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Genetics

Dose-repetition increases the mutagenic effectiveness of *N*-ethyl-*N*-nitrosourea in mouse spermatogonia

(chemical mutagenesis/alkylating agent/mutation/temporary sterility)

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ABSTRACT **In order to maximize the mutagenic effectiveness of *N*-ethyl-*N*-nitrosourea in mouse stem-cell spermatogonia, advantage was taken of the fact that these cells can accumulate mutations from repeated doses given over relatively long time periods. Repeated doses (100 mg/kg) of ethylnitrosourea injected intraperitoneally into male mice at weekly intervals were found to allow adequate survival and fertility with total dosages of 300 and 400 mg/kg. The specific-locus mutation frequencies at these dosages were, respectively, 1.8 and 2.2 times that obtained with the maximal practicable single dose of 250 mg/kg. The mutation frequency induced by a 400 mg/kg dosage of ethylnitrosourea is 12 times the maximal mutation frequency achievable with a single exposure to x-rays and 36 times that reported for procarbazine, the most effective chemical mutagen previously known for mouse stem-cell spermatogonia. Ethylnitrosourea is already the mutagen of choice in deliberate attempts to create mouse models for human disease and in any experiments in which a maximal mutation rate is desired. Repeated-dose regimens similar to the ones reported here should increase the efficiency of such studies.**

One advantage of determining induced mutation rates in stem-cell spermatogonia is that these cells can accumulate mutational lesions throughout the reproductive life span of the animal; therefore, repeated doses of mutagens can be given over relatively long time periods to amass a total dosage higher than that tolerated in a single exposure. Ethylnitrosourea (EtNU) has been shown to be the most effective mutagen for induction of specific-locus mutations in mouse spermatogonia (1, 2). This has led rapidly to its being the mutagen of choice by many investigators (3, 4). Therefore, it seemed useful to test whether repeated doses could be given to yield higher specific-locus mutation rates than those obtained by single exposures.

This paper presents data from specific-locus tests with repeated doses of EtNU and from survival and fertility tests that were conducted prior to the specific-locus tests in order to choose feasible dosage regimens. A brief abstract of the preliminary results on survival and fertility has appeared (5).

MATERIALS AND METHODS

Survival and Fertility Tests. EtNU, synthesized in our laboratory by D. G. Doherty, was dissolved in phosphate buffer (6) adjusted to pH 6. The dose injected intraperitoneally was matched to the body weight of the animals by adjusting the volume of solution injected, which never exceeded 1 ml. Male (101 × C3H)F_1 mice were injected with dosages of 300 or 400 mg of EtNU/kg, given in 100 mg/kg doses. In four separate experiments, the individual doses were spaced 1 day, 2 days, 4 days, or 7 days apart, respectively. Six mice, 12 weeks old at the first injection, were in each dosage group in each experiment. All injections were completed within 1 hr after the EtNU was dissolved. 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 (7). Females were checked for pregnancy once a week until their pregnancy was confirmed. When females reached 1 year of age they were replaced by younger ones.

Main Experiments. On the basis of the results of the survival and fertility tests, two dosage regimens were chosen for the main specific-locus experiments. Male (101 × C3H)F_1 mice were given EtNU doses of 100 mg/kg at weekly intervals, for a total dosage of 300 or 400 mg/kg, in the manner described above. They were 8–9 weeks old at the time of the first injection. Each male was mated to one T female 12 weeks (300 mg/kg group) or 14 weeks (400 mg/kg group) after the final injection and was moved to a new female each week. After each 7-week period, the males were rotated back to the original group of females to start the cycle over again. The offspring were scored for mutations at the seven loci.

RESULTS AND DISCUSSION

Table 1 shows the results of the exploratory survival and fertility tests. Early deaths (within 1 month after the last injection) occurred only in the groups exposed at the two shorter dose interval. Later deaths showed no clear-cut dependence on dose interval. Earlier experiments with male mice of the same strain exposed to a single EtNU dose of 250 mg/kg gave 81% and 41% survival at, respectively, 12 and 15 months after injection. These are considerably larger percentages of survival than those at the corresponding times in the current experiments. However, even in the fractionation experiments, a large number of offspring can be obtained during the weeks before death in which the males are fertile.

Because mutation frequencies were to be determined only for stem-cell spermatogonia, the males were not mated until 7 weeks after the last injection. This is the time required for a differentiating stem cell to reach the ejaculate. A single dose of 100 mg/kg was known to induce a short temporary sterile period as a result of spermatogonial killing; consequently, much longer sterile periods were expected with the doses used here. The results given in Table 1 show that the length of the sterile period is dependent on the total dosage and the injection interval. In the 400 mg/kg group, at the three shorter dose intervals, fertility was never recovered, with the exception of one male in the 4-day-dose-interval group. In the 300 mg/kg groups, the longer the interval between injections, the more males recovered their fertility.

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

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Table 1. Exploratory test of survival and fertility after repeated 100 mg/kg injections of EtNU into male mice

Total dosage, mg/kg	Injection interval, days	No. of mice treated	No. surviving at various times (in months) after last injection					No. with recovered fertility	Sterile period,* days	
			1	6	9	12	15		Median	Range
300	1	6	5	5	5	4	0	0	—	
400	1	6	4	3	3	1	0	0	—	
300	2	6	5	4	4	3	2	2	268.5	260–277
400	2	6	5	5	4	3	2	0	—	
300	4	6	6	4	2	2	1	4	105	91–127
400	4	6	6	6	4	3	0	1	213	
300	7	6	6	4	4	3	1	6	62	45–89
400	7	6	6	5	2	1	0	5	88	83–137

*Interval from 7 weeks after last injection to first conception.

In the 7-day-interval group, the median length of sterile period—i.e., the delay in conceptions occurring from sperm treated as spermatogonia—was 62 days in the 300 mg/kg group and 88 days in the 400 mg/kg group.

From these results, both the 300 and the 400 mg/kg dosages delivered in 100 mg/kg doses at 7-day intervals were determined to be feasible procedures for measuring mutation rates. The survival and fertility results with these doses and fractionation intervals in the main experiments are shown in Table 2. In an earlier experiment with a single EtNU dose of 250 mg/kg given to males of similar age, the median length of the sterile period was 86 days. Thus, with 7-day intervals, even the 400 mg/kg dosage causes no greater delay in return of fertility than that caused by a single 250 mg/kg dose.

The results of the specific-locus tests are given in Table 3. The number of offspring observed in the 400 mg/kg group was less than one-third of that in the 300 mg/kg group, because, with the higher exposure, the temporary sterile period was longer, fewer males survived to become fertile, and these died earlier than the males in the other group.

Allelism tests have been completed for all the mutant animals except three that died before producing enough offspring for a conclusive test. For one of these, the locus involved could not be identified on the basis of phenotype: the mutation appeared to be an intermediate allele at either the *b* or the *p* locus. Table 3 lists the number of "clusters," i.e., mutants with the same allele born to the same sire and, therefore, possibly derived from a single mutational event in a stem cell. However, with the high mutation rate in these experiments, there were 12 cases in which two to four mutations at different loci or to different alleles at the same locus occurred among the offspring of a single male. These mutations were obviously of independent origin; consequently, some of the mutations to the same allele were also likely to have been of independent origin. The approximate confidence limits, calculated as described by Russell *et al.* (2), are based on the minimal estimate of independent mutational events, obtained by counting each cluster as a single mutation. Since some of the clusters probably involved more than one mutational event, the confidence limits given are probably wider than the true values.

The distribution of mutations among the seven loci is shown in Table 4. There were no simultaneous mutations at the closely linked *d* and *se* loci. Furthermore, 46% of the mutations were to alleles intermediate between null and wild type. These findings confirm the conclusion reached earlier that mutations induced by EtNU are probably not multilocus deletions and may be mainly intragenic changes (1, 8).

The mutation rates are shown graphically in Fig. 1 along with the dose–response curve for single doses of EtNU reported by Russell *et al.* (2).

The absolute mutation frequencies at 300 and 400 mg/kg are, respectively, 1.8 and 2.2 times that at a single dose of 250 mg/kg. Each increase is statistically significant ($P < 0.05$) even when the calculations are adjusted for the clusters. The point estimates of the mutation frequencies at 300 and 400 mg/kg are close to a linear fit with the frequency at 100 mg/kg. Dominant skeletal mutations obtained by Selby and Niemann (9) in animals from this same experiment showed an approximately linear increase from 300 to 400 mg/kg.

The repeated-dose regimens used in the present study permit adequate survival and fertility at total dosages higher than can be attained in a single exposure, and the mutation frequencies are correspondingly greater. Therefore, in efforts to produce specific types of mutations, such as mouse models for human disease, or in any experiments in which a maximal mutation rate is desired, a repeated EtNU dosage similar to that used here would appear to be the method of choice. Some modification of the procedure may, of course, be necessary, depending on the survival and fertility responses of the strain of mice used. It is possible that dose intervals longer than 7 days would yield higher survival and fertility. Doses <100 mg/kg are not recommended, because at 50 mg/kg the mutation frequency drops below that expected on a linear fit with the frequency at 100 mg/kg, and 10 doses of 10 mg/kg given at weekly intervals yielded a much lower mutation frequency than that from a single 100 mg/kg dose (10).

With the 400 mg/kg exposure, the mutation frequency for the seven loci is 1 mutation in less than 100 offspring. Therefore, with this dose, the induction of a new mutation

Table 2. Survival and fertility in main experiments with repeated 100 mg/kg injections of EtNU given at 7-day intervals to male mice

Total dosage, mg/kg	No. of mice treated	No. surviving to sire offspring	Sterile period,* days	
			Median	Range
300	22	22	39	36–58
400	23	12	84.5	66–114

*Interval from 7 weeks after last injection to first conception.

Table 3. Mutation frequencies in the offspring of male mice given repeated intraperitoneal 100 mg/kg injections of EtNU at 7-day intervals

Total dosage, mg/kg	No. of offspring	No. of mutant offspring	Mutation frequency × 10^5 per locus	
			Point estimate	Approximate 90% confidence limits*
300	5256	46†	125.0	96.1, 158.9
400	1683	18‡	152.8	95.6, 234.3

*See text for calculation.
†Includes 3 clusters of 2 and one cluster of 5.
‡Includes one cluster each of 2, 3, and 4.

Table 4. Distribution of mutations among the loci

a	*b*	*c*	*d*	*p*	*s*	*se*	Untested
0	7–8	10	10–14	16–24	4	3	1

Where a range is given, the higher number represents all the mutants scored, and the lower number assumes (probably incorrectly, see text) that every cluster was the result of a single mutational event.

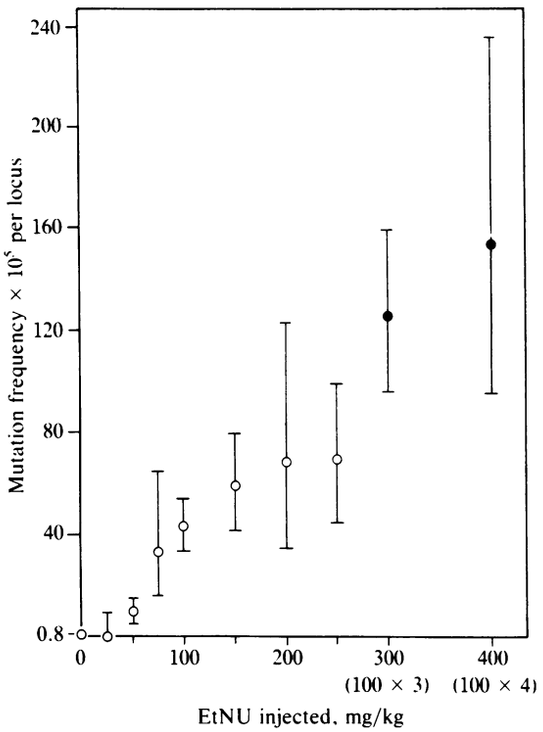

FIG. 1. Frequencies and approximate 90% confidence limits of EtNU-induced specific-locus mutations in mouse stem-cell spermatogonia. ●, Repeated doses of 100 mg/kg injected at weekly intervals; ○, earlier results with single doses, reported by Russell *et al.* (2).

that can be induced at a rate equal to the average of the seven loci used here should occur once in less than 700 offspring scored. Of course, the spread in mutation frequencies among the different loci (Table 4) indicates that some loci are mutated less frequently, and others more frequently, than this.

The mutation frequency obtained with the 400 mg/kg exposure is of a magnitude that seemed out of reach just a few years ago when radiation was the most effective mutagen known in the mouse. The mutation frequency induced by an EtNU dosage of 400 mg/kg is 12 times the maximum mutation frequency achievable with a single exposure of x-rays (11) and 36 times that reported for procarbazine, the chemical mutagen previously thought most effective for mouse stem-cell spermatogonia (12).

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