Assessment of the genotoxicity of trichloroethylene in the in vivo micronucleus assay by inhalation exposure

J. W. Wilmer¹, P. J. Spencer², N. Ball³* and J. S. Bus³

¹Wilmer Tox Consulting, Langnau am Albis, Switzerland; ²The Dow Chemical Company, Midland, MI, USA; ³Center for Toxicology and Mechanistic Biology, Exponent®, Midland, MI, USA

*To whom correspondence should be addressed. Tel: +1 9896331828; fax: +1 9896389305; Email: nball@dow.com

Received on November 8, 2013; revised on February 3, 2014; accepted on February 10, 2014

The in vivo genotoxic potential of trichloroethylene (TCE) was evaluated by examining the incidence of micronucleated polychromatolythic erythrocytes (MN-PCEs) in the bone marrow. Groups of male CD rats were exposed by inhalation to targeted concentrations of 0 (negative control), 50, 500, 2500 or 5000 ppm for 6 consecutive hours on a single day. The exposure concentrations were selected to overlap those employed by a published study that reported a 2- to 3-fold increase in the frequency of micronuclei in male rats following a single inhalation exposure to 5, 500 and 5000 ppm TCE for 6 h but not following repeated exposure to similar concentrations. In addition, any treatment-related findings were assessed in the context of potential TCE-induced hypothermia. Clinical signs consistent with marked TCE-induced sedation were observed in rats exposed to 5000 ppm and subsequently three rats died prior to the end of the 6 h exposure period. No remarkable changes in body temperature were observed in surviving animals monitored with transponders before and after exposures. There were no statistically significant increases in the frequencies of MN-PCEs in groups treated with the test material as compared to the negative controls. The positive control animals showed a significant increase in the frequency of MN-PCEs and a decrease in the relative proportion of PCEs among erythrocytes as compared to the negative control animals. There were no statistically significant differences in the per cent PCEs in groups treated with the test material. As no increase in the incidence of micronuclei was observed in any of the TCE exposure groups, kinetochore analyses were not performed. Under the experimental conditions used, TCE was considered to be negative in the rat bone marrow micronucleus test.

Introduction

Trichloroethylene (TCE), a non-flammable, colourless liquid has been in commerce worldwide for >70 years. Today, TCE is mainly used as chemical intermediate in the production of fluorochemicals, as process solvent in the manufacturing of pharmaceuticals and other chemicals, and as solvent for degreasing processes, particularly in the metal fabricating and aircraft/aerospace industry.

Liver, lung and kidney tumours are the major findings reported in lifetime studies in rodents exposed to TCE, either by gavage or by inhalation. For recent reviews, see the EU Risk Assessment (1) and the EPA-IRIS document on TCE (2). In 2012, IARC reclassified TCE as a human carcinogen based on sufficient evidence for kidney cancer and limited evidence for liver cancer and non-Hodgkins lymphoma (3).

In order to explore the mode of action of tumour induction in animal bioassays, TCE and its metabolites have been extensively studied for potential mutagenicity and genotoxicity. Comprehensive reviews of the mutagenicity of TCE and its metabolites have been published by Moore and Harrington-Brock (4), in the EU Risk Assessment Report (1) and in the EPA-IRIS Document on TCE (2).

There is no convincing evidence that pure TCE is mutagenic in bacterial test systems for gene mutations (e.g. Ames test). Most of the available data are somewhat confounded by the fact that TCE is often stabilised with a very low concentration of epichlorohydrin or 1,2-epoxybutane, both known to be potent mutagens (1,2,4). Apart from a single Ames test in which a limited (<2) induction was found in Salmonella typhimurium TA100 in the presence of S9 (5), all Ames tests with epoxide-free TCE showed no induction of the mutation frequency (4).

Some evidence of weak induction of mutational damage (mitotic recombination and aneuploidy) was observed in Aspergillus nidulans with epoxide-free TCE using a 24 h exposure via the vapour phase (6). In other studies in fungal or yeast systems, including host-mediated assays, TCE gave conflicting results or studies were confounded by the presence of epoxide stabilisers.

Epoxide-free TCE is positive in a mouse lymphoma gene mutation assay (MLA), only in the presence of an exogenous metabolising system (S9) at dose levels 250 and 500 nl/ml, which were highly toxic to the cells. Relative total growth is only 6% at the highest dose level tested and no colony sizing was performed in this study (7). In another study, TCE was also reported to be positive in the MLA in the presence of S9, but no details were presented about dose levels and testing results (8).

In addition, TCE has been assessed in vitro for the induction of chromosome aberrations (CAs), sister chromatid exchanges (SCEs), micronucleus formation, unscheduled DNA synthesis (UDS) and DNA fragmentation (9–11). Epoxide-free TCE was negative in a well-conducted in vitro CA test in Chinese hamster ovary cells (9,11). Equivocal results were observed in UDS and SCE assays (4). In DNA strand-breakage studies, weak positive results were observed only at very high doses (12). Several of the available in vitro studies showed technical deficiencies. Therefore, it is not possible to draw a firm conclusion concerning the in vitro genotoxicity of TCE in these assays.

Interpretation of in vivo genotoxicity studies of TCE has been the focus of much attention within the scientific and regulatory communities. Negative results were observed in an inhalation transgenic (LacZ) mouse study by Douglas et al. (13), in several CA tests both by the oral and inhalation route (14,15), in the micronucleus study (intraperitoneal route) by Shelby et al. (16) in an in vivo–in vitro replicative DNA synthesis test with hepatocytes prepared from male B6C3F1 mice (17), and in a comet assay (inhalation route) in rat kidney by...
Clay (18). In the latter study, and contrary to the findings of Robbiano et al. (19), TCE exposure of rats by inhalation did not induce DNA damage in rat kidney. Oral administration of the putative cytotoxic and genotoxic metabolite of TCE, S-(1,2-dichlorovinyl)-l-cysteine (DCVC), has been shown to be renal toxic in rats (20). However, despite evidence of systemic distribution and toxicity of DCVC to rodent kidney, DCVC was negative in a kidney comet assay following high-dose treatment in rats (18). This is an important finding as it has been hypothesised that TCE-induced kidney cancer may be mediated through genotoxic metabolite(s) generated locally in the kidney via β-lalase metabolism of DCVC (21,22). In addition, this hypothesis has been further challenged by findings indicating that the putative DNA reactive metabolite formed from β-lalase metabolism of DCVC, chlorothioketene, did not form adducts with DNA bases after in vitro incubation in aqueous solutions (23).

Contrary to these negative findings, both Kligerman et al. (24) and Robbiano et al. (19) identified evidence of genotoxicity in micronucleus assays. In the study by Kligerman et al. (24), groups of five male CD rats or C57BL/6J mice received a single 6h exposure to reagent grade TCE (purity >99%; no information is available about stabilisers) by the inhalation route at nominal concentrations of 0, 5, 500 or 5000 ppm. Additionally, groups of five male rats received four consecutive daily 6h exposures at nominal concentrations of 0, 5, 50 or 500 ppm. A single, 6h TCE exposure induced a statistically significant and dose-related increase in the numbers of micronucleated polychromatic erythrocyes (MN-PCEs) in rats. In contrast, TCE had no effect in rats on the numbers of MN-PCEs following repeated exposure (4 days) or on the numbers of micronucleated peripheral blood lymphocytes (PBLs), following either a single or repeated exposure. Similarly, TCE had no effect on the numbers of micronuclei in mice splenocytes or PCEs. No changes in the frequency of CAs and SCEs were reported in the TCE-exposed rats (PBLs) or mice (splenocytes). The authors speculated that the observed pattern of genetic alterations was consistent with chemicals affecting the spindle apparatus of the cell to produce aneuploidy.

In a single oral dose study by Robbiano et al. (19), a statistically significant increase in the average frequency of DNA breaks and/or alkali labile sites and a statistically significant increase in the average of micronuclei frequency were observed in rats that had undergone unilateral nephrectomy and folic acid injection to stimulate cell proliferation in the remaining kidney. In summary, there does not appear to be a consistent, reproducible profile of activity emerging from the many in vivo genotoxicity assays that have been conducted on TCE in the past. However, while it appears that TCE is not genotoxic in vivo, there are some positive findings, particularly those of Kligerman et al. (24) in the micronucleus test that warrant further investigation.

The purpose of the current inhalation micronucleus study was to determine the clastogenic and aneugenic capability of TCE in the rat bone marrow PCEs, in order to address the observations in the study by Kligerman et al. (24). As there is an increasing amount of literature indicating that compounds that cause either significant increases in or decreases in core body temperature for a sustained period may induce increases in micronucleated PCEs (25–27), changes in body temperature were examined in this study as a possible confounder to any TCE-associated response.

Materials and methods

Chemicals

TCE (synonyms: 1,1,2-TCE, HI-TRI solvent; CAS number: 79-01-6), a colourless liquid, was obtained from The Dow Chemical Company, Freeport, TX, USA (QK13019201). Its purity (99.97%) was determined by gas chromatography/thermal conductivity detector (Vander Kamp, Putzig, and Langer, unpublished work). Cyclophosphamide monohydrate (CP) and vinblastine sulphate salt (Vinblastine) were both obtained from Sigma, St Louis, MO, USA. CP was used as a solution in distilled water; Vinblastine was prepared fresh in saline solution.

Animals and husbandry

Male rats, CD(Crl:CD(SD)IGS BR) (referred to as CD), were purchased from Charles River Laboratories (Portage, MI, USA). The animals were 8 weeks old upon their arrival at the laboratory; body weights (BW) varied between 200 and 220 g. They were housed two per cage in stainless steel cages, in rooms designed to maintain adequate conditions (temperature, humidity and photocycle) and acclimated to the laboratory for at least 7 days prior to the start of the study. A 12h light/dark photocycle was maintained for all animal rooms with lights on at 6:00 a.m. and off at 6:00 p.m. Room air was exchanged at ~12–15 times/h. Animals were provided LabDiet® Certified Rodent Diet #5002 (PMI Nutrition International, St Louis, MO, USA) in pelleted form. Animals were uniquely identified with subcutaneously implanted transponders (BioMedic Data Systems, Seaford, DE, USA), which were correlated to unique alphanumeric identification numbers. The rats were randomised by a stratified randomisation procedure using BW and distributed into the various treatment groups.

During randomisation, one animal from each of the control and TCE exposure groups was selected and surgically fitted with a radio-telemetry device (TA10TA-F20; Data Sciences International, St Paul, MN, USA). Each radio-telemetry device was uniquely identified and mapped to an individual animal for data capture.

The study was conducted to meet the US EPA (28), EC (29) and OECD (30) guidelines for the in vivo mammalian micronucleus test under conditions of Good Laboratory Practice (GLP) and in accordance with the Institutional Animal Care and Use Committee final rules (IACUC, 1989).

Experimental design

Male CD rats were exposed by inhalation to targeted concentrations of 0 (negative control), 50, 500, 2500 and 5000 ppm for 6 consecutive hours on a single day. CP (the positive control for the micronucleus assay) was dissolved in distilled water and administered by gavage once at a dose level of 24mg/kg BW. The kinetochore inducing positive control, vinblastine, was administered as a single intraperitoneal injection of 4mg/kg BW in a dosing volume of 1ml/kg, ~19h before sacrifice. Vinblastine is a known aneugenic agent in this assay (31).

There were six rats/group/sampling time, with an additional rat in each group implanted with a radio-telemetry device to monitor temperature changes throughout the exposure and to be used as a replacement in the event of deaths occurring among the groups (Table 1). The rats were observed daily and signs of toxicity or deaths occurring during the observation period were recorded. Approximately 19 and 48h after end of the 6h inhalation exposure, bone marrow samples were collected from all animals except CP-treated animals where bone marrow samples were only collected 19h after dosing. Bone marrow samples were not collected from the additional telemetry rats with the exception of a telemetry animal in the 5000 ppm exposure group (48h) since there were three deaths among the exposed animals of this group.

Exposures to TCE

The animals were exposed to filtered air or TCE vapours in 2 or 4 m³ stainless steel and glass Rochester-type whole-body exposure chambers (2 m³ chambers: 1.3 m x 1.2 m wide x 1.2 m deep with a pyramidal top and bottom; 4 m³ chambers: 1.5 m x 1.5 m wide x 1.3 m deep with a pyramidal top and bottom). Chamber airflow was maintained at ~450 l (2 m³ chambers) or 900 l (4 m³ chamber) per minute. This flow rate was sufficient to provide the normal concentration of oxygen to the animals and 12–15 calculated air changes per hour. The chambers were operated at a slightly negative pressure, relative to the surrounding area.

Vapour generation system

The various concentrations of TCE were generated using the glass J-tube method (32). Liquid test material was pumped into glass J-tube assemblies (one per exposure chamber) and vapourised by the flow of nitrogen gas passing through the head bed of the glass J-tube. The nitrogen was heated with a flameless heat torch (FHT-4; Master Appliance Corporation, Racine, WI,
Assessment of the genotoxicity of trichloroethylene

Vehicle control 6 6 1
50 ppm 6 6 1
100 ppm 6 6 1
2500 ppm 6 6 1
5000 ppm 6 6 1
Cyclophosphamide (CP) (24 mg/kg) 6
Vinblastine (4 mg/kg) 6

For temperature characterisation only, no bone marrow collected.
*Bone marrow harvested from this animal due to increased mortality at this dose level.
*Positive control for micronucleus test, administered via oral gavage.
*Positive control for kinetochore analyses, administered via intraperitoneal injection.

USA) to the minimum extent necessary to vapourise the test material. The vapourised test material and carrier gas were mixed and diluted with supply air to achieve a total flow of 450 or 900 l/min at the desired test chamber concentration.

Chamber monitoring

The chamber concentrations of TCE, measured approximately in the centre of the breathing zone of the animals, were determined at least once per hour with a Miran 1A infrared spectrophotometer (Foxboro/Wilks, South Norwalk, CT, USA). The nominal concentration of the test material in each chamber was estimated based on the amount of test material used and the total airflow through the chamber. Prior to the start of the study, each of the chambers was checked to ensure that a uniform distribution of vapour was present throughout the breathing zone of the animals.

Environmental conditions during exposure

Airflow through the chamber was determined with a manometer, which measures the pressure drop across a calibrated orifice plate and was maintained at ~450 or 900 l/min (2 and 4 m³ chambers, respectively). Chamber temperature and relative humidity data were recorded from a thermometer and hygrometer (Brooklyn Thermometer Co., Inc., Farmingdale, NY, USA) stationed in the interior of each chamber. Airflow data, chamber temperature and relative humidity were recorded once per hour during the exposure period.

Animal observations and BW determination

The rats were observed for positive signs of toxicity immediately prior to and after the exposure and once a day on the subsequent days. The rats were weighed prior to exposure and on the day of their scheduled sacrifice in order to assess the extent of stress experienced by the animals following treatment (33).

Body temperature data collection

Body temperatures were monitored during the study using the programmable transponders subcutaneously implanted in each animal. The temperatures were recorded immediately prior to exposure, immediately after exposure, ~3h after exposure and at 19 and 48 h post-exposure. During the inhalation exposures, body temperatures were monitored in animals fitted with the radio-telemetry device. Temperatures were sampled at 5 min intervals for 22 h pre-exposure, over the 6 h exposure period and for 19 h post-exposure to produce a body temperature profile that showed the onset, duration and resolution of any treatment-related alterations in body temperature.

Bone marrow sampling

Micronucleus assay. At the end of the specified interval following treatment, the animals were euthanised using central nervous system depression following exposure to carbon dioxide in a closed chamber. The bone marrow was removed from each femur separately by aspiration into 1 ml of fetal bovine serum (GIBCO, Grand Island, NY, USA). The samples used for direct bone marrow smears were centrifuged at ~1000 r.p.m. for 5 min; the supernatant was drawn off, leaving a small amount of serum with the remaining cell pellet. The cell pellet was resuspended in remaining serum and wedge smears were prepared on microscope slides using small portions of the cell suspension. The slides were fixed in methanol and stained with acridine orange (Sigma). The remaining bone marrow cell suspension was combined with the bone marrow from the other femur and used to prepare slides for kinetochore analysis.

Kinetochore. Slides for kinetochore analysis were prepared from the pooled bone marrow pellet resuspended in 5 ml of fetal bovine serum for each animal. The erythrocytes were separated from the rest of the bone marrow cells using column filtration. A filtration column using a 20 ml syringe containing equal parts of Sigma cell type 50 cellulose and alpha cellulose fibre (Sigma) was used. Hank’s balanced salt solution (GIBCO) was used to elute the cells. The eluate was passed through a 12 micron mesh cloth (Small Parts, Inc., Miami Lakes, FL, USA) and washed with McCoy’s serum-free medium (GIBCO). Cell counts were completed and the volume of the cell suspension was adjusted to deliver 1.5–2×10⁶ cells per cytospin circle where possible. Approximately 200 µl of the cell suspension was added to the cytospin cups for cytocentrifugation at 1400 r.p.m. for 5 min. The slides were fixed in cold methanol, air dried and stored in the refrigerator until staining as reported in Gudi et al. (31).

Slides for MN-PCEs were prepared and stained by a modification of the method of Heddle et al. (32). The micronucleus test was performed on bone marrow smears as described by Heddle et al. (32). Cells were stained with acridine orange (Sigma). All smears were processed using 2 ml of 200 µl of acridine orange (Sigma) was used. Hank’s balanced salt solution (GIBCO) was used to elute the cells. The eluate was passed through a 12 micron mesh cloth (Small Parts, Inc., Miami Lakes, FL, USA) and washed with McCoy’s serum-free medium (GIBCO). Cell counts were completed and the volume of the cell suspension was adjusted to deliver 1.5–2×10⁶ cells per cytospin circle where possible. Approximately 200 µl of the cell suspension was added to the cytospin cups for cytocentrifugation at 1400 r.p.m. for 5 min. The slides were fixed in cold methanol, air dried and stored in the refrigerator until staining as reported in Gudi et al. (31).

Slide scoring for MN-PCEs

To control for bias, all slides were coded, scored and decoded upon completion. A microscope fitted with a blue excitation filter 460–490 nm combined with a suppression filter of 520 nm was used to analyse the slides. Two thousand PCEs were examined from each animal and the number of MN-PCEs was recorded. The ratio of PCEs to normochromatric erythrocytes (NCEs) in the bone marrow was determined by examining 200 erythrocytes and was expressed as PCEs × 100/PCEs + NCEs.

Statistics

Means and standard deviations were reported for BW, body temperatures obtained by transponder and chamber concentration, temperature, humidity and airflow. Body temperatures obtained with the radio-telemetry device were graphically depicted to produce a temperature profile and qualitatively evaluated for treatment-related effects. The raw data on the counts of MN-PCEs for each animal were first transformed by adding one to each count and then taking the natural log of the adjusted number. The transformed MN-PCEs data and the data on per cent PCEs were analysed separately by a one-way analysis of variance (34). Pairwise comparisons of treated versus control groups were done, if the dose effect was significant, by Dunnett’s t-test, one sided (upper) for MN-PCEs and two sided for the per cent PCEs (34). Linear dose-related trend tests were performed only if any of the pairwise comparisons yielded significant differences. The r level at which all tests were conducted was 0.05. The final interpretation of biological significance of the responses was based on both statistical outcome and scientific judgment.

Results

Prior to the start of the study, the distribution of TCE vapour was determined in each of the exposure chambers from eight different sampling points within the breathing zone of the animals. Actual mean chamber concentration values for the study were 0, 53, 521, 2579 and 4677 ppm. Actual chamber concentration values deviated by only 3–6% from the targeted values of 0, 50, 500, 2500 and 5000 ppm.

There was no significant effect on the BW of the animals. The high dose was clearly toxic, with 3 animals out of 13 dying during the exposure period. The surviving animals in the high-dose group exhibited clinical signs consistent with marked sedation of the central nervous system and included decreased activity and incoordination, repetitive head bobbing, tremors and altered respiration and posture. Due to the mortality in the top dose, the telemetry animal was used to assess micronuclei formation at the 19 h time point. Clinical signs decreased in severity and incidence at 2500 ppm with only a few animals exhibiting decreased activity and incoordination. There were no indications of toxicity upon daily observation during the in-life portion of the micronucleus test at concentrations of 0, 50 and 500 ppm.
No remarkable changes in body temperature were detected for any of the TCE exposure groups as monitored by transponders prior to and after exposure. Data collected via radiotelemetry devices are depicted for the control and the 5000 ppm exposure group in Figure 1. Body temperature profiles revealed the typical rodent thermoregulatory pattern with frequent temperature fluctuations in the range of 35.5–38°C during the 22 hr pre-exposure monitoring period. The range of pre-exposure body temperature as measured by telemetry corresponded well with transponder recorded mean pre-exposure body temperatures. No alteration in thermoregulatory pattern occurred for any animals during the data collection interval with the exception of the rat exposed to 5000 ppm TCE. At 5000 ppm TCE, a small (~2.5°C) but notable drop in body temperature occurred shortly after the start of exposure and was accompanied by an amelioration of the typical thermoregulatory fluctuation pattern. The normal thermoregulatory pattern was restored quickly upon cessation of exposure. The transient drop in body temperature did not reach the threshold for hypothermia that must be achieved to result in the formation of micronuclei (25). The degree of body temperature decrement observed at 5000 ppm TCE was, therefore, considered inconsequential to the results of the micronucleus test.

Data on the frequencies of MN-PCEs and per cent PCEs observed in the various treatment groups are presented in Table II. There were no significant differences in mean MN-PCE frequencies between the groups treated with the test material and the negative controls (19 hr, control: 3.1% versus 5000 ppm: 2.7%; 48 hr, control: 1.8% versus 5000 ppm: 2.3%). The adequacy of the experimental conditions for the detection of induced micronuclei was ascertained from the observation of a significant increase in the frequencies of MN-PCEs in the positive control group (Table II). Due to the lack of an increased incidence of MN-PCEs, the slides prepared for the kinetochore analysis were not evaluated.

The per cent PCE values observed in the test material-treated animals were not significantly different from the negative control values (Table II). The per cent PCE values of the positive control animals were found to be significantly lower than those of the negative control animals. The slides prepared for the kinetochore analysis were not evaluated since the TCE exposure did not induce significant increases in the number of MN-PCEs.

Discussion
A single 6 hr inhalation exposure to 5000 ppm TCE caused marked toxicity and deaths in male rats. The severity of clinical signs and the deaths at the highest exposure concentration confirm achievement, if not exceedance, of a maximum tolerated dose and the systemic bioavailability of the test material to the target tissue, bone marrow. Based upon the results of this study, it is concluded that TCE does not induce a significant increase in the frequencies of bone marrow MN-PCEs of male CD-1 rats, following a single 6 hr inhalation exposure at concentrations ranging from 50 to 5000 ppm.

Our study, conducted in the same strain of rats as used by Kligerman et al., did not replicate the small increase in MN-PCE frequency as previously reported in this study (24). This failure to replicate the previous findings could be due to the fact that
Table II. Summary of MN-PCE frequencies (%) and per cent PCEs

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Sacrifice time point (h)</th>
<th>MN</th>
<th>Mean</th>
<th>SD</th>
<th>% PCEs</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19</td>
<td>6</td>
<td>3.1</td>
<td>1.1</td>
<td>62.7</td>
<td>14.4</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>19</td>
<td>6</td>
<td>3.0</td>
<td>0.9</td>
<td>67.9</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>19</td>
<td>6</td>
<td>3.0</td>
<td>1.8</td>
<td>57.3</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>19</td>
<td>6</td>
<td>2.9</td>
<td>0.9</td>
<td>58.9</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>19</td>
<td>5f</td>
<td>2.7</td>
<td>0.6</td>
<td>56.9</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>6000</td>
<td>19</td>
<td>5f</td>
<td>2.3</td>
<td>0.8</td>
<td>51.8</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>CP 24*</td>
<td>19</td>
<td>6</td>
<td>41.1</td>
<td>16.8</td>
<td>43.2</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>

MN, micronuclei.

*a*Three deaths occurred among animals exposed to 5000 ppm animals/sacrifice.

*b*Three deaths occurred among animals exposed to 5000 ppm animals/sacrifice.

*c*Rats were dosed with the vehicle (distilled water).

*d*Deaths occurred among animals exposed to 5000 ppm animals/sacrifice.

*e*Deaths occurred among animals exposed to 5000 ppm animals/sacrifice.

*f*Deaths occurred among animals exposed to 5000 ppm animals/sacrifice.

A summary of the MN-PCE frequencies (%) and per cent PCEs is provided in Table II.

Kligerman *et al.* only identified weak/equivocal evidence of TCE-induced genotoxic activity in rats receiving a single 6 h exposure of 5000 ppm and no evidence of genotoxic activity in rats receiving a four daily exposures up to 500 ppm. Although Kligerman *et al.* (24). This failure to replicate the previous findings could be due to the fact that Kligerman *et al.* only identified weak/equivocal evidence of TCE-induced genotoxic activity in rats receiving a single 6 h exposure of 5000 ppm and no evidence of genotoxic activity in rats receiving a four daily exposures up to 500 ppm. Although Kligerman *et al.* (24) reported significant elevations in the incidence of bone marrow MN-PCEs following single 6h exposures to 5, 50 and 5000 ppm TCE (3.2 ± 1.2%, 4.4 ± 0.4% and 6.6 ± 0.4% MN-PCEs, respectively, control = 1.7 ± 0.8%), the toxicological significance of these findings is tempered by the observation that the control incidence in the single exposure studies was over 50% lower than the control incidence in the repeated exposure component of the study (4.0 ± 1.9%). This suggests that the increased frequency of micronuclei in the low- and mid-dose TCE single exposure groups may have arisen by chance alone and that both values are well within the range of the negative controls reported in this study and further indicates only equivocal evidence of a high-dose specific TCE-induced MN-PCE response at 5000 ppm. In addition, there was no evidence of genotoxicity in the MN-PBL (micronuclei in PBLs), CA and SCE investigations conducted in the same animals. Thus, since the current study has not been able to replicate the findings of Kligerman *et al.* (24), the conclusion of the EU Risk assessment and by Moore and Harrington-Brock that the results can be considered as equivocal, is justified.

The study by Robbiano *et al.* (19) can only be considered to be of limited value in contributing to an assessment of the genotoxicity of TCE in the intact animal due to the very high oral dose (½ of the LD₅₀) and the non-standard methodology that was used (1). Moreover, the oral route is not a common route of exposure to TCE.

Evaluation of genotoxicity data requires a weight-of-evidence approach that includes consideration of the various types of genetic damage that can occur, the quality of the various studies and consideration of both positive and negative results. There is generally consensus that positive results in the Ames test are due to epoxide stabilisers used in TCE in the past. Negative results have been reported in well-conducted *in vitro* CA assays. In the *in vitro* MLA, positive effects of TCE were reported in the presence of S9 at dose levels, which were highly toxic to the cells. Besides the equivocal and/or high-dose specific positive results reported in the above micronucleus studies, a series of other *in vivo* studies with TCE have shown clear negative results. The current inhalation micronucleus assay with TCE is clearly negative. It could be argued that the bone marrow micronucleus assay is not predictive for establishing a potential genotoxic mode of action of TCE for kidney tumours through genotoxic metabolite(s) generated locally in the kidney via β-lyase metabolism of DCVC. However, this hypothesis is significantly flawed as TCE (by inhalation) and DCVC (by oral exposure) are not genotoxic in the comet assay in rat kidney (18). Moreover, mice have a much higher metabolic capacity for DCVC than rats but do not exhibit kidney cancer, and there is little evidence of such metabolism in human kidney (20,35).

It is more likely that TCE-induced kidney tumours are the result of direct cytotoxicity and sustained regenerative cell proliferation, which would imply a threshold for kidney cancer (35,36).

In conclusion, the present inhalation micronucleus study in rats conducted with a high-purity sample of TCE (without epoxide stabilisers), and according to internationally accepted guidelines and under GLP, provides further evidence that TCE is not genotoxic *in vivo*.

**Funding**

The work was supported by The Dow Chemical Company.

Conflict of interest statement: N.B. and P.J.S. are employed by a company (The Dow Chemical Company) that manufactures Trichloroethylene. J.S.B. and J.W.W. provide toxicology consulting services to a company (The Dow Chemical Company) which manufactures trichloroethylene.

**References**


