A Host-Mediated Microbial Assay for the Detection of Mutagenic Compounds (33666)

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(Introduced by J. Reilly)

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Assay systems for the study or detection of chemical mutagens utilize indicators ranging from microorganisms to mammals (1–3). Microbial tests (4) have a number of advantages since one can observe several generations and large populations over a short period of time. Bacterial systems, however, tend to give false negatives for compounds which are metabolized to an active state of mammals (5, 6), and give misleading results for compounds which are rendered less active in the host. The following microbial assay, which uses a murine host to mediate the test agent, represents one attempt at eliminating these difficulties in a simple but reliable test for mutagens.

Materials and Methods. Three histidine auxotrophs (7) of *Salmonella typhimurium*, strain C207 (a frameshift mutant) and strains C340 and G46 (nonsense mutants), were maintained on tryptone agar slants. Swiss albino mice (Flander's strain), 18–23 g, were used throughout, and were treated in groups of three. Test compounds were dissolved in saline wherever possible. When necessary, dimethyl sulfoxide or ethanol was used, along with appropriate controls. Final concentrations represented a compromise between solubility, mouse toxicity, and organism toxicity.

For the host-mediated test, tryptone broth was inoculated from an agar slant culture of *S. typhimurium* and incubated in a reciprocating shaker for 2 hr at 37°. This culture was diluted 1:4 with saline (final OD_{660} approximately 0.1) and 2 ml of the resulting suspension was injected intraperitoneally into the mouse.

Each mouse was then given the first of three intramuscular injections (0.1 ml) of the test compound; the remainder was administered at 1-hr intervals. All mice were sacrificed 30 min after the third injection. Each mouse then received 1 ml of saline intraperitoneally and as much fluid as possible was aseptically removed from the peritoneum.

Tenfold serial dilutions (10^{-1} to 10^{-7}) of each peritoneal fluid sample were made in saline. The four highest dilutions were plated on minimal agar with a histidine overlay to give the total *Salmonella* cell count, and the three lowest dilutions were plated on minimal agar without histidine for mutant growth.

Plates (15 × 100-mm plastic petri dishes) contained a base layer of 20 ml minimal agar (8) with 0.5% glucose. A standard pour plate technique was used: 0.1 ml of the proper dilution and 2 ml of molten 0.6% agar were added to a sterile tube, mixed, and poured over the surface of a base plate. The histidine overlay contained 0.1 ml of 0.1 *M* L-histidine/40 ml of agar. Plates were incubated at 37° for 48 hr, and the ratio of mutants:total cells (mutant frequency or MF) was determined.

A modification of the Szybalski (4) method was used for the *in vitro* tests. Molten 0.6% agar (2 ml) was added to 0.1 ml of an overnight broth culture of *Salmonella* and the mixture was poured over the surface of a minimal agar base plate. Positive results consisted of mutant colonies in a ring formation around the sample of test compound after incubation.

Results. In vitro *tests.* Streptozotocin, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NNG), and 2-aminopurine nitrate (AP) were positive in the standard plate test using *S. typhimurium*, G46. The remainder of the test compounds were negative.

Effect of procedure on organism viability.

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TABLE I. Effect of Streptozotocin (250 μg/injection) on Mutant Frequency of *Salmonella typhimurium* Implanted in Mice.[a]

Trial	Mice[b]	Mean mutant frequency ($\times 10^{-4}$)
1	10:2	1.7
2	10:2	1.6
3	10:2	1.7
4	10:2	3.1
5	10:1	2.1
6	7:4	2.3

[a] Control mutant frequency approximately 1×10^{-8}.

[b] No. treated : no. controls.

To determine the effect of the *in vivo* procedure on organism viability, one group of mice was given the standard intraperitoneal injection of diluted *S. typhimurium*, G46, without the subsequent mutagen injections. Pairs of mice were sacrificed at hourly intervals, and the number of viable organisms (mean value) per milliliter of peritoneal fluid was determined as previously described. The diluted culture, before injection, contained 4×10^6 organisms/ml and approximately 50% were recovered immediately after injection ($t = 0$), while at 2.5 hr (the normal recovery time) the titer was 1×10^7 organisms/ml.

Test reliability. Streptozotocin, at 250 μg/ml, markedly increased the *S. typhimurium*, G46, mutant frequency with the method described. This compound was tested further with a total of 70 mice (57 treated and 13 controls) in six separate trials to determine the reliability of the procedure (Table I). The mean mutant frequency had a range of 1.7 to 3.1×10^{-4}. In each trial, the mean mutant frequency (MF) with streptozotocin was increased at least 50-fold over the controls (control MF was approximately 1×10^{-8}), with an average increase of 4000-fold for the entire group.

Mutagens tested. Table II records the results of testing 19 compounds against *S typhimurium*, G46, in the host-mediated assay. Streptozotocin, NNG, and dimethylnitrosamine were all active.

Figure 1 shows a dose–response curve for NNG in a concentration of 200–1000 μg/injection. A dose–response curve of streptozotocin in a range of 50–600 μg/injection also displayed a linear relationship, but leveled off at $MF_t/MF_c = 1400$ for the 400–600 μg range.

Dimethylnitrosamine (DMNA) was consistently nonmutagenic in the standard plate test, but displayed activity in the host-mediated assay. The mutant frequency from five separate trials with 0.1 ml of 10%

TABLE II. Summary of Results from Host-Mediated Assay.

Compound[a]	Highest conc tested[b]	Results[c]
1. 2-Aminopurine nitrate	1200	—
2. Caffeine	1000	—
3. Theophylline	1000	—
4. 6-Azauridine	1000	—
5. Triacetyl 6-azauridine	1000	—
6. 5-Bromodeoxyuridine	1000	—
7. 5-Fluorodeoxyuridine	2000	—
8. Captan	400	—
9. Mitomycin C	100	—
10. Chloramphenicol	100	—
11. Streptozotocin	600	+
12. Neocarzinostatin	500	—
13. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine	1000	+
14. Triethylenemelamine	100	—
15. LSD	10	—
16. Patulin	500	—
17. Quinacrine	1000	—
18. Hydroxylamine	1200	—
19. Dimethylnitrosamine	10% (v/v)	+

[a] Compounds 1, 4, 5, and 6 were obtained from Calbiochem, Los Angeles, Calif.; 2 and 17 from Nutritional Biochemicals Corp., Cleveland, Ohio; 3 from Matheson, Coleman and Bell, East Rutherford, N. J.; 7 from Hoffmann-La Roche, Nutley, N. J.; 8 from California Chemical Co., Richmond, Calif.; 9 and 12 from Parke-Davis Co., Detroit, Mich.; 11 and 16 from Aldrich Chemical Co., Milwaukee, Wis.; 14 from Lederle Laboratories, Pearl River, N. Y.; 15 from the Bureau of Drug Abuse Control, FDA; 18 from Fisher Scientific Co., Pittsburgh, Pa.; and 19 from Eastman Organic Chemicals, Rochester, N. Y.

[b] μg per 0.1 ml injection.

[c] (—), no increase in mutant frequency; (+), more than 10-fold increase.

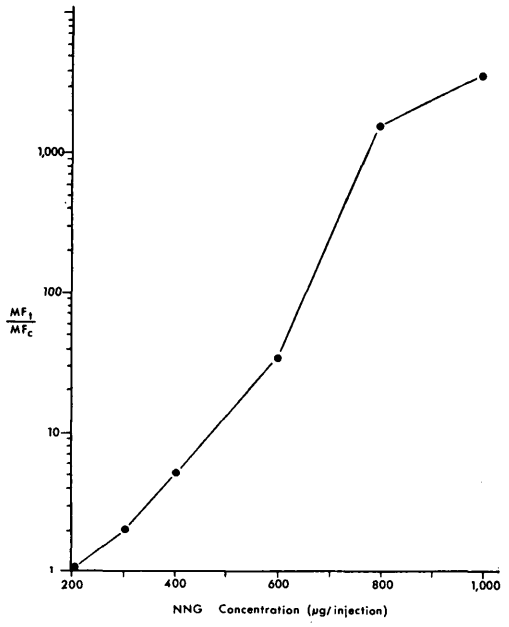

FIG. 1. Effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NNG) on mutant frequency of *S. typhimurium*, G46, in mice: MF_t = mutant frequency in treated mice; MF_c = mutant frequency in control mice.

DMNA was 1.3×10^{-6}; the lowest concentration that gave a positive response was 0.1% ($MF = 1.2 \times 10^{-7}$). When the MF after one injection of 10% DMNA was determined with time as a parameter, the maximal effect was noted at 120 min (Fig. 2).

Additional strains tested. S. typhimurium C340, was tested in the host-mediated system with streptozotocin at 50, 100, and 200 μg/injection. The increase in the number of revertants to histidine independence was 370-, 700-, and 870-fold, respectively. Strain C207 was negative for streptozotocin.

Escherichia coli, strains 532 and SD4-73 (streptomycin-dependent), were also tested. The saline used to dilute before injection and the complete agar (tryptone) contained 200 μg/ml of streptomycin sulfate. Streptozotocin increased the mutant frequency of strain 532, twentyfold and SD4-73, sixtyfold. Mitomycin C and triethylenemelamine, both positive in the plate test, were negative in the *in vivo* test. Difficulties were encountered in recovering large numbers of viable *E. coli*. The *Salmonella* strains adapted more easily to this system, and the spontaneous mutant frequency to prototrophy was lower and more stable.

Discussion. The preliminary experiments described here confirm the feasibility of a chemical mutagen detection system using a bacterial indicator and mediated by a mammalian host. Repeated trials with one of the positive compounds, streptozotocin, reveal that test results are reproducible. A histogram of mutant frequency versus frequency of occurrence indicated that the results were from the same homogenous population and were not randomly distributed.

Of the three compounds positive in the G46 plate test, NNG and streptozotocin were active in the host-mediated system, while AP was inactive. The negative is most probably due to detoxification, but a nonsystemic distribution or suboptimal concentration is also possible. A negative result would also be obtained following a point mutation at a locus different from that used in defining the auxotroph; i.e., a negative result only indicates the absence of a mutation at the locus being examined and does not preclude other mutations (7). The probability of detecting mutagenic activity could thus be increased by using an organism(s) with more than one marker.

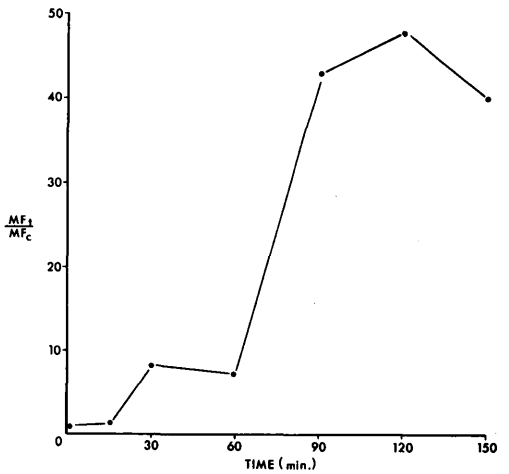

FIG. 2. Effect of time on the mutant frequency of *S. typhimurium*, G46, in mice after administration of 0.1 ml of 10% dimethylnitrosamine: MF_t = mutant frequency in treated mice; MF_c = mutant frequency in control mice.

Although a large part of the methodology was established using *S. typhimurium* G46, other organisms may also be used. Preliminary results indicate that implantation, recovery, and mutant detection are feasible using various organisms, strains, and markers, as well as different genes within an operon.

Dimethylnitrosamine is a potent carcinogen which fails to give evidence of mutagenicity *in vitro*, although it methylates protein, RNA, and DNA *in vivo* (6) and is mutagenic after hydroxylation (9). Its activity in the host-mediated test is especially pertinent since (a) this is the first instance of microbial mutagenic action due to DMNA after passage through an intact host, and (b) it illustrates the unique property of this test—the ability to detect mutagenic activity in metabolites of apparantly nonmutagenic compounds.

While an increase in mutant frequency suggests a mutational event, the role of selective forces must be evaluated. A compound which selectively kills auxotrophs or stimulates spontaneously arising prototrophs would result in a false positive. This source of error could be eliminated by the use of reconstruction experiments on each compound which substantially increases mutant frequency.

The role of selection could be further minimized by showing that (a) slopes of growth curves for auxotrophs and prototrophs are identical, and (b) the time in which mutants arose in treated animals is less than that which would be required for the propagation of the same number of mutants spontaneously. Each of these experiments has been conducted with the three compounds tentatively identified as mutagens, and selection by them has been completely eliminated.

Mutation rates, which could be determined using a zero-point or fluctuation method adapted to the animal system, would also minimize selection. These are also subject to limitations, however, since the concentration relative to the organism could not be determined, and it is not possible to recover 100% of the organisms originally injected.

The method described here has obvious advantages over simpler tests (4) since these results are semiquantitative and the activation–detoxification reactions become an integral part of the system. Compared with the more complex tests (10, 11) less time and expense and fewer manipulations are required. In addition, since a change in phenotype constitutes a positive response, this test can detect more subtle nucleic acid alterations than the widely used dominant lethal systems (10, 11) which primarily detect polyfunctional alkylating agents, and thus score NNG as negative, in contrast to our results. The haploid genetic complement also eliminates the masking of a recessive mutation by a dominant gene.

Summary. A procedure was established whereby a microbial indicator was incorporated in an animal system to identify and characterize mutagenic agents. The organisms, histidine auxotrophs of *Salmonella typhimurium*, were injected into mice intraperitoneally, and the test compound was administered intramuscularly. Mutagenesis, indicated by an increase in the incidence of prototrophs in the population, was induced by dimethylnitrosamine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and streptozotocin. Such a system appears capable of detecting microbial mutagenic activity in compounds which remain or become physiologically active after administration.

The authors wish to acknowledge the expert technical assistance of Mr. Alfred DeNunzio and the capable photographic aid of Mrs. Holdine Roginski.

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Received Oct. 17, 1968. P.S.E.B.M., 1969, Vol. 130.