B. 18. DNA DAMAGE AND REPAIR - UNSCHEDULED DNA SYNTHESIS - MAMMALIAN CELLS *IN VITRO*

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The Unscheduled DNA Synthesis (UDS) test measures the DNA repair synthesis after excision and removal of a stretch of DNA containing the region of damage induced by chemical and physical agents. The test is based on the incorporation of tritium labelled thymidine (^{3}H-TdR) into the DNA of mammalian cells which are not in the S phase of the cell cycle. The uptake of ^{3}H-TdR may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from the treated cells. Mammalian cells in culture, unless primary rat hepatocytes are used, are treated with the test agent with and without an exogenous metabolic activation system. UDS may also be measured in *in vivo* systems.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Test chemicals and control or reference substances should be prepared in growth medium or dissolved or suspended in appropriare vehicles and then further diluted in growth medium for use in the assay. The final concentration of the vehicle should not affect cell viability.

Primary cultures of rat hepatocytes, human lymphocytes or established cell lines (e.g. human diploid fibroblasts) may be used in the assay.

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

Test conditions

Number of cultures

At least two cell cultures for autoradiography and six cultures (or less if scientifically justified) for LSC UDS determinations are necessary for each experimental point.

Use of negative and positive controls

Concurrent positive and negative (untreated and/or vehicle) controls with and without metabolic activation should be included in each experiment.

Examples of positive controls for the rat hepatocyte assay include 7,12- dimethylbenzathracene (7,12-DMBA) or 2-acetylaminofluorene (2-AAF). In the case of established cell lines 4-nitroquinoline-N-oxide (4-NQO) is an example of a positive control for both the autoradiographic and LSC assays performed without metabolic activation; N-dimethylnitrosamine is an example of a positive control compound when metabolic activation systems are used.

Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response should be used. The highest concentration should elicit some cytotoxic effects. Relatively water-insoluble compounds should be tested up to the limit of solubility. For freely water-soluble non-toxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis.

Cells

Appropriate growth media, CO_2 concentration, temperature and humidity should be used in maintaining cultures. Established cell lines should be periodically checked for *Mycoplasma* contamination.

Metabolic activation

A metabolic activation system is not used with primary hepatocyte cultures. Established cell lines and lymphocytes are exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

Procedure

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density, and incubated at 37 °C.

Short-term cultures of rat hepatocytes are established by allowing freshly dissociated hepatocytes in an appropriate medium to attach themselves to the growing surface.

Human lymphocyte cultures are set up using appropriate techniques.

Treatment of the cultures with the test substance

Primary rat hepatocytes

Freshly isolated rat hepatocytes are treated with the test substance in a medium containing ^{3}H-TdR for an appropriate length of time. At the end of the treatment period, medium should be drained off the cells, which are then rinsed fixed and dried. Slides should be dipped in autoradiographic emulsion (alternative stripping film may be used), exposed, developed, stained and counted.

Established cell lines and lymphocytes

Autoradiographic techniques: Cell cultures are exposed to the test substance for appropriate durarions followed by treatment with ^{3}H-TdR. The times will be governed by the nature of the substance, the activity of metabolising systems and the type of cells. To detect the peak of UDS, ^{3}H-TdR should be added either simultaneously with the test subsrance or within a few minutes after exposure to the test substance. The choice between these two procedures will be influenced by possible interactions between test substance and ^{3}H-TdR. In order to discriminate between UDS and semi-conservative DNA replication, the latter can be inhibited, for example, by the use of an arginine-deficient medium, low serum content or by hydroxyurea in the culture medium.

LSC measurements of UDS: Prior to treatment with test substance, entry of cells into S-phase should be blocked as described above; cells should then be exposed to test chemical as described for autoradiography. At the end of the incubation period, DNA should be extracted from the cells and the total DNA content, and the extent of ^{3}H-TdR, incorporation determined.

It should be noted that, where human lymphocytes are used in the above techniques, the suppression of semi-conservative DNA replication is unnecessary in unstimulated cultures.

Analysis

Autoradiographic determinations

In determining UDS in cells in culture, Sphase nuclei are not counted. At least 50 cells per concentration should be counted. Slides should be coded before counting. Several widely separated random fields should be counted on each slide. The amount of ^{3}H-TdR incorporation in the cytoplasm should be determined by counting three nucleus-sized areas in the cyptoplasm of each cell counted.

LSC determinations

An adequate number of cultures should be used at each concentration and in the controls in LSC UDS determinations.

All results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form.

2.1. Autoradiographic determinations

The extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains found over the cell nucleus should be recorded separately.

Mean, median and mode may be used to describe the distribution of the extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains per nucleus.

2.2. LSC determinations

For LSC determinations, ^{3}H-TdR incorporation should be reported as dpm/µg DNA. The mean dpm/µg DNA with standard deviation may be used to describe the distribution of incorporation.

Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

-cells used, density and passage number at time of treatment, number of cell cultures,

-methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration,

-test substance, vehicle, concentrations and rationale for selection of concentrations used in the assay,

-details of metabolic activation systems,

-treatment schedule,

-positive and negative controls,

-autoradiographic technique used,

-procedures used to block entry of cells into S-phase,

-procedures used for DNA extraction and determination of total DNA content in LSC determination,

-dose/response relationship, where possible,

-statistical evaluation,

-discussion of results,

-interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.