OECD GUIDELINE FOR TESTING OF CHEMICALS

476

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"Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Solid, liquid, vapour or gaseous test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- Melting point/boiling point
- pH (where appropriate)
- Vapour pressure data (if available)

• Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and the CHO and V-79 lines of Chinese hamster cells. In these cell lines the most commonly used systems measure mutation at the thymidine kinase (TK), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and Na^+/K^+ ATPase loci. The TK and HPRT mutational systems detect base pair mutations, frameshift mutations and small deletions; the Na^+/K^+ ATPase system detects base pair mutations only.

• Definitions

A forward mutation assay detects a gene mutation from the parental type to the mutant form which gives rise to a change in an enzymatic or functional protein.

Base pair mutagens are agents which cause a base change in the DNA.

Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Phenotypic expression time is a period during which unaltered gene products are depleted from newly mutated cells.

- Reference substances

The following are examples of the type of substance which might be used as a positive control:

- ethyl methanesulphonate
- hycanthone
- N-nitroso-dimethylamine
- 2-acetylaminofluorene
- 7, 12-dimethylbenzanthracene

- Principle of the test method

Cells are exposed to the test substance, both with and without metabolic activation, for a suitable period of time and are subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection.

Cells deficient in thymidine kinase (TK), due to the forward mutation $TK^+ \rightarrow TK^-$ are resistant to the cytotoxic effects of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The deficiency of the "salvage" enzyme thymidine kinase means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. However, in the presence of thymidine kinase, BrdU, FdU or TFT are incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity.

Thus mutant cells are able to proliferate in the presence of BrdU, FdU or TFT, whereas normal cells, which contain thymidine kinase, are not.

Similarly, cells deficient in HPRT are selected by resistance to 8-azaguanine (AG) or 6-thioguanine (TG), and cells with altered Na^+/K^+ ATPase are selected by resistance to ouabain.

B. DESCRIPTION OF THE TEST PROCEDURE

Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony-forming ability or growth rate of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each selected locus, to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency. After a suitable incubation time, cell colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in non-selective medium to derive the mutant frequency.

- Preparations

Cells

A variety of cell types are available for use in this assay including subclones of L5178Y, CHO cells or V-79 cells. Cell types used in this assay should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a low spontaneous mutant frequency. Cells should be checked for Mycoplasma contamination and may be periodically checked for karyotype stability.

Media and culture conditions

Appropriate culture media and incubation conditions (culture vessels, temperature, CO_2 concentration and humidity) should be used.

Media should be chosen according to the selective systems and cell type used in the assay.

Test substances

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle should not interfere with cell viability or growth rate.

Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system.

- Test conditions

Exposure conditions

The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures and concentrations of test substance used should reflect these defined parameters. The number of cells per culture is based on the expected background mutant frequency; a general guide is to use a number which is ten times the inverse of this frequency.

Several concentrations (usually at least four) of the test substance should be used. These should yield a concentration-related toxic effect. The highest concentration should produce a low level of survival, and the survival in the lowest concentration should approximate the negative control. Cytotoxicity should be determined after treatment with the test substanceboth in the presence and in the absence of an exogenous metabolic activation system. Relatively insoluble substances should be tested up to their limit of solubility under culture conditions. For freely soluble, non-toxic substances, the highest concentration used should be determined on a case-by-case basis.

Controls

Positive and negative (untreated and/or vehicle) controls should be included in each experiment. When metabolic activation is used, the positive control substance must be known to require such activation.

- Performance of the test

Cells should be exposed to the test substance both with and without metabolic activation. Exposure should be for a suitable period of time, in most cases one to five hours is effective; exposure time may be extended over one or more cell cycles.

At the end of the exposure period, cells are washed and cultured to determine viability and to allow for expression of the mutant phenotype.

At the end of the expression period, which should be sufficient to allow near optimal phenotypic expression of induced mutants, cells are grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency, respectively.

Results should be confirmed in an independent experiment.

3. DATA AND REPORTING

- Treatment of results

Data should be presented in tabular form. Individual colony counts for the treated and control groups should be presented for both mutation induction and survival. Survival and cloning efficiencies should be given as a percentage of the controls. Mutant frequency should be expressed as number of mutants per number of surviving cells.

Data should be evaluated by appropriate statistical methods.

- Evaluation of results

There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the mutant frequency. Another may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

A test substance producing neither a statistically significant concentration-related increase in the mutant frequency nor a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

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

- Test report

The test report should also include the following information:

- cell type used, number of cell cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subcultures and feeding schedules, if appropriate), selective agent(s)
- methods used to enumerate numbers of viable and mutant cells
- dose-response relationship, where possible

- Interpretation of results

Positive results for an *in vitro* mammalian cell gene mutation test indicate that a substance induces gene mutations in the cultured mammalian cells used. Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

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4. LITERATURE

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