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Genetics

Effect of dose fractionation on the ethylnitrosourea induction of specific-locus mutations in mouse spermatogonia

(chemical mutagenesis/genetic repair/risk assessment/alkylating agent)

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ABSTRACT **As measured by specific-locus mutations in mouse spermatogonia, fractionating a dose of 100 mg of ethylnitrosourea per kg of body weight into doses of 10 mg/kg injected intraperitoneally at weekly intervals greatly reduces the mutation frequency compared with that from a single dose of 100 mg/kg. Because there is independent evidence that the doses of 10 and 100 mg/kg reach the germ cells in amounts directly proportional to the injected dose, the lower mutational response with the fractionated dose is attributed to repair. The induced mutation rate expected from a single 10-mg/kg dose (on the assumption that this would be 1/10th the rate observed after 10 such doses) would be only 75% of the spontaneous mutation rate. Mouse spermatogonia apparently have an efficient repair system that is effective even against a potent mutagen.**

A companion paper (1) reports the results obtained from two series of experiments exploring the shape of the dose–response curve for specific-locus mutations induced in mouse spermatogonia by *N*-ethyl-*N*-nitrosourea (ENU). The second series involved doses of 25, 50, 75, and 100 mg/kg. When the animals in these experiments had been producing offspring for approximately one year, it appeared (and has since been confirmed with the completion of the experiments) that the mutation frequencies at the lower doses would fall below a linear interpolation between the control and 100-mg/kg frequencies. It was clearly desirable to find out what would happen at still lower doses. However, the mutation frequencies at 25 and 50 mg/kg were already so low that it was probable that single-dose experiments below 25 mg/kg would require prohibitively large numbers of offspring to determine reliable mutation rates. To avoid this difficulty, an experiment with a fractionated dose was started. Doses of 10 mg/kg were injected at weekly intervals for a total dose of 100 mg/kg, the rationale being that doses spaced a week apart would be solely additive and, therefore, that 1/10th of the mutation frequency obtained with this exposure might approximate what would be expected from a single 10-mg/kg dose. This experiment is still proceeding, but the results obtained to date are informative, and a progress report is therefore presented here. A brief account of an earlier stage in the experiment was presented in a symposium paper (2).

MATERIALS AND METHODS

ENU, synthesized in our laboratory by D. G. Doherty, was dissolved in phosphate buffer (3) adjusted to pH 6. The dose injected intraperitoneally was matched to the body weight of the animal by adjusting the volume of solution injected, which approximated 1 ml. Wild-type (101 × C3H)F_1 male mice were injected with a dose of 10 mg/kg. One week later, they received another dose of 10 mg/kg, and this was repeated at weekly intervals for a total dose of 100 mg/kg. The animals were ≈5 weeks old at the time of the first injection and, therefore, ≈14 weeks old when the last injection was given. All injections were completed within 1 hr after dissolving the chemical. Seven weeks after the final injection, these males were mated to females of our standard specific-locus test strain (T), which is homozygous for seven marker genes (4). Each male was mated to two females and moved to two new females each week. After each 7-wk period, the males were rotated back to the original group of females to start the cycle over again. Because all the offspring came from conceptions occurring more than 7 wk after the end of the injections, they were derived from cells that had been exposed to the chemical in the spermatogonial stem-cell stage. The offspring were examined for mutations at the seven loci.

RESULTS AND DISCUSSION

The results are given in Table 1. In the fractionated-dose experiment, allelism has been confirmed by breeding tests for eight of the nine mutations, thereby establishing that the mutations occurred at the specific loci. The one so far untested is judged by its phenotype to be another specific-locus mutation. Data for the first of the two single-dose 100 mg/kg experiments listed in the table come from Russell *et al.* (1). Because a new batch of ENU from a different source was used for the fractionation experiment, data from another single-dose 100-mg/kg experiment using ENU from this source are included, for comparison, even though allelism tests for these mutations are not yet completed.

In the fractionation experiment, the animals were ≈5 wk old at the beginning of the series of injections and 14 wk old at the end whereas, in the single-dose experiments, the animals were 10.5–14.5 wk old at injection. However, we have unpublished data on mutation frequencies in males injected with 100-mg/kg doses at 4 or 8 wk of age, neither of which shows a statistically significant difference from the mutation frequencies in the animals injected at 10.5–14.5 wk.

The results are shown graphically in Fig. 1. Because allelism tests have not been completed in the smaller of the two single-dose experiments, only the data from the larger one are plotted. The 90% confidence limits were calculated as described in Russell *et al.* (1).

The mutation rate with the fractionated dose is significantly higher than the control rate ($P < 1 \times 10^{-5}$). It is quite apparent that fractionation of the dose gives a mutation rate that is significantly lower than that produced by the single dose ($P < 1 \times 10^{-9}$). The induced mutation frequency with the fractionated dose is only 13% of that with the single exposure.

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Abbreviation: ENU, *N*-ethyl-*N*-nitrosourea.

Table 1. Mutation frequency at seven specific loci in the offspring of male mice injected intraperitoneally with fractionated or single doses of ENU

Source of ENU	Dose, mg/kg	Offspring, no.	Mutant offspring, no.	Mutations $\times 10^5$ per locus	Induced mutations* $\times 10^5$ per locus
—	0†	531,500	28	0.75	—
D. G. Doherty	10 × 10	19,991	9‡	6.4	5.7
Bio-Clinical Laboratories	100	21,235	64§	43.1	42.3
D. G. Doherty	100	3,679	12	46.6	45.8

* Experimental minus control.
† Historical control.
‡ Includes one possible cluster of two.
§ Includes 11 possible clusters of two. See, however, Russell *et al.* (1).

Although some unforeseen complexities may be involved in an animal's response to fractionating the dose, it seems likely, with the fractions spaced 1 wk apart, that 1/10th of the induced mutation frequency obtained with ten 10-mg/kg doses can be used as an estimate of the effect of a single 10-mg/kg injection. On this assumption, the mutation frequency induced by a 10-mg/kg dose would be 0.57×10^{-5} per locus, which is only 75% of the spontaneous mutation rate. It is clear that an experiment to determine the mutagenic effect of a single dose of 10 mg/kg would require a prohibitively large number of offspring.

It is pointed out in the companion paper (1) that there is evidence that, over the range of 10–100 mg/kg, the amount of ENU reaching the germ cells is directly proportional to the injected dose. Therefore, the reduced mutagenic effect when the dose is fractionated is presumably due to a repair process that is swamped when 100 mg/kg is given in a single injection. Is this a general phenomenon for chemical mutagenesis in mammalian germ cells? The only other test of fractionation effects of chemicals on specific-locus mutation induction in mouse spermatogonia was carried out with procarbazine (5). The mutation rate from two doses of 300 mg/kg given 24 hr apart did not differ significantly from that from a single 600-mg/kg dose ($P = 0.32$). Six doses of 100 mg/kg separated by 24-hr intervals yielded 2 mutations in 20,621 offspring, compared with 16 mutations in 45,413 offspring from the single 600-mg/kg dose. The difference is on the borderline of statistical significance ($P = 0.048$, one-tailed test) but is in the same direction as the response here for ENU. No information is provided on whether the compound is reaching the testis in amounts proportional to the injected dose.

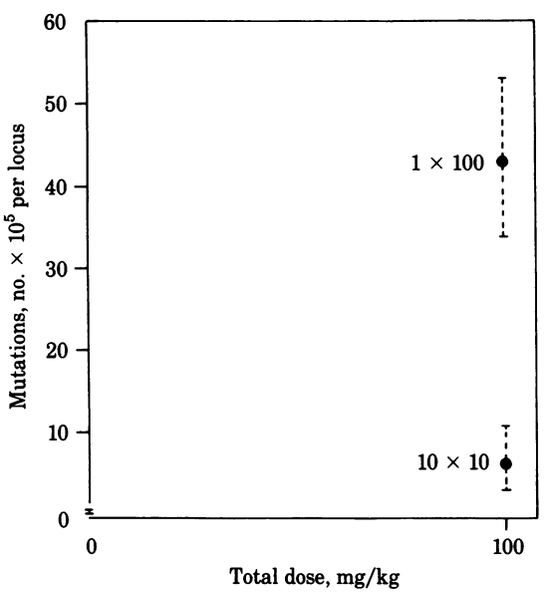

FIG. 1. Frequencies and 90% confidence limits for specific-locus mutations induced by single and fractionated doses of ENU in mouse spermatogonia. The interval between dose fractions was 1 wk.

The fact that many chemicals that are potent mutagens in other biological systems have given low or zero mutation frequencies in mouse spermatogonia, even when it is known that they or their active metabolites reach the testis, has strongly suggested the existence of effective repair processes in mouse spermatogonia. The fractionation effect reported here, and the dose–response relationship described in the companion paper (1), coupled with the evidence that ENU reaches the germ cells in amounts proportional to the injected dose, strengthen the view that mammalian spermatogonia may have an efficient repair system that is effective even against a potent mutagen. It is of consequence to note that ENU is a compound that mice have presumably not encountered before in their evolutionary history.

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