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Molecular Targets, DNA Breakage, DNA Repair: Their Roles in Mutation Induction in Mammalian Germ Cells

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OVERVIEW

Variability in genetic sensitivity among different germ-cell stages in the mammal to various mutagens could be the result of how much chemical reaches the different stages, what molecular targets may be affected in the different stages and whether or not repair of lesions occurs. Several chemicals have been found to bind very strongly to protamine in late-spermatid and early-spermatozoa stages in the mouse. The chemicals also produce their greatest genetic damage in these same germ-cell stages. While chemical binding to DNA has not been correlated with the level of induced genetic damage, DNA breakage in the sensitive stages has been shown to increase. This DNA breakage is believed to indirectly result from chemical binding to sulfhydryl groups in protamine which prevents normal chromatin condensation within the sperm nucleus.

INTRODUCTION

In studies of the genetic effects of chemical agents in mice, the exposure given to the animals (by injection, inhalation, skin application, etc.) is accurately known. However, in general, little or nothing is known about the molecular dose of the chemical that actually reaches the different germ-cell stages (see Fig. 1).

The variability in genetic sensitivity among different germ-cell stages could be the result of how much chemical reaches the different stages, what molecular targets the chemical may interact with in different stages, and whether or not repair of lesions in specific germ-cell stages is possible.

In particular, the chemical mutagens ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), ethylene oxide (EtO) and

acrylamide (AA) have all been shown, in genetic studies, to produce very many more dominant lethal mutations and heritable translocations in late spermatids and early spermatozoa than in other germ-cell stages in male mice.(EMS [Ehling et al, 1968; Generoso and Russell, 1969]; MMS [Ehling et al, 1968; Ehling, 1980]; EtO [Generoso et al, 1980]; AA [Shelby et al, 1986,1987]). We have, subsequently, used all 4 of these chemical mutagens in radioactive form to find out where they are binding within the germ cells and to see what correlations may exist between the extent of chemical binding in different stages and the amount of induced genetic damage.

In addition, three of these chemicals have been studied for their ability to induce DNA breakage in different germ-cell stages of male mice using an alkaline elution technique. (MMS [Sega et al, 1986]; EtO [Sega and Generoso, 1988]; AA [G.A. Sega and E.E. Generoso, manuscript submitted to Mutation Research]). This technique makes use of filters to differentiate DNA strands on the basis of their single-strand size in a high pH solution (Kohn, 1979). The filters act to physically impede the passage of long DNA strands. Thus, if single-strand breaks have been induced, the smaller DNA fragments will pass more quickly through the filter than will undamaged DNA when an alkaline solvent is slowly pumped through the filter.

All of the chemicals have also been studied for their ability to induce DNA repair in mouse germ cells. (MMS and EMS [Sega and Sotomayor, 1982]; EtO [Sega et al, 1988]; AA [G.A. Sega, manuscript in preparation]). The occurrence of DNA repair in the germ cells of treated animals is monitored following testicular injection of ^{3}H-thymidine ([^{3}H]dThd). If DNA repair has occurred in meiotic or post-meiotic stages, the [^{3}H]dThd will be incorporated into cells in which no scheduled DNA synthesis normally takes place. Thus, the repair is detected by an unscheduled DNA synthesis (UDS) response in the affected stages.

RESULTS

Chemical binding to whole germ cells

Mice from the same strains used in genetic experiments were exposed to radioactively labeled EMS, MMS, EtO or AA. Sperm moving through the reproductive tracts were recovered from the animals at different times, up to three weeks, after exposure and assayed for how much chemical was bound (alkylation). With chemicals such as EMS, MMS, EtO and AA, that are powerful mutagens in late spermatids and early spermatozoa, we found that there was a dramatic increase in the amount of chemical bound to these stages (at least an order of magnitude more binding than in other stages). Thus, there was a correlation between increased genetic damage and increased levels of chemical binding to the sensitive stages.

Fig. 2 shows the binding pattern of AA in sperm recovered from the vasa deferentia over a three-week period after exposure. The same basic pattern of binding was found with EMS, MMS and EtO. The binding reaches a peak in the recovered sperm between ~8 to 12 days after exposure and represents germ cells that were late spermatids to early spermatozoa at the time of treatment (Sega and Sotomayor, 1982).

Chemical binding to germ-cell DNA

To further characterize the molecular nature of the lesions in the germ cells, DNA was extracted from the sperm recovered from the vasa deferentia at different times after treatment and assayed for the amount of chemical bound to it. It was found that the amount of chemical binding to DNA represented only a very small

fraction of the total chemical binding to the germ cells. Furthermore, there was no increase in DNA alkylation in the most sensitive stages, late spermatids and early spermatozoa. Fig. 2 also shows the pattern of binding of AA to the DNA in the developing spermiogenic stages. Similar results were found with EMS, MMS and EtO.

Protamine as a possible target for alkylation

These surprising results forced us to look for alternative molecular targets for mutagenesis within the germ cells. It was known that in mid- to late-spermatid stages of mammals, the usual chromosomal proteins (histones) are replaced with small, very basic proteins (protamines) that contain more than 50% of the amino acid arginine (Monesi,1965; Bellvé et al, 1975; Balhorn et al, 1977). In addition, mammalian protamines contain cysteine (Bedford and Calvin, 1974; Calvin, 1975), and it is the cross-linking of the cysteine amino acids in protamine (through disulfide-bond formation) that gives mammalian spermatozoa their keratin-like properties (similar to some of the properties of fingernails and hair). Because protamine contains nucleophilic sites (e.g., the -SH group in cysteine), we reasoned that it might be a target for attack by chemicals such as EMS, MMS, EtO and AA.

Chemical binding to germ-cell protamine

When we purified protamine from sperm recovered from the caudal epididymis (an area of the reproductive tract that immediately precedes the vas) at different times after chemical treatment we were excited to find that the level of mutagen that was bound to protamine increased greatly in the most sensitive germ-cell stages (late spermatids - early spermatozoa). The amount of mutagen binding to protamine exactly paralleled the

total amount of binding to the sperm. In fact, with chemicals such as EMS, MMS, EtO and AA that have their greatest effect in late spermatids and early spermatozoa, we have found that almost all of the binding in the sensitive stages can be attributed to interaction with protamine.

Fig. 3 shows the binding pattern of AA in sperm recovered from the caudal epididymides over a three-week period after exposure. Since sperm from the caudal epididymides take about two days to reach the vas (Sega and Sotomayor, 1982), the alkylation pattern of the sperm recovered from the caudal epididymides is shifted toward earlier times. Taking this time shift into account, the alkylation pattern that was seen with the vas sperm is well reproduced with the epididymal sperm.

Also shown in Fig. 3 is the AA binding to sperm protamine. Final protamine purification was carried out on an HPLC column and detected at 214 nm. The amount of protamine recovered was then expressed in units of OD_{214}-min, i.e., the area under the protamine peaks recovered from the HPLC column. One OD_{214}-min unit represents the protamine from ~22 x 10^6 sperm. Within experimental uncertainty, all of the sperm alkylation can be accounted for by the protamine alkylation. The same basic observations were made with EMS, MMS and EtO.

Chemical binding to cysteine within the protamine

In order to determine where on the protamine the chemicals were binding, samples of alkylated protamine were acid-hydrolyzed. By analysis of the protamine hydrolyzates on an amino acid analyzer and by thin-layer chromatography, we have been able to show that these types of chemicals do, in fact, bind to the sulfur in cysteine. We have shown that with MMS at least 80% of the protamine adducts are S-methylcysteine.(Sega and Owens, 1983) and

AA has been found to yield the adduct S-carboxyethylcysteine, as well as a second, as yet unidentified adduct (Sega et al, 1989).

DNA Breakage

Germ-cell DNA from chemically-treated and control animals was pre-labeled with [^{3}H]dThd and [^{14}C]dThd, respectively. At various times after mutagen treatment, sperm are gently recovered from the vasa of one treated and one control animal, pooled, placed on a polycarbonate filter and lysed. The DNA from the sperm is denatured using an alkaline buffer (pH = 12.2) and eluted through the filter over a 15-hour period. Radioactivity in collected fractions is determined by liquid scintillation counting (Sega , and Generoso, 1988; Sega et al., 1986). The difference between the amount of [^{3}H]dThd- and [^{14}C]dThd-labeled DNA eluted gives a relative measure of the amount of single-strand breaks in the treated DNA.

Typical of the pattern of DNA breakage induced in developing germ-cell stages by these chemicals is that shown for EtO in Fig. 4. During the first week after exposure there is a gradual increase in the amount of DNA eluted from the sperm of the EtO-treated animals. The amount of DNA then peaks between days 7 and 13. Starting at about the end of the second week after exposure, the elution of the treated DNA begins to decrease. By 23 days posttreatment there is barely a detectable difference between the amount of treated- and control-sperm DNA eluted.

DNA Repair

We have made several detailed studies of the meiotic and postmeiotic germ-cell stages that undergo DNA repair (as measured by UDS) when exposed to mutagens (EMS: (Sega, 1974);

cyclophosphamide and mitomen: [Sotomayor et al, 1978]; MMS and X-rays: [Sotomayor et al, 1979]; AA [G.A. Sega, paper in preparation]). In all of these studies the basic finding has been that mutagens induce UDS in meiotic stages and in postmeiotic stages up to about midspermatids. No UDS has been detected in late spermatids and spermatozoa.

Figure 5 shows the pattern of the UDS response induced in different germ-cell stages of the mouse after an i.p.-injected dose of 250 mg EMS/kg (the [^{3}H]dThd was given at the same time as the EMS). For about the first two weeks after treatment, there is no unscheduled presence of [^{3}H]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 [^{3}H]dThd. These sperm represent germ-cell stages that were treated as midspermatids to early meiotic stages.

The absence of UDS in mature spermatozoa to late spermatid stages is not due to failure of EMS (or other chemicals) to alkylate DNA in these stages. In our chemical dosimetry studies we have found that the DNA in these germ-cell stages is being alkylated (Sega , and Owens, 1978; Sega , and Owens, 1983; Sega , and Owens, 1987; Sega et al., 1989). The germ-cell stages that exhibit 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 probable that the DNA lesions present in these cells have become inaccessible to the enzymatic system that 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 that produces the UDS response may be lost at this time.

DISCUSSION

All of these findings have led us to postulate a model for the binding of certain chemicals to mouse chromatin in germ-cell stages that are sensitive to the induction of chromosome breakage and dominant lethality (Sega et al., 1989). In normal nuclear condensation of the developing spermatids, the sulfhydryl groups of cysteine cross-link to form disulfide bridges in the chromatin. However, if chemical binding to a nucleophilic sulfhydryl group occurs before a disulfide bond has formed, cross-linking of the sulfhydryl groups may not take place. This could lead to stresses in the chromatin structure that eventually produce either a single- or double-strand DNA break. The end result would be a dominant-lethal mutation or chromosome translocation.

The patterns of DNA-strand breakage observed in mouse sperm over a 3-week period after exposure to MMS (Sega et al., 1986), EtO (Sega , and Generoso, 1988) and AA (G.A. Sega and E.E. Generoso, manuscript submitted to Mutation Res.) all support the above model. In each case, the DNA breakage is greatest in germ-cell stages having the highest levels of protamine alkylation. (Increased DNA breakage in the late-spermatid and early-spermatozoa stages is not correlated with greater DNA alkylation.)

Sperm recovered in the third week after chemical treatment show a relatively rapid decrease, for successive days, in the amount of DNA that eluted through the filters (refer to Fig. 4 for the elution pattern observed after EtO exposure). Our molecular dosimetry experiments with all four chemicals have also shown a great reduction in protamine alkylation by the middle of the third week after exposure. We attribute the decrease in sperm DNA elution during this period to two factors. First, the sperm protamine sampled in the third week was synthesized after the chemical mutagens had disappeared from the testes. Thus, the

protamine in these sperm was not heavily alkylated and chromatin breakage was subsequently reduced. Second, the sperm recovered in this time period were derived from stages capable of DNA repair at the time of treatment (Sega , and Sotomayor, 1982), so that any DNA lesions that might otherwise have given rise to DNA-strand breaks, were repaired before the alkaline elution assay.

Clearly not all mutagenic chemicals act by the above mechanism, and other molecular targets may be important in other germ-cell stages. However, our observations of how some chemicals bind strongly to sperm protamine in mammals gives a new dimension to our understanding of mutational processes in mammalian germ cells.

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LEGENDS

Fig. 1 External exposure versus molecular dose: The amount of chemical (circles) to which an animal is exposed can be accurately established, but its distribution within molecular targets in different tissues is more difficult to analyze. Smaller boxes shown within "mouse" (large box) represent different tissues. Levels of binding in each tissue can vary, depending on such factors as transport, metabolic conversion to more (or less) potent chemical species, and repair. The effects of chemicals on targets within the germ cells is our principal interest.

Fig. 2 Alkylation of sperm heads and sperm DNA taken from the vasa deferentia during a 3-week period following i.p. injection of 125 mg [^{14}C]AA/kg. 10^6 alkylations per sperm head (■———■); alkylation/10^6 deoxynucleotides (●---●). Error bars represent ± 1 SD. (From Sega, et al, 1989.)

Fig. 3 Alkylation of sperm heads and sperm protamine recovered from the caudal epididymides during a 3-week period following i.p. injection of 125 mg [^{14}C]AA/kg. 10^6 alkylations per sperm head (■———■); 10^{14} alkylations / OD_{214}-min (O---O). Error bars represent ± 1 SD. (From Sega, et al, 1989.)

Fig. 4 Pattern of sperm DNA elution over a 23-day period following exposure to 100 mg EtO/kg. The difference between the amount of [^{3}H]dThd-labeled DNA and the [^{14}C]dThd-labeled DNA eluted has been subtracted from all the data points. Beyond the end of the second week, spermatozoa recovered from the vasa were, at the time of treatment, in germ-cell stages capable of DNA repair (Sega , and Generoso, 1988). Error bars represent ± 1 SEM.

Fig. 5 Unscheduled uptake of [^{3}H]dThd into meiotic and postmeiotic germ-cell stages of male mice after testicular injection of [^{3}H]dThd and i.p. injection of 250 mg EMS/kg. Uptake of [^{3}H]dThd was measured in sperm passing through the vas deferens. (●) [^{3}H]dThd activity in sperm heads from EMS-treated animals; (□) [^{3}H]dThd activity in sperm heads from control animals. Also indicated is the dominant-lethal frequency pattern obtained with EMS (the maximum dominant lethality is around 50-60% with 250 mg EMS/kg (Generoso , and Russell, 1969). Adapted from Sega and Sotomayor, 1982.

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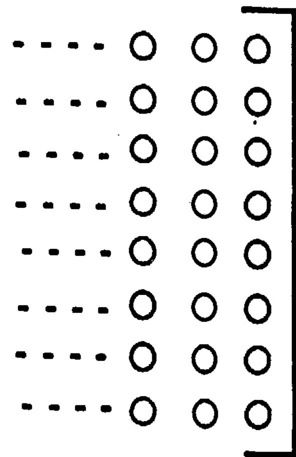
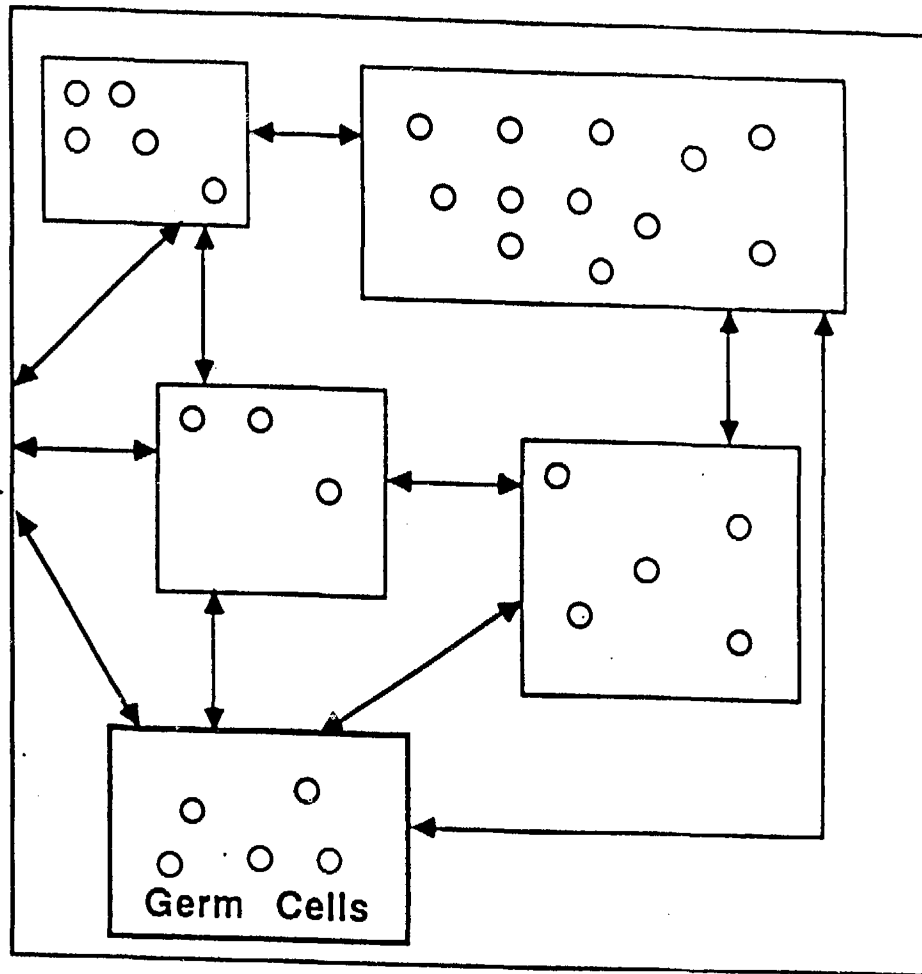
Mutagen
"Exposure"





Mouse

Fig. 1

Sperm DNA alkylation by acrylamide

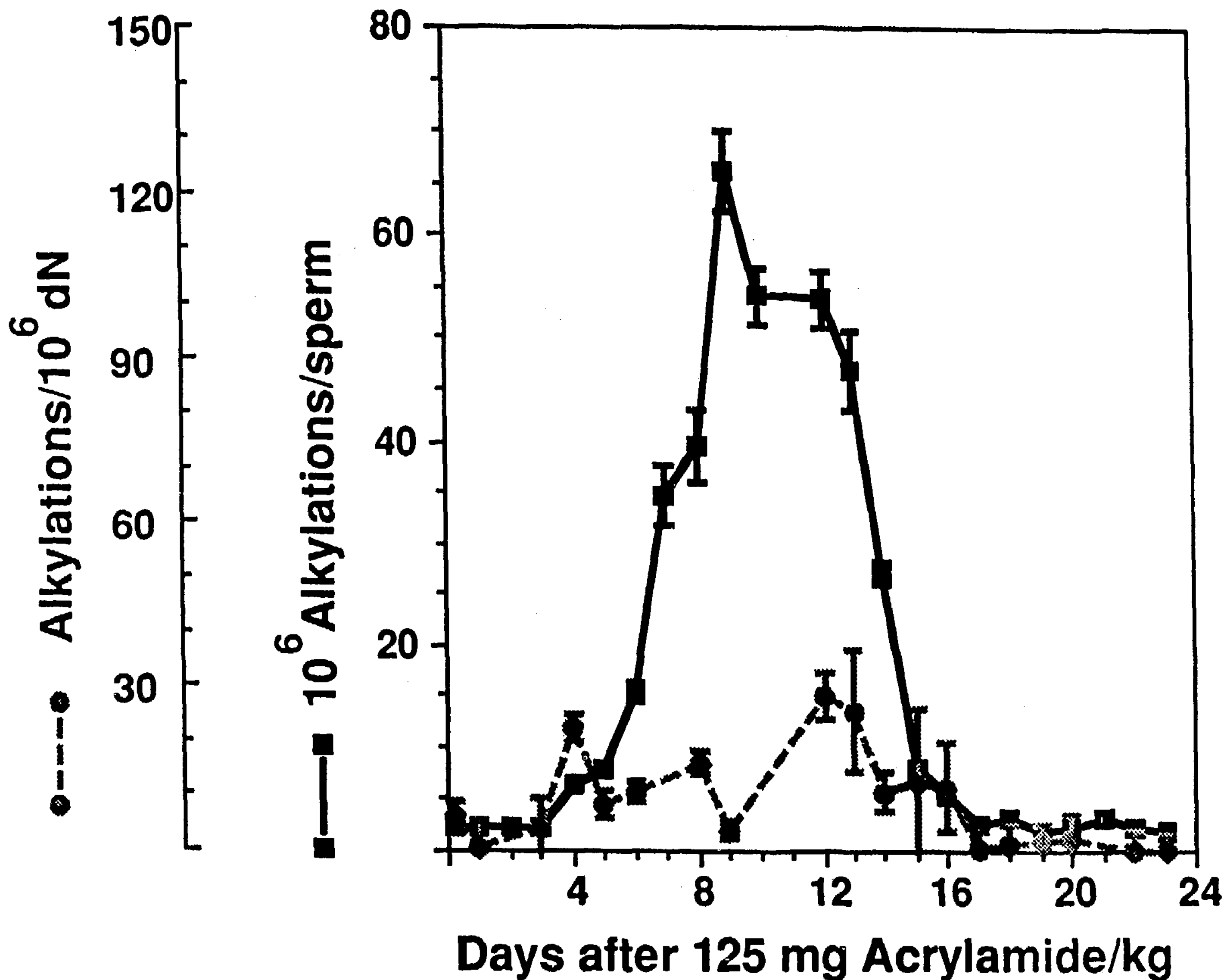


Fig 2

Sperm protamine alkylation by acrylamide

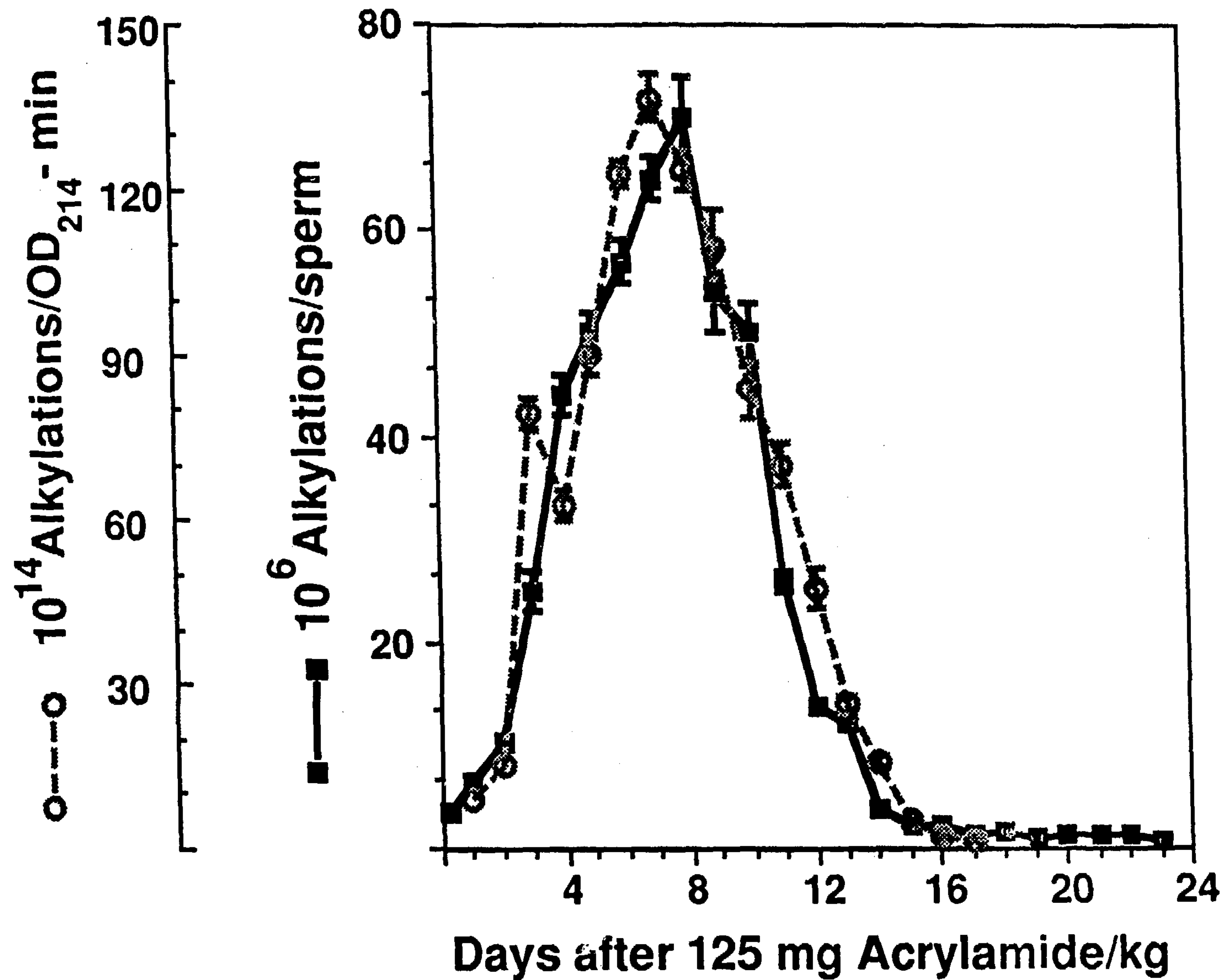


Fig 3

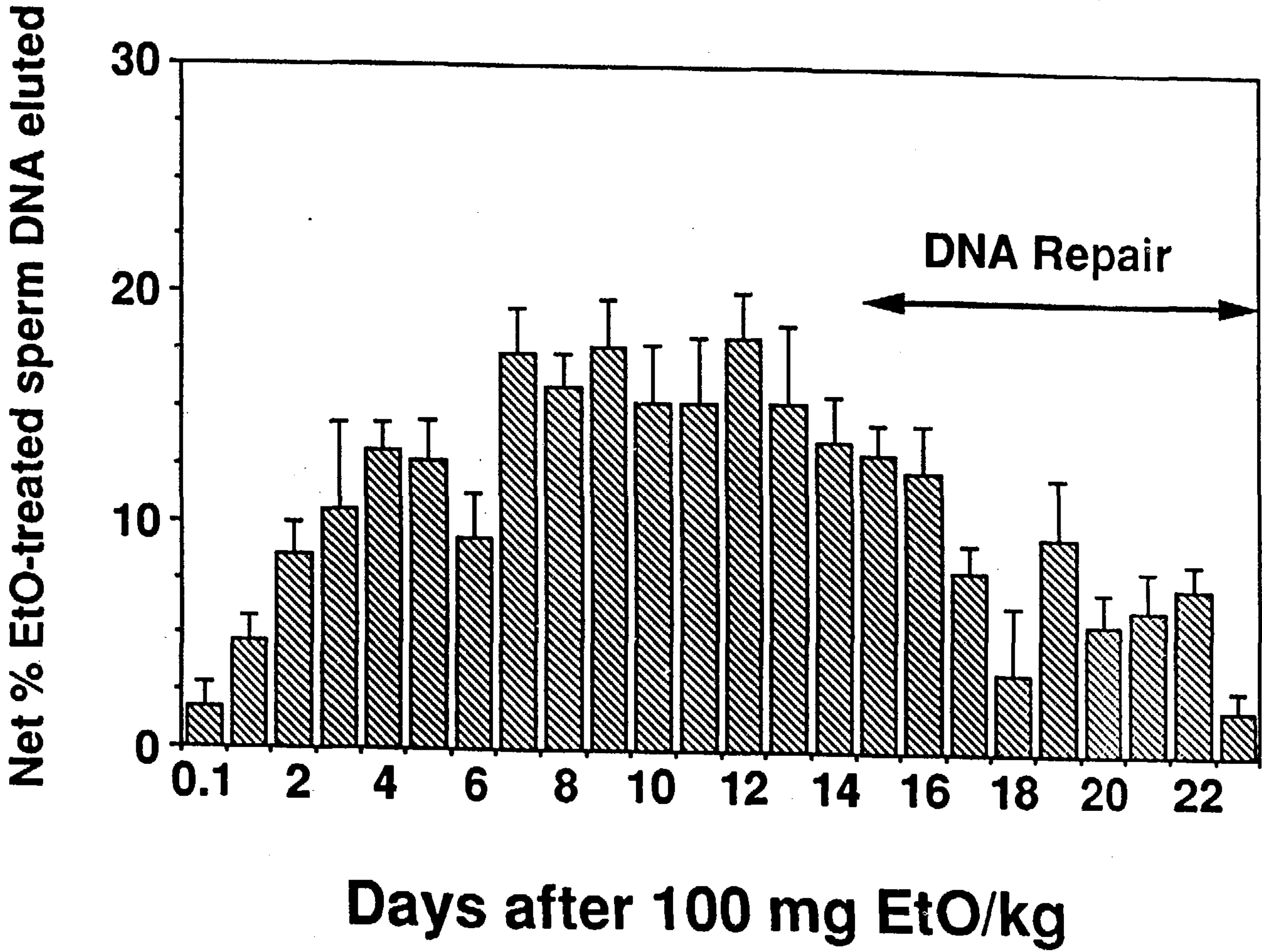


Fig 4

EMS-induced Unscheduled DNA Synthesis

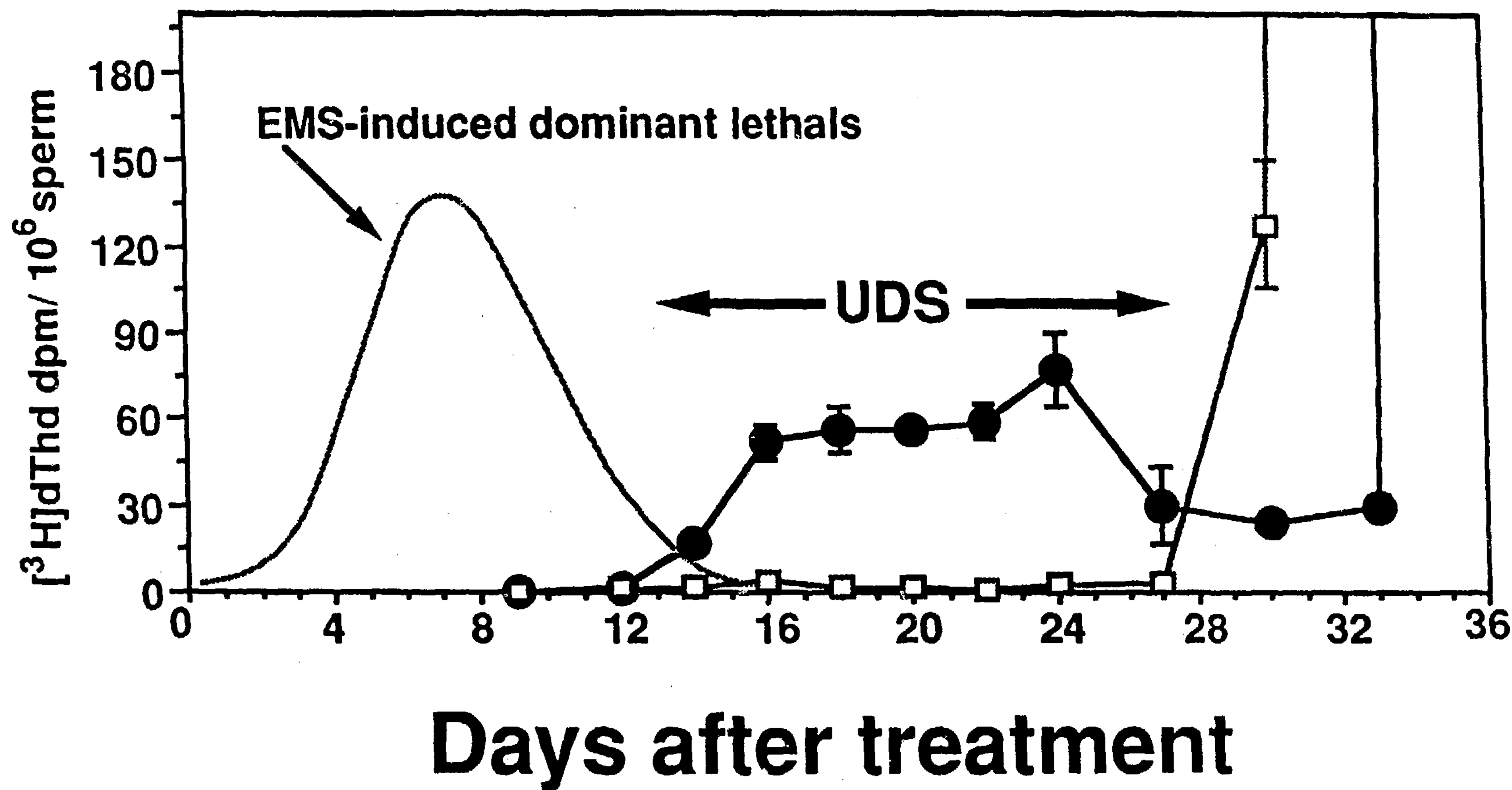


Fig. 5