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Genetics

Dose–response curve for ethylnitrosourea-induced specific-locus mutations in mouse spermatogonia

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

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ABSTRACT **The extreme mutagenic effectiveness of *N*-ethyl-*N*-nitrosourea in the mouse has permitted the accumulation of the most extensive dose–response data yet obtained for chemical induction of specific-locus mutations in spermatogonia. In the lower portion of the curve, below a dose of 100 mg/kg, the data fall statistically significantly below a maximum likelihood fit to a straight line. Independent evidence indicates that, over this dose range, ethylnitrosourea reaches the testis in amounts directly proportional to the injected dose. It is concluded that, despite the mutagenic effectiveness of ethylnitrosourea, the spermatogonia are apparently capable of repairing at least a major part of the mutational damage when the repair process is not swamped by a high dose. This finding is important both in basic studies on the mutagenic action of chemicals in mammals and in risk estimation.**

In 1979, it was shown (1) with the specific-locus method that *N*-ethyl-*N*-nitrosourea (ENU) is, by more than an order of magnitude, a more effective mutagen in mouse spermatogonia than any other compound tested. It was pointed out that ENU could, therefore, serve as a model chemical in exploring, with relative ease, the various factors—such as dose, dose fractionation, sex, and cell stage—that might affect mutagenic action. Preliminary findings on the effects of these and other factors have been reported (2–7). A more detailed dose–response curve than that obtained for any other compound tested for transmitted mutations in the mouse is now available and is presented here.

MATERIALS AND METHODS

ENU (purchased from Bio-Clinical Laboratories, Bohemia, NY) was dissolved in phosphate buffer (8) 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 for all doses. Wild-type (101 × C3H)F_1 male mice 11.5–14.5 wk old were injected with a single dose. The experiment was done in two series, the first with doses of 100, 150, 200, and 250 mg/kg, and the second, 11 wk later, with doses of 25, 50, 75, and 100 mg/kg. The same crystalline batch of ENU, kept in a dessicator under refrigeration, was used throughout but, to check for deterioration of the chemical or other change in the conditions, the 100-mg/kg dose was included in both series. All injections were completed within 2 hr after the chemical was dissolved. The injected males were mated to females of our standard specific-locus test strain (T), which is homozygous for seven marker genes (9). Each male was mated to a group of either two or four females and moved to a new group of 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. All the offspring reported here came from conceptions occurring more than 7 wk after injection, thus ensuring that they were derived from cells that had been exposed to the chemical in spermatogonial stem-cell stage. The offspring were scored for mutations at the seven loci.

RESULTS AND DISCUSSION

The results are shown in Table 1. The mutant offspring, identified by phenotype, were bred to appropriate genetic stocks of mice to confirm the presumed allelism of the mutations. The allelism of all the observed mutations at the 50-, 75-, and 200-mg/kg doses has been established by the breeding tests. At the 100-, 150-, and 250-mg/kg doses, respectively, seven, one, and four of the observed mutant animals were not tested for allelism. Two were sterile, and the rest died either before breeding or before producing enough offspring for a conclusive test. However, 5 of these 12 each belonged to a cluster of 2 mutants from the same treated sire (see below) and 1 belonged to a cluster of 3 mutants. In each case, allelism of the mutant sib(s) was established. It is highly likely, on the basis of phenotype alone, that allelism of the mutations in the remaining six untested animals was correctly identified.

The question has been raised (10) as to whether the figures given for our historical control, which were also cited in an earlier paper on ENU results (1), might have included some preexisting mutants. In response, it can be pointed out that, not only in the historical control, but in all our past and current experiments with the specific-locus method, the number of offspring raised from each wild-type parent is more than adequate to determine that this parent is not heterozygous at any of the specific loci and, therefore, that it is not carrying a preexisting mutation at these loci. In the present experimental series, the average number of young per treated male was more than 500.

The occurrence of some sibships containing two or three mutations to the same allele at the same locus provides evidence of clustering—i.e., the derivation of mutant sibs from a single mutated spermatogonium. This is undoubtedly what has happened in some cases, especially at the higher doses of ENU, where only a small proportion of the spermatogonia survive. However, in the present data, the number of offspring for each male was so large, and the mutation frequency induced by ENU was so high, that it is probable that some of the apparent clusters were actually independent mutational events. One piece of evidence for this, from the larger of the two 100-mg/kg experiments, is as follows. Considering the three loci at which mutation is most frequent—namely, *b*, *d*, and *p*—there were seven cases in which two mutants to the same allele occurred in the offspring of one male but also seven cases in which two mutants to *different* alleles at the same locus occurred in the offspring of one male. The mutations to different alleles were obviously of independent origin, so some of the mutations to the same allele were probably of independent origin as well. As would

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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 ENU

Dose, mg/kg	Offspring, no.	Mutant offspring observed, no.	Minimum of independent mutational events,* no.	Mutations $\times 10^5$ per locus	
0†	531,500	28	28	0.75	
25	3,687	0	0	0.0	
50	15,204	10	10	9.4	
75	3,015	7	5	33.2	
100	2,317	9	7	55.5	43.1
100	18,918	55	44	41.5	
150	7,715	32	26	59.3	
200	2,080	10	7	68.7	
250	6,547	32	21	69.8	

* Based on number of apparent clusters; e.g., at the second 100-mg/kg dose, there were 11 cases of two mutants involving the same allele born to the same sire. There was one "cluster" of three at each of the 75-, 150-, and 250-mg/kg exposures. All others were of two mutants. In this column, the members of an apparent cluster are assumed to arise from a single mutational event. See text for an explanation of why this may not always be true in these data.

† Historical control.

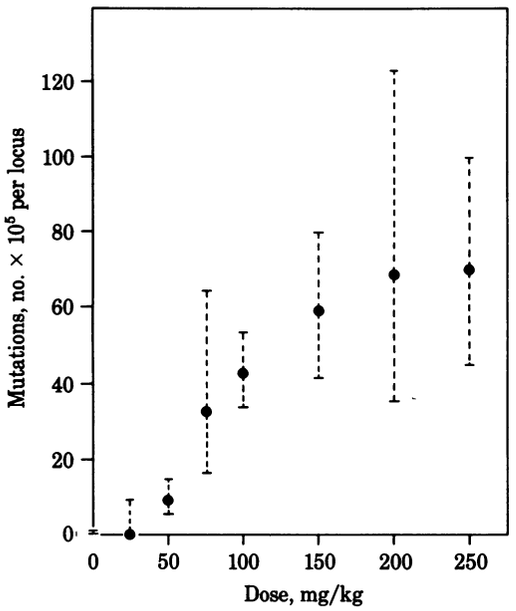

FIG. 1. Frequencies and 90% confidence limits of ENU-induced specific-locus mutations in mouse spermatogonia.

be expected, there is less evidence of this kind at the 250-mg/kg dose, at which a smaller proportion of spermatogonia survive. The column in Table 1 headed "Minimum of independent mutational events" is based on the assumption that each apparent cluster of two or three mutants derives from a single mutational event. In view of the evidence presented above, it seems likely that the true number of independent mutational events is somewhat higher than the figures given in this column.

The mutation frequency in the last column of Table 1 is calculated on the basis of the number of observed mutants divided by (*i*) the number of offspring and (*ii*) seven to give the per locus rate. Other authors (10) have calculated mutation rates from our data by counting clusters as single mutants. This is incorrect, giving a biased underestimate of the true mutation rate, because it fails to take into account that there are clusters of nonmutants as well as of mutants.

As shown in Table 1, emphasis has been placed on obtaining data at the 50-, 100-, 150-, and 250-mg/kg doses. Small samples were collected at the other doses to check the possibility of major departures from a smooth dose–response curve.

The results for the two experiments at 100-mg/kg are presented separately. This is the dose point of overlap between the first series of experiments at the higher doses and the second series at the lower doses. The difference between the two mutation rates is not statistically significant. The slightly higher rate in the 100-mg/kg experiment from the second series tends to alleviate any concern that the ENU might have decomposed significantly in the 11-wk interval between the two series of experiments.

The data of Table 1, with the addition of 90% confidence limits, are shown graphically in Fig. 1. The confidence limits are based not on the number of observed mutations but on the minimum number of independent mutational events in a number of offspring adjusted to give the same point estimate of the mutation rate as that calculated from the observed mutants in the total offspring. Thus, the 90% confidence limits for the 250-mg/kg experiment are based on 21 mutations in 6,547 × 21/32 = 4,296 offspring—i.e., the number of offspring that are assumed to represent independent observations. This is admittedly a rough approximation, but it is probably a conservative estimate in the sense that the confidence limits obtained will tend to be wider than the true values, because some of the apparent clusters, here taken as single events, may, as was pointed out above, have involved independent mutations. The tables of Crow and Gardner (11) were used for computing the confidence limits.

The dose–response curve appears to be S-shaped. Statistical departure from linearity was tested separately for the portions of the curve above and below the apparent point of inflection. In the upper part of the curve, the data show no statistically significant departure from a straight line, fitted by the method of maximum likelihood, through the control and the points at 100 mg/kg and above. Nevertheless, the progressive decrease in the point estimates of the mutation rate at all doses >100 mg/kg, the fact that three other experiments at 250 mg/kg (ref. 1 and unpublished data) fall below a linear extrapolation from the 0- and the 100-mg/kg points, and the observation that another experiment at the 100-mg/kg dose (12) is yielding a somewhat higher mutation rate than that shown in Fig. 1 tend to raise the expectation that more extensive data may show a statistically significant flattening of the curve above ≈100 mg/kg.

Whether or not the response flattens out at the 250-mg/kg dose, it is clear that the mutation frequency has not decreased below that at 100 mg/kg, as might have been expected from the extent of spermatogonial killing. With x-rays (13, 14) and procarbazine (15), when the spermatogonial killing reaches high levels, the mutation frequency falls to less than that observed at some lower doses. This is attributed to a lower mutational sensitivity of the resistant spermatogonia that survive the higher dose (13, 14). The amount of spermatogonial killing at the 250-mg/kg dose of ENU, as judged by the length of the temporary sterile period, is similar to, or even greater than, that observed for x-ray and procarbazine doses that show the decrease in mutation rate to less than that seen at lower doses. The explanation for this difference between ENU and x-ray results may lie in the nature of the cell killing by ENU, which E. F. Oakberg (personal communication) finds to be markedly different from that with x-rays. The selective killing of specific stages of the mitotic cycle is less marked with ENU than with radiation. Thus, those cells that survive may not be ones as resistant to mutation induction as are the survivors from x-irradiation.

In the lower portion of the dose–response curve, at doses of 100 mg/kg and below, the data show a statistically significant departure from linearity. Two separate approximate χ^2 tests for goodness of fit, based on departures from a maximum likelihood fit to a straight line, gave P values of 0.027 and 0.0065. The drop below linearity as the dose is decreased raises the possibility that the proportion of injected chemical reaching the testis decreases with decreasing dose. Because ENU is a reactive and unstable compound, this seems, at first sight, to be a likely explanation. However, there is strong evidence against it. V. Carricarte and G. Sega (personal communication) have measured the amount of unscheduled DNA synthesis occurring in mouse spermatids after intraperitoneal injection of ENU. Over the range from 100 to at least 10 mg/kg, and possibly lower, unscheduled DNA synthesis is directly proportional to the amount of ENU injected. This finding suggests that the decrease in mutation rate below linearity in this range of doses is not due to failure of the chemical to reach the testis in proportionate amounts. The other obvious and presumably most likely conclusion is a capacity of the spermatogonia to repair some genetic damage when the repair process is not swamped by a high dose.

An attempt to determine what happens at still lower doses than those reported here is being made by the use of fractionated doses. Information obtained to date is presented in a companion paper (12).

A limited dose–response curve for specific-locus mutations in the mouse has been reported for procarbazine (15), but there the data were not extensive enough to determine the shape of the curve at low doses. Thus, the present data on ENU represent the most extensive dose–response data yet obtained for chemical induction of transmitted specific-locus mutations in a mammal. Because this test involves the most effective mutagen known in mouse stem-cell spermatogonia, it is noteworthy that, despite this effectiveness of ENU, there is strong evidence that the spermatogonia are apparently capable of repairing at least a major part of the mutational damage induced at lower doses. This finding contributes to an understanding of basic mechanisms of mutation induction by chemicals in mammals, and it has important application to risk estimation.

Note Added in Proof. An additional study by G. Sega (personal communication) has shown that DNA ethylation in the testis after intraperitoneal injection of tritium-labeled ENU is directly proportional to injected dose over the range of 10 to 100 mg/kg. This greatly strengthens the conclusion that the greater than proportional reduction in mutation frequency at low doses of ENU is due to repair.

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