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Project Summary

Skeletal Variants as an Indicator of Biological Effects of Environmental Contaminants

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The project consisted of an attempt at developing a Skeletal Variant Assay System (SVAS) for mice whereby it might be possible to detect subtle biological effects of *in utero* exposure to noxious substances even in the absence of gross malformations, by an examination of a series of variations in development in adult skeletons.

The compounds tested included several pesticides and a number of known teratogens. Animals were CD-1 mice. Doses were chosen to include potentially teratogenic and no effect levels; they were administered by the most appropriate route and at appropriate times; choices were culled from published protocols wherever these were available. Toxicity tests were performed when necessary. Vehicle controls and untreated controls were included in each series.

Eighty-eight variants were selected for study. They were all qualitative in nature to increase ease of typing, objectivity, and reliability. Most were polymorphic in CD-1 mice and thus what was measured was change in frequency of occurrence in response to treatment.

In many cases a prenatal study was also performed in order to provide comparability with traditional teratogen studies. A series of fetal skeletal parameters was included in the prenatal studies.

A series of adult skeletons of four-way outcross mice which had been selected for susceptibility and resistance to the teratogen trypan blue were available and were also examined for the SVAS variants as a separate confirming study.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The experimental design was very similar for all compounds. Mice were purchased as needed and maintained for, usually, three to six weeks before the start of the experiments, in shoebox-type cages, five animals of one sex per cage, at 72 ± 2°F, relative humidity at 50 ± 5%, on light:dark regime of 18hr:6hr, with Purina Mouse Chow and tap water available *ad libitum* Groups of five females were mated with a single male at 4:00 p.m. and checked for vaginal plugs the following morning Mated females were isolated to experimental pens, with five females per pen, each from a different mating, receiving the same treatment. Each male contributed to different experimental groups

The order of the experimental pens was randomized on the shelves.

Females were isolated after their last treatment and bore and raised their litters in isolation. Litters were counted and weighed within one day of birth and examined for gross abnormalities. They were again examined, sexed, and weighed at weaning and allowed to remain together until sacrifice at 62 ± 2 days of age. Litters were killed, identified with unique numbers, processed for alizarin staining of articulated skeletons, examined for a battery of 44 variants, reprocessed for study of an additional battery of 44 variants in papain-digested, disarticulated skeletons, and stored.

Frequencies of each variant in each experimental group were calculated and each experimental was compared with Untreated and Vehicle Control values for each variant. Of interest were the number of variants which differed in the experimental groups, the magnitude and direction of the differences, and the specific variants which were affected.

Statistical analysis initially involved both Mann-Whitney U tests on mean litter percent derived frequencies with significance being defined as $P<.05$ and t-tests on arcsin square-root transformed frequencies with significance being defined at $P<.01$ to compensate for Type I errors It was subsequently felt that the Mann-Whitney test discarded potentially useful data and the t-test became the standard test of significance for the preliminary analyses.

In the prenatal studies fetuses were dissected on the 18th day of gestation, examined for gross malformations, weighed, and preserved in alcohol or formalin for alizarin staining Fetal skeletons were studied for ossification of cervical centra, caudal vertebrae, number of metatarsals and metacarpals, number of phalangeal rows, and number of ribs and sternebrae.

All of the variants which occurred in these experiments were photographed as were additional representative specimens of interest, thus providing a permanent record. Some specimens were photographed through a dissecting microscope. A photographic atlas of variants is presented in the project report.

Conclusions

Skeletons of CD-1 mice were examined in a systematic way at $62 + 2$ days postnatal for a series of 88 normally occurring variations. Subjects had been exposed *in utero*, by treatment of their dams, to one of a group of pesticides—Trifluralin (TFL), 2,4,5-T (245-T), Captan, Maneb, Dicamethrin (DMT); or known teratogens—Thalidomide, trypan blue (TB), acetazolamide (ACZM), bromodeoxyuridine (BUDR), diphenylhydantoin (DPH), cortisone (CORT), at both high and no-effect levels. Frequencies of occurrence of each variant in each treatment group were contrasted with vehicle (VEH) control and untreated (UNTD) values. Sixty-eight of the skeletal variants were useful in these studies; the others occurred rarely or not at all.

Prenatal exposure to 2,4,5-T, TFL, DMT, TB, ACZM, and BUDR could be detected by the existence of substantial numbers of skeletal variants the frequencies of which were significantly different from VEH and UNTD controls, at the higher dose levels. Prenatal exposure to Captan, Thalidomide, cortisone, diphenylhydantoin, and probably Maneb, could not be detected by this criterion. This suggests that the SVAS is a sensitive, yet not indiscriminate, method for detecting prenatal exposure to a variety of noxious substances, even in the absence of gross malformations. Considering the particular variants which were affected, it would appear that a smaller subgroup could serve as a reasonable screen for detecting subtle biological effects, by virtue of their having responded in a large number of the experimental series. These include the presence of an interfrontal bone, the existence of parted frontals and preoptic sutures in the skull, imperfect transverse foramina (F.T.I.) of the atlas, reduction of articular processes of the thoracic vertebrae, occurrence of 27 rather than the usual 26 presacral vertebrae, sacral fusions in the vertebral column, possible carpal fusions in the appendicular skeleton, and the occurrence of 14 ribs.

Some variants were only affected by teratogenic or high dose levels. These included malformations of the ribs, and fusions in the lumbar vertebrae and in the transverse processes of the sacral vertebrae. Some of these might be considered cryptic malformations. In most of the series there were variants which were enormously affected only by one or two compounds. These included Interfrontal and Carpal Fusions in TB and BUDR experiments, F.T.I. of the atlas by TB, Accessory Parietal and 27 presacral vertebrae (on day 8 only) by ACZM, fusions of dorsal processes of sacral vertebrae by 2,4,5-T and ACZM, fusions of the vertebral body in sacral vertebrae by ACZM (especially in the days 9-11 treatment) and by TB, the occurrence of fewer than 30 caudal vertebrae in the 245-T, TFL and perhaps ACZM treated groups, and caudal fusions by ACZM and TB. Most of the treatment effects on the important variants in all studies resulted in increases in frequencies of occurrence.

In many of the experiments there were also variants which were uniquely affected. These included: Fused Frontals, Frontal Foramen Double and a really major increase in absence of prominent dorsal spine of the second thoracic vertebra in 245-T, doubling of the posterior foramen minus in the palatal bone by TFL, F.T.I of C3-C6 by DMT, dyssymphysis of the atlas, malformations and dyssymphyses of the cervical vertebrae by TB (also in Captan treated animals). Accessory Parietal and Abnormal Metoptic Roots by ACZM on day 8, lumbar fusions and malformations in both ACZM regimes and in BUDR treatments on day 9.

Replication of experiments with TB, ACZM, and BUDR resulted in good to excellent agreement between the two series. Considerations of spectrum and magnitude of response as well as idiosyncratic or specific responses add strength to the ability of the SVAS to detect prenatal exposure to 245-T, TFL, DMT, TB, ACZM, and BUDR.

Prenatal studies with most of the compounds were in essential agreement with published results; DPH was neither teratogenic nor fetotoxic in these studies and was also negative in the SVAS; cortisone, although it had no effect on the skeletons of survivors, was a potent cleft palate producer. From the prenatal studies there was also the suggestion that ossification of the centra of the cervical vertebrae and of caudal vertebrae might be indicative of exposure to high doses of all or nearly all of the compounds.

Finally, examination of available skeletons from adult mice in several generations of a four-way outcross mouse population selected for susceptibility or resistance to trypan blue teratogenesis as well as an unselected line revealed better agreement across generations for each line that was seen in different batches of CD-1 mice. The variability of different "batches" of CD-1 mice require an examination of untreated animals in each group of animals purchased.

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***Neil Chernoff** is the EPA Project Officer (see below).*
The complete report, entitled "Skeletal Variants as an Indicator of Biological Effects on Environmental Contaminants," (Order No. PB 81-186 025; Cost: $17.00, subject to change) will be available only from:
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Springfield, VA 22161
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