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Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. III: Sensitivity of human cell types to known genotoxic agents

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ABSTRACT

We have demonstrated previously that the seemingly high rate of "false" or "misleading" positive results from *in vitro* micronucleus assays (MNvit) was greater when rodent derived cell lines and certain toxicity measures, such as relative cell count or replication index, were used. These studies suggested that the use of a human cell type with functional p53 and a toxicity measure that included a function of cell proliferation could dramatically reduce the detection of misleading positive results.

A reduced "false positive rate" should not be at the expense of a loss of sensitivity of the assay. Therefore, we have investigated the sensitivity of the MNvit assay to known genotoxic agents using three cell types shown previously to be less prone to misleading positives, namely human lymphocytes (HuLy), TK6 and HepG2 cells. The 17 chemicals are well characterised and are from a list of chemicals known to produce positive results in *in vitro* mammalian cell assays.

These data demonstrated a high sensitivity of the assay in which TK6 and HuLy cells were employed, such that 15 out of the 17 chemicals were correctly identified. By contrast, the use of HepG2 cells resulted in far fewer than expected positive responses.

In conclusion, using TK6 and HuLy cells in preference to long established rodent cell lines in order to improve specificity does not compromise the sensitivity of the MNvit to detect known genotoxic agents.

1. Introduction

The generation of positive responses in *in vitro* mammalian cell tests for genotoxicity with chemicals that are not DNA-reactive, and do not induce genotoxicity or cancer *in vivo*, has been debated in workshops and several key publications over the last few years [4–7]. These positive responses include chemicals that act on DNA indirectly and may be expected to give positive results *in vitro e.g.* through generation of low levels of reactive oxygen but where negative results are obtained *in vivo* due to differences in plasma protein, antioxidant availability and relative exposure. They also include chemicals that disturb the physiological conditions of the cells in culture, perturb the normal functioning of cellular processes and lead to artefactual positive responses at non-physiological levels of toxicity, concentration or pH.

These positive responses are termed "false" or "misleading positives" as they give a misleading response compared to that *in vivo*. Thus the confidence in the *in vitro* genotoxicity test battery is reduced and more weight is placed on follow up *in vivo* tests, potentially increasing unnecessary experimental animal use.

For cosmetics and personal care industries, a European ban on *in vivo* genetic toxicology testing and marketing of cosmetic products or ingredients, effective March 2009 [8], has resulted in cosmetics manufacturers having to rely on *in vitro* testing for genotoxicity safety assessment of new ingredients.

Previous publications have estimated the incidence of "misleading" positive results at up to 80%, under certain conditions

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(particularly when combining tests in a battery) [7]. Improvements to the *in vitro* tests have been sought to improve specificity (*i.e.* accurately giving negative results with chemicals that are non-carcinogenic and non-genotoxic *in vivo*) without compromising sensitivity (*i.e.* without reducing the ability to give positive results with carcinogens and *in vivo* genotoxins). Fowler et al. [1,2] reported that the occurrence of misleading positive responses in the *in vitro* micronucleus assay (MNvit) could be dramatically reduced by careful selection of cell type, in particular using human cells/lines and avoiding long established, often p53-defective, rodent cell lines, and by using a measure of toxicity that takes into account population doubling or division rate rather than simple cell counts.

These chemicals were chosen because they were non-carcinogenic and negative in *in vivo* genotoxicity tests and, for an accurate prediction of that lack of genotoxic activity, should have been negative *in vitro*. Several of these chemicals were later found to have mechanisms that would be expected to give a positive response *in vitro* mainly *via* generation of low levels of reactive oxygen species [1]. As such these chemicals were considered *in vitro* positives; all other chemicals were expected to be negative and as such were suitable for estimating the specificity of each cell type tested.

A special issue of Mutation Research [9] focussing on cytotoxicity measures in the *in vitro* micronucleus test reported data from a collaborative trial involving 12 different laboratories, and showed that for 14 known genotoxic agents, relative population doubling (RPD), relative increase in cell counts (RICC), relative cell counts (RCC) and relative replication index (RI) all selected concentrations for micronucleus analysis that gave rise to expected positive responses across a variety of different commonly used cell types in both the presence and absence of cytochalasin B. Choosing measures of cytotoxicity based on cell proliferation does not therefore reduce the sensitivity of the *in vitro* micronucleus test to detect known genotoxic agents.

In the studies described here we have investigated 17 chemicals from Kirkland et al. [3] that are well characterised genotoxic agents with a variety of modes of action (MOAs) tested using the MNvit assay in TK6, human lymphocytes (HuLy) and HepG2 cells. The objective of the work was to ensure that by switching from long established rodent cell lines to human cell types in order to improve specificity, this does not compromise the sensitivity of the MNvit assay to detect known genotoxic agents.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, unless otherwise stated, were obtained from Sigma, UK at the highest available purity (typically >99%) and formulated in either sterile water or reagent grade DMSO (Sigma, UK) depending on solubility. Stock solutions were prepared approximately 3 h prior to treatment at either 10 fold higher than the final concentration for aqueous solvent or 100 fold higher for DMSO such that the final solvent concentrations were 10% for aqueous solvents and 1% for DMSO. Test solutions were added to culture media and treated by media replacement.

From the 20 chemicals identified by Kirkland et al. [3] as accepted genotoxic agents expected to give positive results in mammalian cell assays, 17 (listed in Table 1) were tested in TK6, HuLy and HepG2 cells. The remaining three chemicals not tested as part of this study were:

- Dimethylnitrosamine, which was only available from the supplier at concentrations that allowed testing to a maximum of 50 μg/mL, where no toxicity or MN were induced.
- Azidothymidine, since it could not be sourced during the experimental phase.
- PhIP·HCl (2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine), which was also difficult to source.

The 17 tested chemicals had been categorised [10] as giving positive responses in one or more *in vitro* mammalian cell genotoxicity tests due to direct or indirect DNA reactive mechanisms (*i.e.* aneuploidy, topoisomerase inhibition *etc.*). Most of these chemicals are also well known mutagenic carcinogens.

Table 1
Chemicals selected for testing alongside treatment conditions (recovery times vary according to cell type).

Chemical	CAS no.	S-9	Treatment times (h)
Cadmium chloride	10108-64-2	–	3
Sodium arsenite	7784-46-5	–	3
2-Acetylaminofluorene	53-96-3	+	3
2,4-Diaminotoluene	95-80-7	+	3
IQ (2-amino-3methylimidazo [4,5-f]quinoline)	30516-87-1	+	3
Chloramphenicol	56-75-7	–	3
7,12-Dimethylbenzanthracene	57-87-6	+	3
Taxol	3309-62-4	–	24
Cyclophosphamide	6055-19-2	+	3
p-chloroaniline	106-47-8	–	3
Aflatioxin B1	1162-65-8	+	3
ENU	759-73-9	–	3
Etoposide	33419-42-0	–	3
Hydroquinone	123-31-9	–	24[a]
MMS	66-27-3	–	3
Cisplatin	15663-27-1	–	3
Benzo[a]pyrene	50-32-8	+	3

[a] With additional 24 h recovery phase (weak aneugen).

In this study, the micronucleus assay was performed in the presence of cytochalasin B and replication index (RI) was used to estimate toxicity, these choices were due to the inclusion of HuLy cells which are a heterogeneous population of cells not suited to large scale automated counting.

Despite the fact that HepG2 cells may have some metabolic activities, all experiments were performed under comparable conditions *i.e.* for those chemicals that required metabolic activation, rat liver S-9 was added to all cell types, including HepG2.

In order to assess the functioning and performance of the individual micronucleus experiments the genotoxic chemicals 4-nitroquinoline-1-oxide (0.25 μg/mL and 0.5 μg/mL) and cyclophosphamide (6 μg/mL and 8 μg/mL) were used as positive controls in the absence and presence of S-9 metabolic activation respectively in every test performed. Data from positive controls are not presented. However data on test chemicals are only presented for valid experiments in which positive control chemicals clearly demonstrated a positive response in the assay.

2.2. Cell culture

To maintain karyotypic stability, for every experiment performed with TK6 and HepG2, fresh cultures were reconstituted from stocks preserved in liquid nitrogen.

TK6 cells were obtained from the European Collection of Cell Cultures (ECACC) and were maintained in tissue culture flasks containing RPMI 1640 medium (Gibco, Paisley, Scotland) containing glutamax™ and supplemented with 10% FCS and 100 μg/mL penicillin plus 100 U/mL streptomycin. TK6 were found to have an average generation time of 16 h and a modal chromosome number of 47. For TK6 cells growing in suspension, 10 cm^3 vented cap tubes were seeded with approximately 1×10^5 cells/mL in 5 mL culture medium. The cells were incubated at 37 ± 1 °C in an atmosphere of 5% (v/v) CO_2 in air, and were usually treated on the following day.

HepG2 cells were obtained from ECACC and were maintained in Eagle's minimum essential medium (EMEM) with 1% (v/v) L-glutamine and supplemented with 10% FCS and 100 U/mL penicillin plus 100 U/mL streptomycin. The modal chromosome number for the clone of HepG2 cells used was 52 and the average generation time was calculated to be 26 h. The cells were incubated at 37 ± 1 °C in an atmosphere of 5% (v/v) CO_2 in air, and were usually treated two days later, by which time cultures were approximately 50–60% confluent.

Lymphocyte (whole blood) cultures were prepared from three healthy, non-smoking male/female volunteers for each experiment from a pool of around 30 individuals. No volunteer was suspected of any virus infection or exposed to high levels of radiation or hazardous chemicals. As part of suitability for blood donation, the measured cell cycle time of the lymphocytes from the donors used fell within the range 13 ± 1.5 h. Average generation time was calculated from BrdU staining and the number of cells in their first, second and third divisions over a set time. This analysis was performed annually for each donor. For each experiment, an appropriate volume of whole blood was drawn from the peripheral circulation of three donors of the same sex into heparinised tubes two days prior to culture initiation. Blood was stored refrigerated and pooled prior to use using equal volumes from each donor. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL of pooled heparinised blood into 9.0 mL HEPES-buffered RPMI medium containing 20% (v/v) heat inactivated foetal calf serum and 50 μg/mL gentamycin,