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Academy of Sciences, Washington, DC (1977).

(3) World Health Organization. *Principles for the Testing of Drugs for Teratogenicity*. WHO Technical Report Series No. 364. (Geneva: World Health Organization, (1967).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19077, May 20, 1987]

Subpart F—Genetic Toxicity

§ 798.5195 Mouse biochemical specific locus test.

(a) *Purpose*. The mouse biochemical specific locus test (MBSL) may be used to detect and quantitate mutations originating in the germ line of a mammalian species.

(b) *Definitions*. (1) A biochemical specific locus mutation is a genetic change resulting from a DNA lesion causing alterations in proteins that can be detected by electrophoretic methods.

(2) The germ line is comprised of the cells in the gonads of higher eukaryotes, which are the carriers of the genetic information for the species.

(c) *Reference substances*. Not applicable.

(d) *Test method*—(1) *Principle*. The principle of the MBSL is that heritable damage to the genome can be detected by electrophoretic analysis of proteins in the tissues of the progeny of mice treated with germ cell mutagens.

(2) *Description*. For technical reasons, males rather than females are generally treated with the test chemical. Treated males are then mated to untreated females to produce F1 progeny. Both blood and kidney samples are taken from progeny for electrophoretic analysis. Up to 33 loci can be examined by starch-gel electrophoresis and broad-range isoelectric focussing. Mutants are identified by variations from the normal electrophoretic pattern. Presumed mutants are bred to confirm the genetic nature of the change.

(3) *Animal selection*—(i) *Species and strain*. Mice shall be used as the test species. Although the biochemical specific locus test could be performed in a number of in bred strains, in the most frequently used cross, C57BL/6 females are mated to DBA/2 males to produce

(C57BL/6×DBA/2) F1 progeny for screening.

(ii) *Age*. Healthy, sexually-mature (at least 8 weeks old) animals shall be used for treatment and breeding.

(iii) *Number*. A decision on the minimum number of treated animals should take into account possible effects of the test chemical on the fertility of the treated animals. Other considerations should include:

(A) The production of concurrent spontaneous controls.

(B) The use of positive controls.

(C) The power of the test.

(4) *Control groups*—(i) *Concurrent controls*. An appropriate number of concurrent control loci shall be analyzed in each experiment. These should be partly derived from matings of untreated animals (from 5 to 20 percent of the treated matings), although some data on control loci can be taken from the study of the alleles transmitted from the untreated parent in the experimental cross. However, any laboratory which has had no prior experience with the test shall produce a spontaneous control sample of about 5,000 progeny animals and a positive control (using 100 mg/kg ethylnitrosourea) sample of at least 1,200 offspring.

(ii) *Historical controls*. Long-term, accumulated spontaneous control data (currently, 1 mutation in 1,200,000 control loci screened) are available for comparative purposes.

(5) *Test chemicals*—(i) *Vehicle*. When possible, test chemicals shall be dissolved or suspended in distilled water or buffered isotonic saline. Water-insoluble chemicals shall be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels*. Usually, only one dose need be tested. This should be the maximum tolerated dose (MTD), the highest dose tolerated without toxic effects. Any temporary sterility induced due to elimination of spermatogonia at this dose must be of only moderate duration, as determined by are turn of males to fertility within 80 days after

treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) *Route of administration.* Acceptable routes of administration include, but are not limited to, gavage, inhalation, and mixture with food or water, and intraperitoneal or intravenous injections.

(e) *Test performance*—(1) *Treatment and mating.* Male DBA/2 mice shall be treated with the test chemical and mated to virgin C57BL/6 females immediately after cessation of treatment. Each treated male shall be mated to new virgin C57BL/6 females each week. Each pairing will continue for a week until the next week's mating is to begin. This mating schedule permits sampling of all post-spermatogonial stages of germ-cell development during the first 7 weeks after exposure. Spermatogonial stem cells are studied thereafter. Repeated mating cycles should be conducted until sufficient offspring have been obtained to meet the power criterion of the assay for spermatogonial stem cells.

(2) *Examination of offspring*—(i) *Birth and weaning.* Offspring shall be examined at birth and at weaning for externally detectable changes in morphology and behavior; these could be due to dominant mutations. Such characteristics may include, but are not limited to, variations in coat color, appearance of eyes, size (in which case weighing of variant animals and littermates should be carried out), fur texture, etc. Gross changes in external form and behavior shall also be sought. Scrutiny of such visible characteristics of all animals shall be made during all subsequent manipulations of the animals.

(ii) *Tissue sampling.* Blood (about 0.1 mL) and one kidney shall be removed from progeny mice under anesthesia. Both tissues are then prepared for analysis by electrophoresis.

(iii) *Electrophoresis.* The gene products of 6 loci shall be analyzed in the blood sample by broad-range isoelectric focussing and of 27 loci in the kidney sample by starch-gel electrophoresis and enzyme-specific staining. Details on these procedures are included in paragraphs (g)(1) through (g)(3) of this section.

(iv) *Mutant identification.* Presumptive electrophoretic mutants shall be identified by variation from the normal electrophoretic banding patterns. Reruns of all variant samples shall be performed to confirm the presence of altered banding patterns. Samples from parents of progeny exhibiting banding pattern variations shall be assayed to determine whether the variant was induced by the experimental treatment or was pre-existing. All treatment-induced variants are bred to determine the genetic nature of the change.

(f) *Data and reports*—(1) *Treatment of results.* Data shall be presented in tabular form and shall permit independent analysis of cell stage-specific effects, and dose-dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive control data (if employed) and spontaneous control data shall also be tabulated. These concurrent controls shall be added to, as well as compared with, the historical control data.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the frequency of electrophoretic mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of these test points.

(ii) A test chemical which does not produce a statistically significant increase in the frequency of electrophoretic mutations over the spontaneous frequency, or a statistically significant and reproducible positive response for at least one of the test points, is considered nonmutagenic in this system, provided that the sample size is sufficient to exclude a biologically significant increase in mutation frequency.

(iii) Both biological and statistical significance should be considered together in the evaluation.

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(4) *Test evaluation.* (i) Positive results in the MBSL indicate that, under the test conditions, the test chemical induces heritable gene mutations in a mammalian species.

(ii) Negative results indicate that, under the test conditions, the test chemical does not induce heritable genemutations in a mammalian species.

(5) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, and paragraph (h) of this section, the following specific information shall be reported:

(i) Strain, age and weight of animals used; numbers of animals of each sex in experimental and control groups.

(ii) Test chemical vehicle, doses used, rationale for dose selection, and toxicity data, if available.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Number of loci screened for both treated and spontaneous data.

(vi) Criteria for scoring mutants.

(vii) Number of mutants found/locus.

(viii) Loci at which mutations were found.

(ix) Use of concurrent negative and positive controls.

(x) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Personal communication from Susan E. Lewis, Ph.D. to Dr. Michael Cimino, U.S. EPA, OPPT, October 5, 1989.

(2) Johnson, F.M., G.T. Roberts, R.K. Sharma, F.Chasalow, R. Zweidinger, A. Morgan, R.W. Hendren, and S.E.Lewis. "The detection of mutants in mice by electrophoresis: Results of a model induction experiment with procarbazine." *Genetics* 97:113-124 (1981).

(3) Johnson, F.M. and S.E. Lewis. "Mutation rate determinations based on electrophoretic analysis of laboratory mice." *Mutation Research* 82:125-135 (1981a).

(4) Johnson, F.M. and S.E. Lewis. "Electrophoretically detected germinal mutations induced by ethylnitrosourea in the mouse." *Proceedings of the National Academy of Sciences* 78:3138-3141 (1981b).

(5) Lewis, S.E., C. Felton, L.B. Barnett, W. Generoso, N. Cacheiro, and M.D. Shelby. "Dominant visible and electrophoretically expressed mutations induced in male mice exposed to ethylene oxide by inhalation." *Environmental Mutagenesis* 8:867-872 (1986).

(h) *Additional requirements.* Testing facilities conducting the mouse biochemical specific locus test in accordance with this section shall, in addition to adhering to the provisions of §§ 792.190 and 792.195 of this chapter, obtain, adequately identify, and retain for at least 10 years, acceptable 35-mm photographs (and their negatives) of the stained isoelectric-focussing columns and the stained starch-gels obtained following analyses of blood and kidney preparations, respectively, from mutant mice, their siblings, and their parents.

[55 FR 12641, Apr. 5, 1990]

§ 798.5200 Mouse visible specific locus test.

(a) *Purpose.* The mouse visible specific locus test (MSLT) may be used to detect and quantitate mutations in the germ line of a mammalian species.

(b) *Definitions.* (1) A visible specific locus mutation is a genetic change that alters factors responsible for coat color and other visible characteristics of certain mouse strains.

(2) The germ line is the cells in the gonads of higher eukaryotes which are the carriers of the genetic information for the species.

(c) *Reference substances.* Not applicable.

(d) *Test method—(1) Principle.* (i) The principle of the MSLT is to cross individuals who differ with respect to the genes present at certain specific loci, so that a genetic alteration involving the standard gene at any one of these loci will produce an offspring detectably different from the standard heterozygote. The genetic change may be detectable by various means, depending on the loci chosen to be marked.

(ii) Three variations of the method currently exist for detecting newly arising point mutations in mouse germ cells: