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The Use of Motor Coordination Endpoints in the F1 Generation to Evaluate for Effects of Ethylmethanesulfonate on Pre- and Post-Meiotic Sperm Cells of Male Mice

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THE USE OF MOTOR COORDINATION ENDPOINTS IN THE F1 GENERATION TO EVALUATE FOR EFFECTS OF ETHYLMETHANESULFONATE ON PRE- AND POST-MEIOTIC SPERM CELLS OF MALE MICE

by

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Groups of 5 male HA(ICR) mice were injected intraperitoneally with 60, 150, 300, or 600 mg/kg body weight of ethylmethanesulfonate or with a saline control. Each male was mated to two untreated females at two weeks after treatment and then again at five weeks after treatment. The treatment groups were labelled post-meiotic and pre-meiotic relative to the stage of spermatogenesis at time of treatment. Progeny were evaluated by observations of litter size, body weight, negative geotactic response, swimming patterns, limb use, water escape time and open field motor coordination activity. Body weight, geotactic response, limb use and open field motor coordination test results demonstrate that EMS causes a measurable genotoxic effect on both post- and pre-meiotic cells. This effect was qualitatively different between the two treatment groups using the geotactic response, limb use and open field motor coordination endpoints.

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CHAPTER 1

INTRODUCTION

Chemotherapy, exposure to various chemicals in the home, workplace and the environment, and the abuse of licit and illicit drugs necessitates the testing of these agents for possible genotoxic effects (Glowa, Deweese, Natale, Holland & Dews, 1983). The effects of these agents may be revealed in the treated individuals, in their progeny or both. The purpose of this study was the detection of the genotoxic effects of the known mutagen/carcinogen ethylmethanesulfonate (EMS) in progeny of treated male mice using new methods.

Motor coordination (behavioral) testing of the progeny of a parent exposed to a genotoxic agent allows assaying of many gene loci that influence the development and coordination of the musculoskeletal and nervous systems and may detect abnormalities that cannot be seen with currently used morphological and biochemical tests (Butcher, 1976; Imel, 1982; Podraza, 1982). The methods used in this study are comparable in cost to other mammalian transmission genetic tests and, because the tests are noninvasive, subjects can be tested throughout various stages of development (Butcher, 1976). Wimer

(1979) has suggested that all mutagenicity screenings include at least a standardized open field motor coordination test of some kind. The methods of evaluation used in this research and in three previous studies in this laboratory are steps in that direction.

EMS was used to induce damage in pre- and post-meiotic germ cells in male mice by a single intraperitoneal injection of one of several doses. Ethylmethanesulfonate is a strong alkylating agent and has been known for many years to cause point mutations and/or chromosomal aberration in bacteria, plant and animal cells (Aaron et al., 1980; Brusick, 1980; Froese-Gertzen, Knozak, Foster & Nilan, 1963; Loveless & Howarth, 1959; Natarajan & Uphadya, 1964; Sega, 1984). Treatment of male mice with EMS has also been shown to cause sperm abnormalities in the treated individuals and their progeny (Brusick, 1980; Sega, 1984; Soares, Sheridan, Haseman & Segall, 1979; Wyrobek & Bruce, 1975).

The studies in this laboratory have used a battery of motor coordination tests developed by Adams and coworkers to assay the progeny of male rats treated with mitomycin C, procarbazine, ethylnitrosourea or cyclophosphamide (Adams, Fabricant & Legator, 1981; Imel, 1982; Podraza, 1982). Significant deficiencies were noted in motor skills at various stages of development as

described by Fox (1965). The F1 progeny of EMS treated male mice were evaluated in the current study using the same negative geotactic tests and swimming/water escape tests. Open field motor coordination, however, was monitored using the Opto-Varimex 3 Behavior Processor. The computer monitored motor coordination test detected transmission of EMS-induced genetic damage with greater ease and sensitivity than the other parameters used.

CHAPTER II

MATERIALS AND METHODS

Treatments and Management

Groups of five HA(ICR) male mice were injected intraperitoneally with 10 mg/kg saline solution or with 60, 150, 300, or 600 mg of EMS dissolved in 10 ml saline/kg body weight. Weights were recorded prior to treatment and each test with an Ohaus digital display balance. Treatments with different doses were staggered at one week intervals in order to facillitate efficient management for motor coordination testing. Each male was mated with two HA(ICR) females for a period of five days two weeks after injection and again with two other females for a period of five days five weeks after injection. This allowed evaluation of progeny resulting from sperm cells derived from cells treated in "post-meiotic" or "pre-meiotic" stages of spermatogenesis, respectively. Following mating, the females were separated and put in individual cages. When litters became due the cages were inspected daily for pups. When a litter appeared it was counted and then randomly reduced to a maximum of six pups to reduce those variables that could result from varying

litter size (Imel, 1982). On day 14 all pups were marked with a 0.05% solution of picric acid for individual identification. This method of marking proved to be visible throughout the length of the study. Following the day 35 motor coordination tests, the mother was removed from each cage.

Testing Procedure and Apparatus

On days 9 and 13 pups were tested individually for negative geotactic response times. Pups were placed head down on a 56 x 60 cm particle board raised to a 25 degree incline. The time for the pup to complete a 180 degree turn was recorded. Test completion was terminated at 60.0 seconds (Adams, Fabricant & Legator, 1981; Butcher, 1976; Imel, 1982). Mice that slid off the plane were restarted up to five times. If criterion was not achieved, the test was terminated at 60.0 seconds and this score was recorded.

Limb usage in swimming, swimming patterns and water escape times were observed on days 14 and 21. The 38 x 33 x 17 cm polypropylene bottom of a rat breeding cage was used as the water tank. Water was kept at a room temperature of approximately 20 degrees C and at a depth of 9.0 cm, making it even with the top of a 10 x 9 x 9.5 cm brick platform at one end of the tank. Each pup was

systematically scored as to whether it swam in a straight line or in a circular direction and whether it used all of its limbs in swimming or just its hind limbs. In earlier trials two scorers were required (Imel, 1982; Podraza, 1982). With the simplified scoring used in this study one observer was sufficient.

Water escape time was measured as the elapsed time from introduction to the water tank until all four paws were on the brick platform at the opposite end. Maximum time allowed for water escape was 180 seconds. Pups that escaped to the platform too quickly to get accurate limb use observations had their escape times recorded and were returned to the water so that observations could be made of limb usage. Pups that seemed to be drowning were rescued and were assigned a 180.0 second score (Imel, 1982).

Motor coordination analysis was conducted on days 28, 35 and 42 using the Opto-Varimex-3 animal activity monitor and accompanying accessories from Columbus Instruments International Corporation, 950 North Hague Avenue, Columbus, Ohio, 43204. The instrument measures animal activity in both the horizontal and vertical planes by setting up a light beam gridwork. Breaking of a beam by the animal is registered as a count. Continual movement within a beam path can also be timed. Six

categories of activity were measured. They were: (a) number of vertical (rearing on hind legs) movements, (b) number of beam interruptions in horizontal plane (walking or running movements), (c) total walking and running movements, (d) total number of starts and stops (stop was counted as >1.0 sec with no ambulation), (e) total number of movements while stationary (groups of scratching or grooming movements), and (f) total ambulatory time. Each mouse was monitored for 5 minutes total time in a darkened room. Readings were taken at the end of the time from the machine itself and from the printout. Between trials the processor cage was cleaned with 95% ethanol and allowed to dry before introduction of the next mouse.

All tests were run without knowledge as to which treatment group was involved.

Analysis of Data

All data except for those of litter size were analyzed by the Statistical Program for the Social Sciences on the Western Michigan University DEC-10 system (Nie, Hull, Jenkins, Steinbrenner & Bent, 1975). Significant differences are expressed at $p<0.05$. Statistical analyses included calculation of means and ranges, assessments of standard error and deviation and

variance and homogeneity tests. Litter sizes were analyzed using Student's t test with a t=0.01 level of confidence.

Injection of 600 mg/kg body weight of EMS resulted in the death of three of five males. No other deaths were caused by injection of the test substance. A mean of 52.2 +- 0.6 mice were evaluated per doseage group with the exception of the 600 mg/kg body weight where 23.9 +- 0.1 animals were evaluated.

CHAPTER III

RESULTS

Litter sizes

Treatment of post-meiotic cells of the male parents produced average litter sizes ranging from 7.4 for controls to 9.9 in the 60 mg/kg group. The 60 mg/kg body weight group had significantly larger litters than the control group. Progeny were absent in the 600 mg/kg group. Litters from the pre-meiotic stage treatments ranged from 8.3 to 10.6 (Table 1).

Table 1

Mean Litter Sizes of Control and Treatment Groups
Asterisk indicates significant difference at $p < 0.05$.
Doseages are with EMS per kg body weight.

Group	Pre-meiotic	Post-meiotic
0 mg/kg	7.4	8.4
60 mg/kg	9.9*	9.9
150 mg/kg	9.6	10.6
300 mg/kg	7.7	10.3*
600 mg/kg	0.0*	8.2

The number of matings were the same as in the first week

post-treatment groups. The 300 mg/kg body weight group showed a significantly greater and the 60 mg/kg group a significantly smaller litter size than controls. The small differences in litter sizes were not dose-related (Table 1).

Body Weight

The mean body weights of pups from post meiotic treatment matings was significantly lower at 9 and 13 days of age than the control group. On days 14 and 21, only the 60 and 300 mg/kg body weight groups showed significantly lower weights. On days 21 and 28, the 150 mg/kg body weight group showed significantly higher body weights. The 60 and 150 mg/kg body weight groups, while within the normal range on day 28, showed lower weights again on day 35. By day 42, all groups showed weights within the normal range compared to the control group (Figure 1).

In the pre-meiotic treatment groups, body weights were significantly lower than controls in the 60, 300, and 600 mg/kg body weight groups on days 9,13,14,21, and 28. The 150 mg/kg body weight group showed weights in the normal range throughout this time span. By day 35, all groups were within the normal range with the exception of the 600 mg/kg body weight groups which was

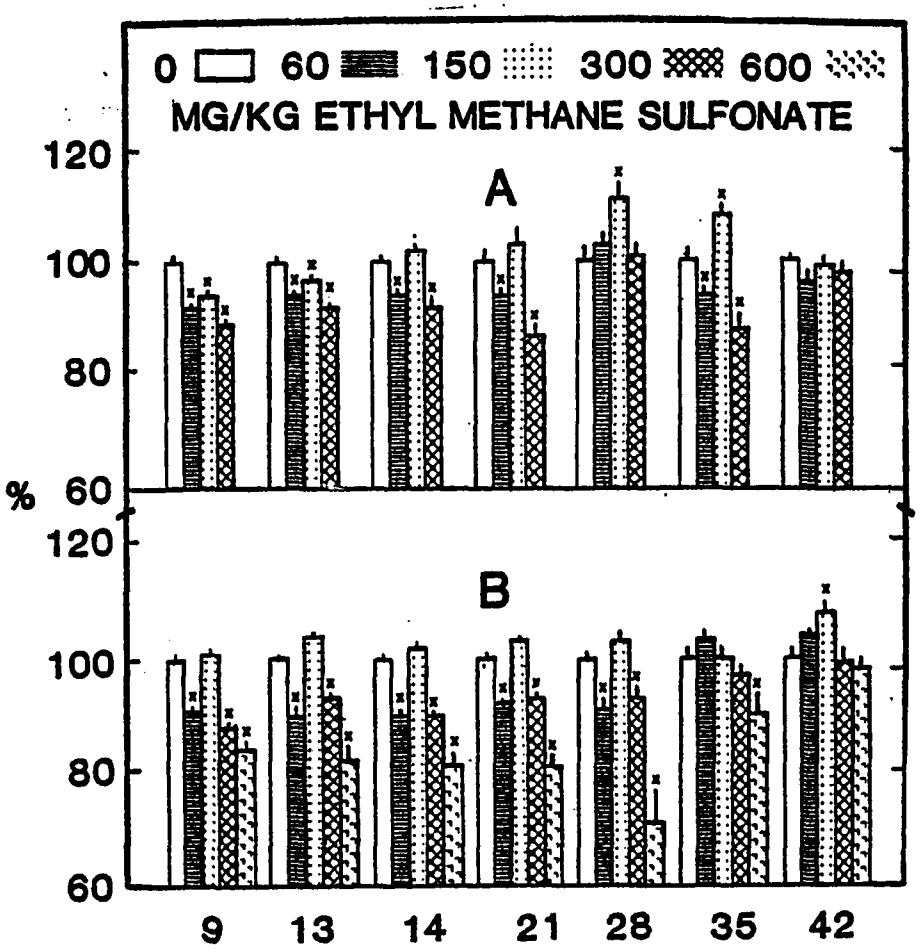

Figure 1. Percentage Body Weights (Concurrent Controls = 100%) at 9, 13, 14, 21, 28, 35 and 42 Days of Age. Panels A and B represent F1 mice from matings following exposure of post- or pre-meiotic cells, respectively. 52.2 +- 0.6 mice were evaluated per dose but only 23.9 +- 0.1 for the 600 mg dose. Vertical bars are SEM; an x indicates significant difference from concurrent controls at the 0.05 level.

significantly higher.

Negative Geotaxis

Day 9 geotactic response testing showed that the 300 mg/kg body weight group from the pre-meiotic treatment mating was significantly faster in reorienting from the head down position than the control group (See Figure 2). In the day 13 negative geotaxis tests all but one pre-meiotic treatment group had significantly shorter reorientation times than the concurrent controls. The 150 mg/kg group did not.

In the post-meiotic treatment groups, only the 150 mg/kg had significantly shorter reorientation times.

Water Escape, Swim Path, Limb Usage

In the post-meiotic treatment groups at day 14 testing, the 60 and 300 mg/kg body weight animals showed significantly less forelimb use in swimming when compared to controls. The pre-meiotic treatment groups did not show significant differences for any of the categories on day 14 testing. Day 21 tests in the first week post-meiotic treatment groups showed recovery to normal limb useage in the 60 and 300 mg/kg body weight groups but significantly less forelimb use in the 150 mg/kg body weight group. All doseage groups in the pre-meiotic

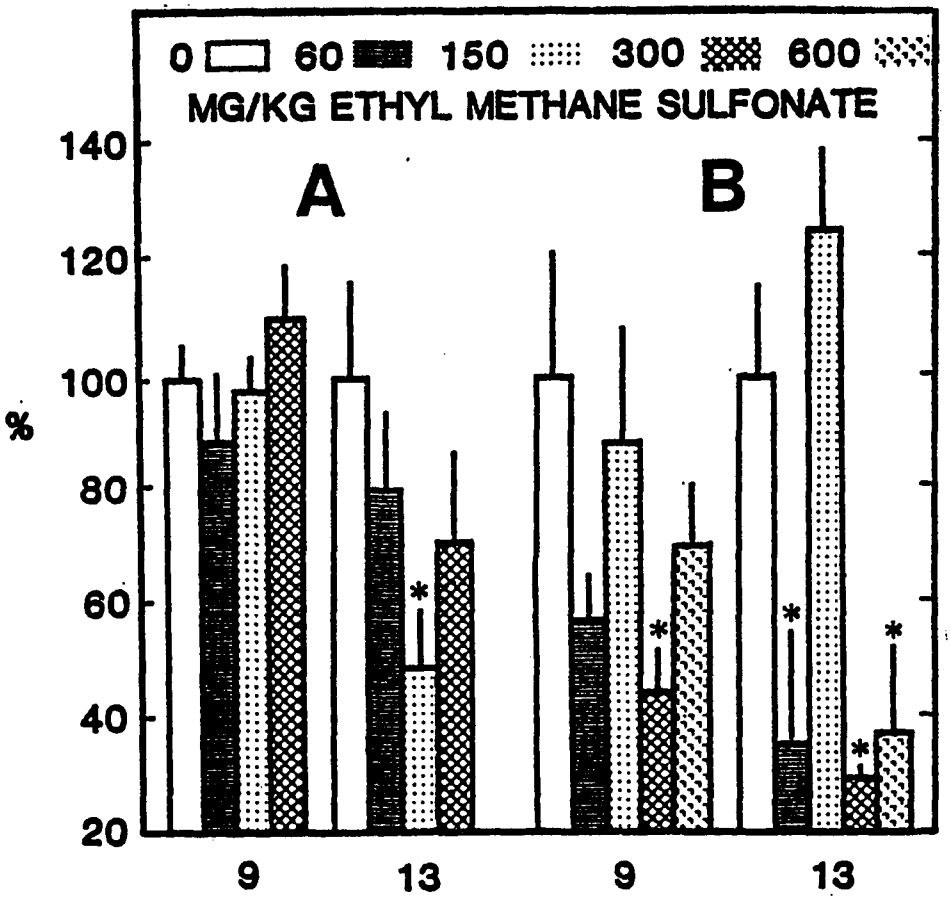

Figure 2. Percentage Geotactic Response (Concurrent Controls = 100%) at 9 and 13 Days of Age. Panels A and B as in Figure 1. 53.6 +- 1.3 mice were evaluated per dose but only 24.0 for the 600 mg/kg dose. Vertical bars are SEM; Asterisk (*) indicates significant difference from the concurrent controls at the 0.05 level.

treatment matings demonstrated significantly less forelimb use compared to controls on day 21 testing.

Open Field Motor Coordination

The post-meiotic treatment mating groups that showed significantly lower values than controls on day 28 testing were; a) 60 mg/kg body weight in categories 1,2, and 4. b) 300 mg/kg body weight in category 1. The day 35 testing showed the following significant differences from the control group in: a) 60 mg/kg body weight low in category 2 b) 150 mg/kg body weight low in category 1 and high in categories 3,4,5 and 6 c) 300 mg/kg body weight group high in categories 3,4,5 and 6. Day 42 testing demonstrated significant differences from the control group in: a) 60 mg/kg body weight showing higher values in categories 1,2,3,4 and 6 b) 150 mg/kg body weight higher values in the same categories as the 60 mg/kg body weight group c) 300 mg/kg body weight higher values in the same categories as the 60 mg/kg body weight group.

The pre-meiotic treatment group scores that were significantly different on day 28 testing were (Figure 5); a) 60 mg/kg body weight significantly higher in category 6 b) 150 mg/kg body weight lower in categories 2,3,4, and 5 c) 300 mg/kg body weight lower in category 1

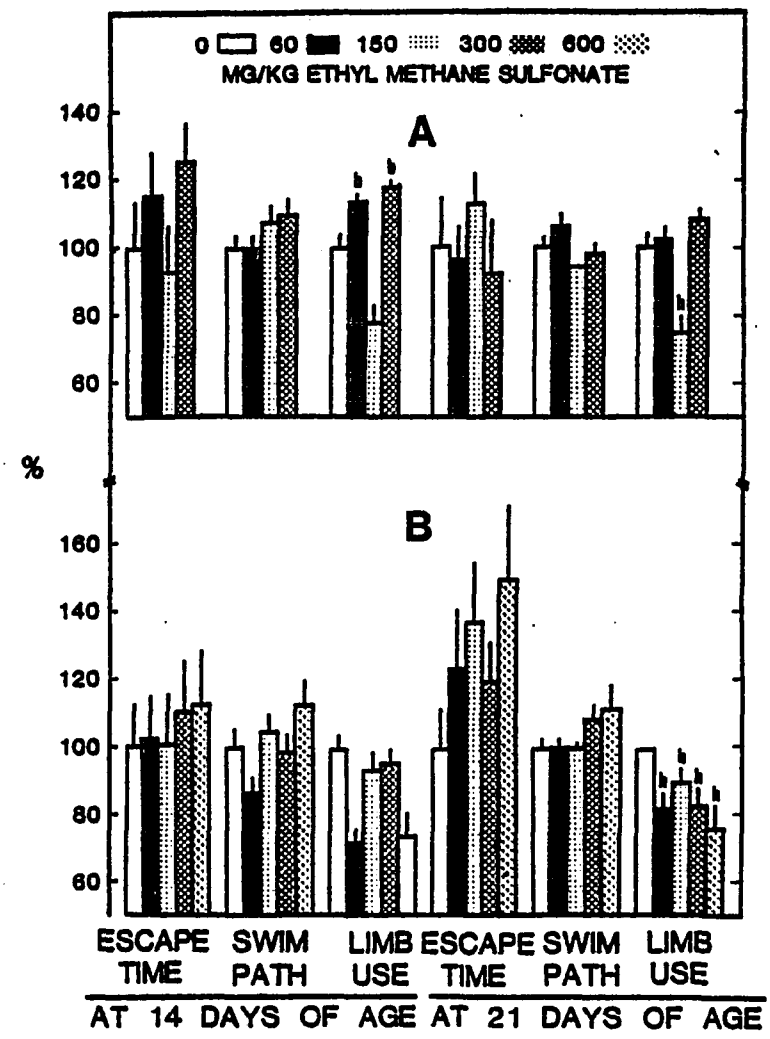

Figure 3. Percentage Water Escape Times, Straight or Circular Swim Paths and Limb Usage (Concurrent Controls = 100%) at 14 and 21 Days of Age. Panels A and B as in Figure 1. 52.9 +- 1.1 mice were evaluated per dose but only 24.0 for the 600 mg/kg dose. Vertical bars are SEM; b indicates significantly greater and h significantly less forelimb use at the 0.05 level.

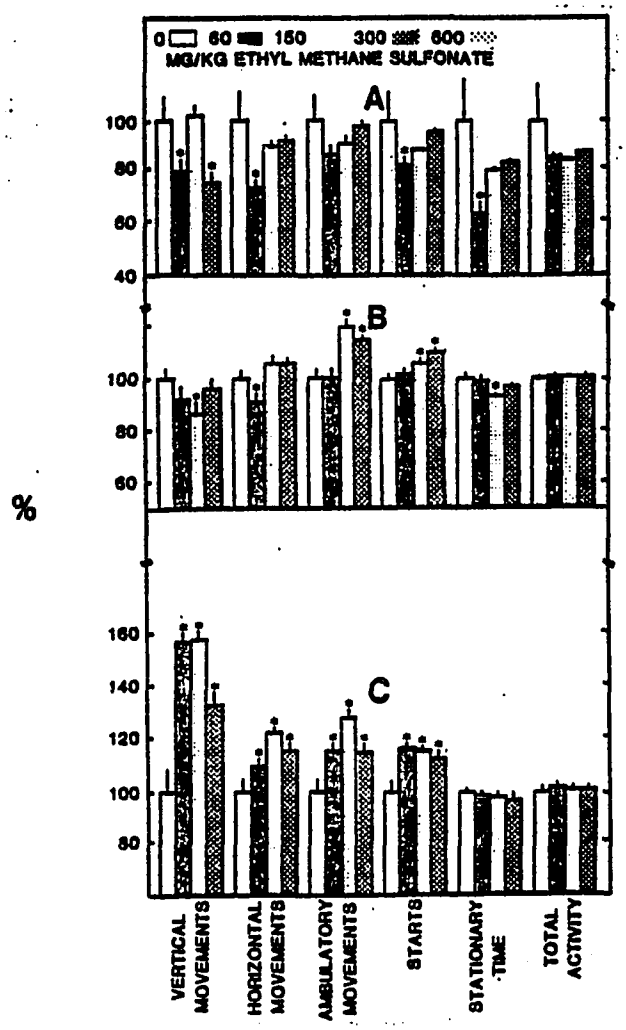

Figure 4. Percentage Motor Coordination Activity (Concurrent Controls = 100%) at 28 (A), 35 (B) and 42 (C) Days of Age. The F1 mice were from matings following exposure of post-meiotic cells. The categories are defined in the text. 50.5 +- 1.3 mice were evaluated per dose but only 22.0 +- 1.5 for the 600 mg/kg dose. Asterisk (*) indicates significant difference at the 0.05 level.

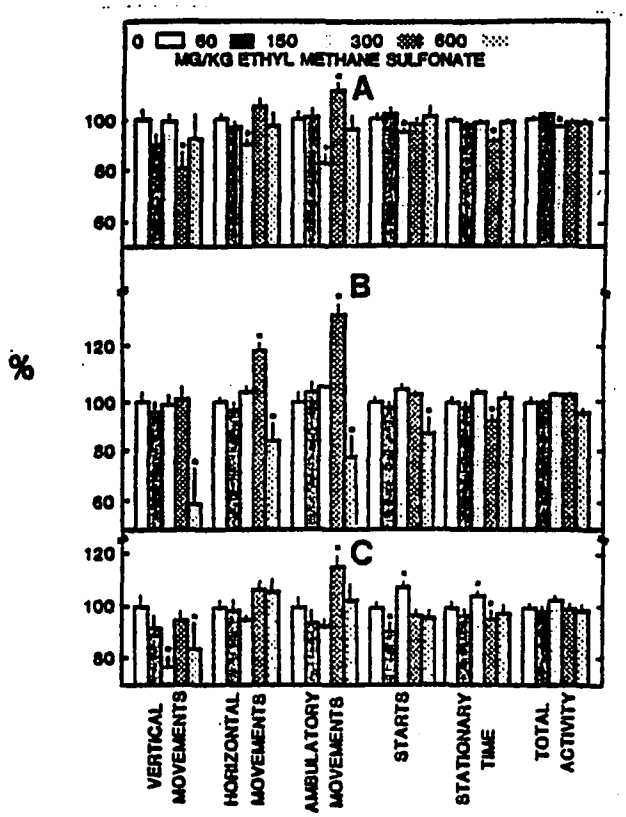

Figure 5. Percentage Motor Coordination Activity (Concurrent Controls = 100%). Panels as in Figure 4. The F1 mice were from matings following exposure of pre-meiotic cells. The categories are defined in the text. 51.1 +- 1.2 mice were evaluated per dose but only 22.0 +- 1.5 for the 600 mg/kg dose. Statistics as in Figure 4.

and higher in categories 3 and 6. Testing on day 35 showed significant differences in: a) 150 kg/mg body weight higher in category 5 b) 300 mg/kg body weight higher in categories 2,3,5 and 6 c) 600 mg/kg body weight lower in categories 1,2,3,4 and 6. The day 42 testing showed significant differences from the control group in: a) 60 mg/kg body weight lower in category 4 b) 150 mg/kg body weight lower in category 1 and higher in category 4 c) 300 mg/kg body weight higher in category 3 d) 600 mg/kg body weight lower in category 1.

The post-meiotic treatment groups showed a total of 29 endpoints out of a possible 54 significantly different with consistently higher values found in categories 1,2,3,5 and 7 at day 42 testing in all groups versus control. The pre-meiotic treatment groups demonstrated 20 significantly different behavioral endpoints out of a possible 63. Day 42 tests for the pre-meiotic treatment group showed 5 significantly different results compared to the 15 noted in the post-meiotic treatment tests.

CHAPTER IV

DISCUSSION

Podraza (1982) found that if treated litter sizes were decreased by dominant lethality to about half of control litter size, the surviving treated litter mates performed better than the control litter mates in early neonatal motor coordination tests. Because litter sizes tended to be similar in control and treated litters and because of the random reduction of all litter sizes to six pups, the differences in the intrauterine and neonatal environments probably did not influence the outcome of the motor coordination tests used here.

The results of this study will be interpreted in terms of how they support one or more of three generalizations. First, the hereditary effect of EMS on male germs cells is expressed in the progeny as a delay of the development of neuromuscular coordination up to the onset of sexual maturity. Second, the two germ cell stages show different sensitivities to EMS. Third, the effect of treatment on post-meiotic and pre-meiotic germ cells results in qualitatively distinguishable phenotypes in the F1 generation.

The data on body weight support the first and

second generalizations. Post-meiotic treatments caused lower body weights at 9 and 13 days of age followed by a trend of body weight gains to reach control levels by the onset of sexual maturity. Pre-meiotic cells were more sensitive than post-meiotic cells.

The geotactic response times showed that pre-meiotic cells were more sensitive to treatments supporting the second and third generalizations.

Limb usage results during swimming supports both the second and third generalizations. The effect on limb usage was generally more pronounced following treatment of pre-meiotic cells. Pre-meiotic treatment groups exhibit a pattern towards infantile limb use. Post-meiotic treatment groups did not demonstrate this pattern, indicating a qualitative difference between EMS effects on the two groups. The scoring of the water escape times and swim path patterns did not reveal any differences between treated and control groups.

The open field motor coordination test results from the post-meiotic treatment groups (Figure 4) showed more infantile motor coordination, i. e., hypoactivity, at the youngest age tested (Figure 4, panel A) followed by a gradual transition towards hyperactivity at 5 weeks of age (Figure 4, panel B). At 6 weeks of age, hyperactivity is predominant in all endpoints measured (Figure 4, panel

C). This observation is unique in that a developmental delay did not progress into normal behavior but was followed by hyperactivity at th sixth week of age, coinciding with the onset of sexual maturity. We did not determine if the hyperactive behavior persisted to a later age.

The pre-meiotic treatment groups showed hypoactivity at the earliest age with a gradual recovery to almost normal activity by the sixth week of age. This result with pre-meiotic cells supports the generalization that an early developmental deficiency may disappear at a later age. These results also provide evidence that a different spectrum of phenotypes results from the treatment of pre- and post-meiotic cells. This difference may be indicative of different types of mutations induced in the two cell populations. Conversely, the observed difference may reflect selection against chromosomal aberration during meiosis. Because motor coordination testing is non-specific in its determination of the gene loci affected it is possible that variance from controls may result from mutation to loci other than those controlling the direct development and coordination of the neuromuscular system. Whether the induced mutation has a direct or indirect effect on the neuro- muscular system is not the issue. The detection of the effects is

the reason for use of the test battery. The tests used here should detect the results of either direct or indirect mutation relative to the neuromuscular system.

Ranking the tests used in this battery as to their contribution toward the formation of useful generalizations gives the following order:

Computerized motor coordination > body weight = limb usage > geotaxis

The measurement of water escape times and swim path scoring did not result in support of any of the generalizations.

The use of motor coordination endpoints as a part of genotoxicity screenings may be helped by the use of mechanized observational equipment. This type of equipment, characterized in this work by the Opto-Varimex Behavioral Processor, helps to eliminate observer bias and can be operated by personnel with a minimum of instruction and/or knowledge in the field of genetic toxicology. It therefore can reduce research costs, especially those involving labor.

It would also be useful to concurrently test subjects treated acutely and chronically with the chemical(s) in question. This might simulate more

accurately possible methods of exposure of humans as pointed out by Imel (1982).

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