlation has been seen between the occurrence of UDS in the germ cells and reduced dominant-lethal frequencies or reduced specific-locus mutation frequencies. It is suggested that the UDS observed in the germ cells may be principally involved in the removal of DNA lesions which, if left, could give rise to subtle genetic damage that current mammalian genetic tests may not be able to detect. Characterization of mouse stocks with reduced UDS capability in their germ cells plus the development of biochemical genetic markers that can measure single amino acid substitutions will likely be necessary before the relationship between UDS in mammalian germ cells and repair of genetic damage can be clearly established.

The first evidence for UDS in mammalian somatic cells was provided by Rasmussen and Painter [17] when they demonstrated that UV radiation induced the uptake of labeled thymidine into the DNA of cultured HeLa and Chinese hamster cells grown in culture. Later, Kofman-Alfaro and Chandley [11], also using in vitro procedures, were able to demonstrate UDS in spermatogenic cells of the mouse after exposure to X-rays or UV. Similar findings were observed in rat germ cells [10] and in human germ cells [5] after in vitro UV exposure.

The induction of UDS in mammalian germ cells can also be studied by in vivo procedures. In the male mouse it is possible to study UDS in vivo in meiotic and post-meiotic germ cell stages by making use of the sequence of events that occur during spermatogenesis and spermiogenesis. In developing male germ cells the last DNA synthesis takes place during a 14-h period [14] in pre-leptotene spermatocytes. After DNA synthesis these spermatocytes continue to develop through a series of germ cell stages for about a 28- to 30-day time period [15, 16, unpublished data of author] before late spermatids leave the testes and enter the caput epididymides. The developing sperm reach the caudal epididymides 2-3 days later, and in 2-3 days more they enter the vas. If the DNA from any meiotic or post-meiotic germ-cell stage is damaged by a physical or chemical agent and UDS is induced, it can be detected by an unscheduled incorporation of [^3H] dThd into the affected germ cell. The unscheduled uptake of [^3H] dThd can be measured either directly, by examining the affected germ-cell stages by use of autoradiography [30], or indirectly, by waiting until the germ cells have matured into sperm in the caudal epididymides and vasa deferentia and then assaying the [^3H] dThd activity contained in several million of these sperm by use of LSC [23, 25, 26].

Significance of UDS in germ cells

The ability of a chemical agent or any of its metabolites to induce a UDS response in mouse germ cells indicates that DNA lesions have been induced and the germ cells are presumably attempting to restore the integrity of the damaged DNA. Although the term "DNA repair" has at times been used synonymously for UDS, it can be misleading. There is no way currently available to know for certain that "repaired" DNA has been restored to its original condition through the process of UDS. We can only say that it does not seem likely that UDS induced in mouse germ cells represents terminal addition of deoxynucleotides to broken ends of DNA, since no terminal transferase has been detected in mouse testes [1, 2].

Also, unpublished data we have collected suggest that excision of alkylated DNA bases is associated with the UDS response. At various times after i.p. injection of [3 H]MMS, testes and vas sperm heads were recovered and DNA was extracted and then hydrolyzed under mild conditions similar to those described by Lawley [12]. The hydrolysates were analyzed on Sephadex G-10 columns to determine guanine and 7 MeG content.

The half-life of 7 MeG in testicular DNA was found to be ~2 days (Fig. 1), which is considerably shorter than its reported half-life of ~6 days in DNA in vitro [13]; this implies that 7 MeG may be enzymatically excised from testicular DNA in vivo. At the same time the half-life of 7 MeG in sperm DNA is ~12 days, if it is assumed that the initial level of DNA alkylation in all of the stages is approximately the same. We have previously noted [24] that 4 h after exposure to EMS the alkylations per deoxynucleotide

in testicular DNA were about the same as those in sperm DNA; recent results with MMS have been similar [Sega, unpublished data]. It therefore does appear that the initial level of DNA alkylation is about the same in different germ-cell stages. The long half-life of 7 MeG in sperm DNA compared with its in vitro half-life of ~6 days may be related to the highly condensed state of the sperm nucleus in which the chromatin is packaged almost as a crystalline structure. The very different half-lives of 7 MeG in sperm and testicular DNA fit in well with our observation that a UDS response can be measured in meiotic and post-meiotic stages found in the testes but not in maturing sperm during a 2-week sampling period following treatment.

General procedures for measuring UDS in mouse germ cells

Male mice are exposed to a chemical or physical agent in exactly the same way they would be in a genetic experiment. The general method of administration of chemicals is by i.p. injection, but other routes such as testicular injections and inhalation have also been used [28]. [^3H] dThd is injected directly into the testes either at the same time or at different times after mutagen treatment. For the testicular injections the mice are anesthetized with Metofane, a small incision is made in the scrotum to visualize the testes, and 36 μl of water containing ~36 μCi of [^3H] dThd is injected into each testis. The incision heals in a few days without any special treatment.

The last scheduled DNA synthesis occurs in primary spermatocytes, and then there is a relatively long time period of about 33–36 days before these spermatocytes complete their passage through a series of meiotic and post-meiotic germ-cell stages to become

mature spermatozoa in the vas. This period involves about 28–30 days for development of germ-cell stages in the testes [15, 16] and another 4–6 days for the maturing sperm to pass through the caput and caudal epididymides and enter the vas. Normally during this extended time period no DNA synthesis occurs. However, if the mutagen being studied is able to produce DNA lesions in any meiotic or post-meiotic germ-cell stages and UDS is induced, it can be detected by the unscheduled uptake of the [^3H] dThd into the affected germ cells. UDS occurring in any stage can be studied indirectly by making use of the timing of spermatogenesis and spermiogenesis in the mouse and recovering sperm from the caudal epididymides or vasa deferentia at the appropriate times after treatment of the males. For example, sperm recovered from the caudal epididymides 16 days after treatment represent germ cells that were mostly in early spermatid stages at the time of treatment.

If a "mutagen exposure—UDS response" curve is desired for a particular germ-cell stage, at the appropriate time after treatment the males are killed and sperm are recovered from the caudal epididymides or vasa deferentia. If it is desired to study all of the germ-cell stages undergoing a UDS response to a particular agent, sperm samples are recovered from the treated males every few days for 5–6 weeks after treatment.

In the recovery of the sperm, the caudal epididymides or vasa deferentia are dissected from the animals, diced, and sonicated to destroy all cell types except for the sperm heads. The tails and midpieces (with their associated mitochondria) are sheared off in this process. After several washes in a Ficoll–SDS solution, a pure population of sperm heads is obtained free of sperm tails, midpieces, somatic cells, or cellular debris [23, 25]. One male typically yields $\sim 7 \times 10^6$ sperm heads from the

vasa deferentia and about twice that number from the caudal epididymides. Several million of these sperm are then assayed by LSC to determine [^3H]dThd activity, and the number of sperm present in each scintillation vial is determined by hemacytometer counts of a diluted sample of the same sperm stock. Finally, the number of [^3H]dThd dpm/ 10^6 sperm heads is calculated. Although LSC is an indirect means of measuring the UDS that had occurred in earlier germ-cell stages, it has the advantage of sensitivity to low levels of induced UDS since millions of sperm heads are sampled.

To make direct observations of the germ-cell stages undergoing UDS autoradiographic procedures are used [28, 30]. These techniques also provide information on the uniformity of the UDS response within a particular germ-cell stage in a single testis and can be used to study the distribution of [^3H]dThd labeling from UDS throughout the chromosomes in diakinesis. However, autoradiographs are hard to quantitate and are not as sensitive as LSC for detecting low levels of induced UDS.

Germ-cell stages that undergo UDS when exposed to mutagenic agents

In our detailed studies of the meiotic and post-meiotic germ-cell stages undergoing UDS when exposed to mutagenic agents, we have used MMS, EMS, cyclophosphamide, mitomen, and X-rays [23, 26, 29, 30]. Gonial cells have not yet been thoroughly studied because of the difficulty in distinguishing UDS from the scheduled DNA synthesis occurring in these germ-cell stages. Other workers studying UDS have used X-rays and UV in an in vitro system with mouse germ cells [11], UV in vitro with rat germ cells [10], and MMS [21] and procarbazine [4] in vivo with the rabbit. In all of these studies the basic finding is that mutagens can induce UDS in meiotic stages and in post-meiotic

stages up to about mid-spermatids. No UDS is detected in late spermatids and sperm cells.

Fig. 2 shows the pattern of the UDS response induced by a 250 mg/kg i. p. injected dose of EMS in different germ-cell stages of the mouse. (The [^3H] dThd was given at the same time as the EMS.) Note that for about the first 2 weeks after treatment there is no unscheduled presence of [^3H] dThd label in the sperm recovered from the vas; these sperm represent germ-cell stages that were treated as mature spermatozoa to late spermatids. In the 3rd and 4th weeks after EMS treatment the vas sperm show the unscheduled presence of [^3H] dThd; these sperm represent germ-cell stages that were treated as mid-spermatids to early meiotic stages.

The absence of UDS in the mature spermatozoa to late spermatid stages is not due to failure of EMS to ethylate DNA in these stages. In our chemical dosimetry studies we have found that the DNA in these germ-cell stages is being alkylated [24, 27]. As we have discussed in an earlier paper [23], the germ-cell stages exhibiting no UDS are those in which protamine has either replaced, or is in the process of replacing, the usual chromosomal histones. At the time of protamine synthesis an extensive condensation of the spermatid nucleus begins. It is possible that the DNA lesions present in these cells have become inaccessible to the enzymatic system which gives rise to UDS in the earlier germ-cell stages. Also, much of the cytoplasm is lost from the spermatids as they develop from mid to late spermatid stages, and the enzymatic system which produces the UDS response may be lost at this time.

ethylation (see Fig. 5). When consideration is taken of the 2- to 3-day shift toward earlier germ-cell stages recovered from the caudal epididymides as compared with those recovered from the vasa deferentia [23], the ethylations per sperm head and the ethylations per A_{206} unit of protamine recovered from the sperm heads both closely parallel the dominant-lethal curve obtained with EMS. (F-5)

These results have led us to suggest the model for the induction of dominant lethals by EMS which is shown in Fig. 6. After protamine synthesis (in maturing spermatid stages), but before cysteine disulfide bonds are formed, EMS could ethylate the sulfhydryl groups and thus effectively block normal disulfide-bond formation. This in turn would prevent normal chromatin condensation in the sperm nucleus, leading to stresses in the chromatin structure that would eventually result in a lethal chromosome break. Some of our recent work (unpublished) with [^3H] MMS has, in fact, shown that S-methyl-L-cysteine is a major alkylation product recovered from mouse sperm protamine. (F-6)

Our findings suggest to us that even in the absence of a UDS response, EMS would probably not be effective in producing dominant lethals in meiotic and post-meiotic stages in which no protamine is present. We feel the UDS response observed in these stages likely involves the restoration of altered deoxynucleotides which might otherwise give rise to subtle genetic damage such as recessive point mutations, but not necessarily to chromosomal aberrations or dominant lethals.

Data on specific-locus mutation frequencies in each of the first 4 weeks of mating following treatment with chemical mutagens are not extensive enough for us to determine if a difference in mutation frequency exists between germ-cell stages showing UDS.

(sampled in the 3rd and 4th weeks of mating after treatment) and those not showing UDS (sampled in the first 2 weeks of mating). However, more data are available for X-rays.

With an acute X-ray exposure of 300 R, specific-locus mutation frequencies have been determined by W. L. Russell for each of the first 4 weeks of mating after treatment [26] (see Table I). The mutation frequency in each of the 4 weeks is at least an order of magnitude higher than the spontaneous frequency, and there is no reduction in the frequency in the 3rd and 4th weeks as compared with the first 2 weeks. In fact, the mutation frequency is higher (at a 90% confidence level) in the 3rd week than it is in the first 2 weeks. There is no indication that UDS results in a reduced specific-locus mutation frequency after X-ray treatment.

L. B. Russell has shown that among the specific-locus mutations induced by X-rays at the "dilute" and "short ear" loci [18] and the albino locus [19] there are a number representing intragenic changes. However, those mutations induced in the stages considered in this paper, namely the post-spermatogonial stages, could all be small deficiencies. Thus, in the case of X-rays, the UDS response we measure in the meiotic and post-meiotic stages may represent restoration of damaged deoxynucleotides and/or single-strand breaks but may have no effect on the removal of specific-locus mutations in the mouse in which small deletions are involved. By the same reasoning, the occurrence of X-ray-induced dominant lethals in germ-cell stages showing a UDS response may indicate that the enzymatic system giving rise to the UDS can handle some types of DNA lesions but not those which could generally give rise to dominant lethals as, for example, double-strand breaks.

Development and characterization of mouse stocks with reduced UDS levels to further study the relationship between UDS and repair of genetic damage in mammalian germ cells

A total of 22 mouse stocks have been tested for the UDS response of their germ cells to MMS (unpublished data). Males from each of the stocks were given the usual testicular injections of [^3H] dThd followed immediately by an i. p. injection of 75 mg MMS/kg, which was known to induce a high level of UDS in several germ-cell stages of the (C3Hf x 101) F_1 hybrid males that we ordinarily use. Sperm from the caudal epididymides, representing early spermatid stages at the time of treatment, were recovered 16 days after treatment and assayed for unscheduled [^3H] dThd incorporation.

Within the experimental uncertainties, most of the 22 stocks showed about the same UDS response in the early spermatids (see Fig. 7). However, there was a significant difference between the stocks showing the highest UDS response and those showing the lowest. The (C3Hf x 101) F_1 hybrids showed one of the highest responses, whereas SEC and BALB/c males showed the lowest UDS responses, only one-third to one-fourth that of the (C3Hf x 101) F_1 males.

BALB/c males were chosen for further studies since their body and testis weights are very similar to (C3Hf x 101) F_1 males of the same age. When other doses of MMS were used there was a consistent reduction in the UDS levels induced in BALB/c germ cells compared with that observed in the germ cells of (C3Hf x 101) F_1 males. This effect is not due to reduced levels of DNA methylation in BALB/c germ cells, because our recent chemical dosimetry studies (unpublished) have shown that the testicular DNA and sperm DNA of both stocks are alkylated to the same extent by equimolar injected doses of [^3H] MMS. Such data at least suggest that the germ cells of BALB/c males may be

less efficient than those of (C3Hf x 101)F₁ males in the removal of DNA methylation products. The eventual characterization of mouse stocks with reduced UDS capability in their germ cells plus the development of biochemical genetic markers that can measure single amino acid substitutions will likely be necessary before the relationship between UDS in mammalian germ cells and repair of genetic damage can be clearly established.

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TABLE I

SPECIFIC-LOCUS MUTATION FREQUENCIES INDUCED IN MEIOTIC AND POST-MEIOTIC GERM-CELL STAGES OF MALE MICE AFTER EXPOSURE TO 300 R OF ACUTE X-RAYS^a

Weeks after 300 R ^b	Progeny scored	Mutants ^c	Mutation frequency ^d (locus/gamete)
1	9,281	13	$2.0 \left(\begin{smallmatrix} 3.3 \\ 1.0 \end{smallmatrix} \right) \times 10^{-4}$
2	9,412	12	$1.8 \left(\begin{smallmatrix} 3.1 \\ 1.0 \end{smallmatrix} \right) \times 10^{-4}$
3	} UDS	10	$3.4 \left(\begin{smallmatrix} 5.9 \\ 1.8 \end{smallmatrix} \right) \times 10^{-4}$
4		4	$1.6 \left(\begin{smallmatrix} 3.9 \\ 0.6 \end{smallmatrix} \right) \times 10^{-4}$
Control	531,500	28	$0.075 \left(\begin{smallmatrix} 0.107 \\ 0.051 \end{smallmatrix} \right) \times 10^{-4}$

^aData of W. L. Russell cited in ref. [26].

^bTreated males were (101 X C3H) F_1 hybrids, 12-21 weeks old.

^cSeven loci were scored for mutation; see ref. [20] for description of mutations.

^dUpper and lower 95% confidence limits of the observed mutation frequencies are shown in parentheses.

Figure Legends

Fig. 1. Comparison of the rates of loss of 7 MeG from vas sperm DNA (●) and testicular DNA (O) after a single i.p. injection of 100 mg [^3H] MMS/kg. Error bars represent ± 1 standard deviation; least-squares fits are indicated.

Fig. 2. Unscheduled uptake of [^3H] dThd into meiotic and postmeiotic germ-cell stages of male mice after testicular injection of [^3H] dThd and i.p. injection of 250 mg EMS/kg, measured in sperm passing through the vasa deferentia. ●, [^3H] dThd activity in sperm heads from EMS-treated animals; O, [^3H] dThd activity in sperm heads from control animals. Also indicated is the dominant-lethal frequency pattern obtained with EMS (the peak in dominant lethals is around 50–60% with 250 mg EMS/kg [7,9]) and the time period during which protamine is synthesized. From ref. [23].

Fig. 3. Relationship between the dominant-lethal frequency patterns induced by EMS [7, 9] and X-rays [6, 22], and the occurrence of UDS in the germ cells of male mice [23, 26].

Fig. 4. Ethylation of sperm heads and sperm DNA taken from the vasa deferentia during a 2-week period following i.p. injection of 200 mg [^3H] EMS/kg. Standard deviations are indicated. From ref. [27].

Fig. 5. Ethylation of sperm heads and sperm protamine taken from the caudal epididymides at various times after i.p. injection of mice with 200 mg [^3H] EMS/kg. From ref. [27].

Fig. 6. Model for the ethylation of mouse sperm chromatin by EMS, leading to chromosome breakage and dominant lethality. The double helices represent double-stranded DNA, and the dashed lines represent the associated sperm protamine. The sulfhydryl (-SH) groups are part of the cysteine residues in the protamine, and the ethylated SH group is represented as Et-S-. In normal nuclear condensation (a), the SH groups of cysteine cross-link to form disulfide bridges in the chromatin. However, if EMS ethylates a nucleophilic SH group before a disulfide bond forms (b), cross-linking of the SH groups may not take place. This could lead to stresses in the chromatin structure and eventually produce a chromosome break either near the region of ethylation (as shown) or away from this region, where stresses in the chromatin might be greater.

Fig. 7. UDS occurring in early spermatid stages of 22 different mouse stocks following exposure to 75 mg MMS/kg. Sperm from the caudal epididymides were assayed for the unscheduled presence of [^3H]dThd 16 days after treatment. These sperm represent early spermatid stages at the time of treatment. Standard deviations are indicated.

36524

Fig. 1

365-25

Fig. 2

32143

Fig. 2

Fig. 4

34075

34076

Fig. 5

32574

Fig. 6

32144-1

Fig. 1