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Prevalidation study of the Syrian hamster embryo (SHE) cell transformation assay at pH 7.0 for assessment of carcinogenic potential of chemicals

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ABSTRACT

The European Centre for the Validation of Alternative Methods (ECVAM) has organised an interlaboratory prevalidation study on the Syrian hamster embryo (SHE) cell transformation assay (CTA) at pH 7.0 for the detection of rodent carcinogens. The SHE CTA at pH 7.0 has been evaluated for its within-laboratory reproducibility, transferability and between-laboratory reproducibility. Four laboratories using the same basic protocol with minor modifications participated in this study and tested a series of six coded-chemicals: four rodent carcinogens (benzo(a)pyrene, 3-methylcholanthrene, 2,4-diaminotoluene and o-toluidine HCl) and two non-carcinogens (anthracene and phthalic anhydride). All the laboratories found the expected results with coded chemicals except for phthalic anhydride which resulted in a different call in only one laboratory. Based on the outcome of this study, it can be concluded that a standardised protocol is available that should be the basis for future use. This protocol and the assay system itself are transferable between laboratories and the SHE CTA at pH 7.0 is reproducible within- and between-laboratories.

1. Introduction

The *in vitro* cell transformation assays (CTAs) have been developed to simulate the process of carcinogenesis and have been proposed as an alternative to long-term rodent experiments for carcinogenicity testing [1]. The Syrian hamster embryo (SHE) cell transformation system is considered to mimic early stages of carcinogenesis whereas the C3H10T1/2 or BALB/c 3T3 mouse cell lines recapitulate later stages of carcinogenesis. The SHE CTA is based on the conversion of normal to neoplastic-like colonies of cells having oncogenic properties and provides a system to detect genotoxic as well as non-genotoxic carcinogens [2–4]. SHE cells are normal diploid cells characterised by (1) the ability to metabolise chemicals, (2) a low spontaneous transformation rate and (3) the rapidity of phenotypic changes (seven days). At pH 7.0, the cells in control colonies grow in an orderly monolayered pattern without overlap. Chemically induced phenotypic changes to a transformed phenotype are distinguished by the alteration of cellular morphology involving a random orientation pattern of growth in three dimensions. Transformed cells after subsequent passages acquire the characteristics of malignant cells and have the ability to produce tumours when injected back into newborn animals of the same species or immunosuppressed animals. The transformed-cell phenotype formation is used as a morphological indicator of cell transformation. The SHE cell system at pH 7.0 has been used to determine the *in vitro* potential of a variety of chemicals including polycyclic aromatic hydrocarbons, nitro compounds, metals, fibers, biotoxins, *etc.* [1,5–12].

In 2007, the Organisation for Economic cooperation and Development (OECD) published a Detailed Review Paper (DRP) for the detection of chemical carcinogens and recommended the CTA as an alternative method to detect carcinogenic chemicals [1]. The SHE assay carried out in the range of pH 7.0–7.35 was evaluated on a

Abbreviations: CTA, cell transformation assay; DMEM-L, Dulbecco's modified Eagle's medium with LeBoeuf's modification; DMSO, dimethylsulfoxide; DRF, dose range finding; DRP, Detailed Review Paper; ECVAM, European Centre for the Validation of Alternative Methods; FBS, foetal bovine serum; MTF, morphological transformation frequency; OECD, Organisation for Economic cooperation and Development; PE, plating efficiency; RPE, relative plating efficiency; SHE, Syrian hamster embryo; SOP, Standard Operating Procedure; VMT, Validation Management Team.

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set of 204 chemicals and the results showed a sensitivity of 92%, a specificity of 66%, a positive predictivity of 88%, a negative predictivity of 75% and a concordance of 85% [1]. Based on the data in the OECD DRP, an analysis of the SHE CTA performances for the detection of human carcinogens demonstrated that the assay (pH 6.7 and pH ≥ 7.0) had 100% sensitivity for 44 inorganic human carcinogens and 82% sensitivity for 11 organic human carcinogens (higher when 24-h exposure was also considered) [13]. In contrast, the Ames assay displays a 42% sensitivity (5/12) in detecting human carcinogens [14].

Although a considerable amount of data on the performances of the assay has been collected over the years, further validation focusing particularly on reproducibility and on the establishment of a standardised protocol, was considered important. Therefore, the European Centre for the Validation of Alternative Methods (ECVAM), after consultation with an expert group, decided to coordinate a prevalidation study. In order to evaluate whether the test would meet the criteria requested by the ECVAM principles on test validity [15], the modular approach of validation was followed [16]. This study focused on the assessment of the following modules: (1) test definition, (2) within-laboratory reproducibility, (3) transferability, and (4) between-laboratory reproducibility. In addition, preliminary information on the predictive capacity of the standardised SHE CTA (pH 7.0) protocol was also produced.

Four independent laboratories from the United States and Europe have participated in the prevalidation exercise. Each test was conducted according to the same agreed protocol. Six coded chemicals (benzo(a)pyrene, 3-methylcholanthrene, 2,4-diaminotoluene, o-toluidine HCl, anthracene and phthalic anhydride) were tested. The data produced by the four laboratories and the statistical analyses are presented.

2. Materials and methods

2.1. Participating laboratories

The four participating laboratories were: UMR CNRS 7146 – LIEBE – University of Metz (Metz, France), which was selected as the lead laboratory because of its experience in performing the assay at pH 7.0; BASF (Ludwigshafen, Germany); Harlan Cytotest Cell Research GmbH (Harlan CCR, Rossdorf, Germany); BioReliance (Rockville, MD, USA).

2.2. Chemical selection

The chemicals tested in this study included four carcinogens: benzo(a)pyrene, 2,4-diaminotoluene, 3-methylcholanthrene, o-toluidine HCl; and two non-carcinogens: anthracene, phthalic anhydride (Table 1). In all experiments, benzo(a)pyrene was used as positive control. The chemical solutions were prepared in dimethylsulfoxide (DMSO) in order to obtain a final DMSO concentration in the medium of 0.2%, which had no effect on cell response. The chemical solutions were diluted in culture medium just before use. Instructions for 3-methylcholanthrene and o-toluidine HCl dose ranges were given by the Validation Management Team (VMT) and were as follows: 0.01–10 μg/mL and 20–1200 μg/mL, respectively. For the other chemicals the laboratories had to choose the dose range themselves based on dose-range finding experiments. All chemicals were purchased from Sigma–Aldrich, coded and distributed to the participating laboratories by Johnson & Johnson Pharmaceutical Research and Development (Beerse, Belgium) and ECVAM (Ispra, Italy).

2.3. Syrian hamster embryo cell culture

Cryopreserved SHE cells were isolated from Syrian golden hamster embryos at day 13 of gestation using the procedure described by Pienta et al. [17] and in accordance with the modifications suggested by Elias et al. [18]. Stock cells have been prepared by the University of Metz laboratory for its own use, and by BioReliance for the use of BASF, Harlan CCR and itself.

2.4. Medium

The medium used for routine cell culture and for the assays was DMEM-L (Dulbecco's modified Eagle's medium with LeBoeuf's modification), a modified formulation of low glucose (1000 mg/L) DMEM containing 4 mM of glutamine and 110 mg/L of sodium pyruvate [19]. No phenol red was added into the culture medium. The pH of DMEM-L was adjusted to 7.0 at 37 °C in a 10 ± 0.5% CO_2 humidified atmosphere with the addition of $NaHCO_3$.

The three participating laboratories BioReliance, BASF and Harlan CCR used a liquid DMEM-L. University of Metz used lyophilised DMEM-L reconstituted with ultrapure water and sterilised by membrane filtration (0.2 μm porosity) after addition of $NaHCO_3$.

2.5. Serum

The complete medium was obtained after addition of 15% foetal bovine serum (FBS Hyclone Perbio AQL25247 for University of Metz and FBS Hyclone Perbio APB20666 for BASF, Harlan CCR and BioReliance). The serum lots were pre-selected for their ability to support optimal cell growth, cloning and transformation by benzo(a)pyrene.

2.6. Cell transformation assay

The detailed CTA protocol is described in this issue [20] and consists of two steps: a preliminary dose range finding (DRF) and the transformation assay. For each chemical the DRF test was based on the evaluation of the relative plating efficiency (RPE) to determine the experimental doses to be used in the transformation assay. The transformation assays included the measurement of cytotoxicity (RPE and colony density/size measurements) and the calculation of the morphological transformation frequency (MTF).

To summarise, 60,000 X-ray irradiated cells (exposed to 50 grays or approximately 5000 rad, to prevent cell division) used to feed target SHE cells were plated in 40 Petri dishes (60-mm) per dose with 2 mL of complete medium. The next day, target SHE cells were seeded with 2 mL of complete medium per dish on the feeder layer of irradiated SHE cells in order to obtain between 25 and 45 colonies per dish, so that at least 1000 colonies could be scored per treatment group. The cells were treated 24 h after target cell seeding with 4 mL of complete medium containing the test chemical. The cells were exposed to the test chemical at 37 °C in a 10 ± 0.5% CO_2 humidified atmosphere for seven days. At the end of the exposure period the medium was removed and the cells were washed with phosphate-buffered saline (PBS), fixed with absolute ethanol or methanol and stained with 10% aqueous Giemsa. After rinsing with tap water, the dishes were air-dried before being scored. Each dish was coded and scored blindly.

The controls used were: DMSO (0.2%), which served as the concurrent vehicle control; benzo(a)pyrene (1–5 μg/mL dissolved in 0.2% DMSO) used as the positive control; feeder cell controls, which corresponded to five dishes seeded with only feeder cells, so as to verify that no formation of colonies occurred. Additionally, the cell culture medium can be used as the concurrent untreated control, although this control is not mandatory.

At least five concentrations per chemical-treated group were scored. Definitive assay doses included, if possible:

- At least one dose which had no effect on PE.
- At least five concentrations selected up to a maximum of 5 mg/mL or 10 mM, whichever was lower and solubility permitting, if the test substance was essentially non-toxic.
- A high dose level tested causing an approximate 50% decrease in RPE or relative colony size/density, regardless of the number of insoluble dose levels, for toxic and insoluble test substances.
- A high dose level tested within twice the visible solubility limit in complete medium for non-toxic and insoluble substances.

The colonies were examined under a stereomicroscope for scoring normal or morphologically transformed phenotypes. The morphologically transformed cells are characterised by a spindle shape, an increased nuclear/cytoplasm ratio and a higher basophilic affinity. These cells have a criss-cross orientation pattern and may be multilayered compared to normal cells [21].

Plating efficiency (PE, [total number of colonies/total number of target cells seeded] × 100), relative plating efficiency (RPE, [PE of treated cells/PE of control cells] × 100), and morphological transformation frequency (MTF, [number of transformed colonies/total number of colonies] × 100) were determined for each concentration and control. Around 1000 colonies were scored per concentration for PE, RPE and MTF determinations in control cells and in treated cells. In the tables of results (Appendix A: Tables A.1–A.6), cytotoxicity of test chemicals is indicated by the RPE (%) and by colony density/size. Sparse colonies were not scored for morphological transformation but were included in the total number of colonies for PE calculation. The column "scorable colonies" corresponds to the total number of colonies minus the sparse colonies. This number was used in the Fisher's exact test to calculate the statistical significance of the increase in morphological transformation.

2.7. Statistical analysis

Results were analysed using the one-sided Fisher's exact test [22] to determine if an increase in morphological transformation was significant compared to vehicle control. The Cochran–Armitage trend test for a positive dose-related response was