A Subchronic Exposure to Trichloroethylene Causes Lipid Peroxidation and Hepatocellular Proliferation in Male B6C3F1 Mouse Liver

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Received October 29, 1997; accepted March 19, 1998


The common groundwater contaminant trichloroethylene (TCE), when given by oral gavage, can produce free radical species during metabolism. Furthermore, TCE end-stage metabolites, trichloroacetic acid and dichloroacetic acid, cause lipid peroxidation in mouse liver. The time courses of lipid peroxidation, free radical generation, and 8-hydroxydeoxyguanosine (8OHdG) formation were used to assess the level of oxidative stress in the liver of B6C3F1 mice dosed orally once daily, 5 days a week for 8 weeks at 0, 400, 800, and 1200 mg/kg TCE in corn oil. Peroxisomal proliferation, cell proliferation, and apoptosis were evaluated at selected times during the study. Lipid peroxidation, as measured by thiobarbituric acid-reactive substances (TBARS), was significantly elevated at the two highest dose levels of TCE on days 6 through 14 of the study. 8OHdG levels were statistically significant in the 1200 mg/kg/day group on days 2, 3, 10, 28, 49, and 56 only. The highest measured free radical load, 307% of oil control, occurred at day 6. A significant increase in cell and peroxisomal proliferation was observed during the same time period in the 1200 mg/kg/day group. Necrosis or an increase in apoptosis was not observed at any dose. The temporal relationship between oxidative stress and cellular response of proliferation, both of which occur and resolve within the same relative time period, suggests that TCE-induced mitogenesis may result from alteration in the liver microenvironment which offers a selective advantage for certain hepatocyte subpopulations.

Trichloroethylene (TCE) is a widely used industrial solvent and degreasing agent. Because it is commonly detected as a groundwater contaminant, its potential to adversely affect human health has been studied and debated for many years. The primary concern about TCE environmental exposures arises from the National Toxicology Program (NTP) studies that have shown TCE to be a rodent hepatocarcinogen when given in high doses by corn oil gavage (NCI, 1976; NTP, 1990). Consistent species differences have been reported. Mice, especially the B6C3F1 strain, develop hepatocellular neoplasms while rats do not (NTP, 1988). Two major TCE metabolites, chloral hydrate (CH) and trichloroacetic acid (TCA), as well as a reported minor metabolite dichloroacetic acid (DCA), also produce liver tumors in mice (Herren-Freund et al., 1987; Daniel et al., 1992; DeAngelo et al., 1991). CH has been found to be genotoxic in a variety of in vitro systems, whereas TCA, DCA, and the parent compound, TCE, are recognized as non-genotoxic carcinogens in the mouse (reviewed in ECETOC, 1994). Tumor dose–response characteristics and species-specific metabolic differences in terms of TCA and/or DCA production may account for TCE’s tumorigenicity (Larson and Bull, 1992a; Templin et al., 1993; reviewed in Goeptar et al., 1995). Significant histopathologic differences have been observed between TCA- and DCA-induced liver lesions following chronic exposure (Bull et al., 1990; Pereira, 1996), leading to the suggestion that each may produce liver tumors by distinct mechanisms. Bull et al. (1990) reported that TCA, unlike DCA, caused large dose-related hepatocellular accumulations of lipofuscin, a lipid membrane peroxidation by-product. Furthermore, TCA chronic treatment resulted in sustained peroxisomal proliferation, whereas this effect was transitory for DCA (Bull et al., 1993).

TCA and DCA have been shown to cause oxidative stress in rodents. Both increase the formation of thiobarbituric acid-reactive substances (TBARS) in a dose response manner in mouse liver following a single oral dose, suggesting that each was capable of yielding a radical species which could initiate lipid peroxidation (Larson and Bull, 1992b). Recently, CH and TCA were shown to generate free radicals and to induce lipid peroxidation in male B6C3F1 mouse liver microsomes (Ni et al., 1996). This laboratory has shown that carbon-centered free radicals are produced in B6C3F1 liver slices exposed to the parent compound TCE (Steel-Goodwin et al., 1996).

The purpose of this study was to evaluate dose–response and temporal characteristics of selected biological endpoints in the...
mouse liver for mice dosed 56 days with TCE given by corn oil gavage. Elevations in endpoints including peroxisome proliferation and lipid peroxidation have been demonstrated elsewhere using in vitro or following acute in vivo TCE, or its chloroacetic acid metabolite, exposures (Bull et al., 1990, 1993; Elcombe et al., 1984). Cell proliferation for subacute TCE gavage exposure has not been adequately characterized. We report the only instance to date where these putative mechanisms in the pathway toward TCE-induced hepatocarcinogenesis are examined in the whole animal given the parent compound under a dosing regimen known to produce tumors in a chronic bioassay. Furthermore, we characterize the extent and time course of hepatocellular proliferation, apoptosis, and indicators of oxidative stress, as well as other relevant pathologic observations.

### MATERIALS AND METHODS

#### Chemical and dosing solutions

Trichloroethylene (99.5±% without antioxidant additives) was obtained from Aldrich Chemical Company (Milwaukee, WI, Lot No. MF 01428EF). Gavage solutions were prepared in corn oil (Mazola, Best Foods, Somerset, NJ). Fresh dosing solutions were prepared each week, analyzed by gas chromatography immediately following preparation, and again at week's end to ensure potency. All gavage solutions were stored at 4°C.

#### Animals, housing, and husbandry

Male hybrid mice (B6C3F1/Crl BR) were maintained under a program of care accredited by the American Association for the Accreditation of Laboratory Animal Care. Twelve-week-old, 25- to 30-g mice were obtained from the Portage, Michigan, production facility of Charles River Laboratories (Wilmington, MA). Upon receipt, the mice were housed five mice per polycarbonate cage on hardwood chip bedding and held in isolation from other rodents for 7 days. The mice were housed in sanitizable rooms providing 10–15 complete fresh air changes per hour while maintaining an air temperature of 20 ± 2°C and 45 ± 5% relative humidity. Electronically controlled full spectrum fluorescent light was provided on a 12:12-h light:dark cycle. Fresh conditioned (reverse osmosis) water and certified rodent chow (No. 5002, Purina Mills, Inc., St. Louis, MO) were available ad libitum.

#### Study Design

Table 1 summarizes the study design and number of animals per dose group. One week prior to the study, mice were implanted with subcutaneous magnetic identification micro-transponders (BMDS, Biomedic 510 15 20 25 30 35 40

#### FIG. 1. Time course for lipid peroxidation in liver tissue following TCE gavage. Lipid peroxidation, as measured by TBARS, in liver tissue was significantly elevated in the 1200 and the 800 mg/kg/day dose groups, starting at day 6. Inset graph shows all dose groups. There is a clear demarcation between the two higher dose groups and the lowest, 400 mg/kg/day, treatment group. All dose group TBARS values approached control levels by day 35. All data points represent means ± SE of independent samples (n=6). *Values significantly (p < 0.05) different from control.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Time points</th>
<th>Total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water control</td>
<td>7</td>
<td>11</td>
<td>77</td>
</tr>
<tr>
<td>Corn oil control</td>
<td>7</td>
<td>11</td>
<td>77</td>
</tr>
<tr>
<td>TCE 400 mg/kg/day</td>
<td>7</td>
<td>11</td>
<td>77</td>
</tr>
<tr>
<td>TCE 800 mg/kg/day</td>
<td>7</td>
<td>11</td>
<td>77</td>
</tr>
<tr>
<td>TCE 1200 mg/kg/day</td>
<td>7</td>
<td>11</td>
<td>77</td>
</tr>
</tbody>
</table>
Data Systems, Maywood, NJ) as per the manufacturer's instructions. Mice were then randomly assigned to treatment groups and were housed in the same room under the conditions stated above. To conform to the weekly dosing regimen used in the previous studies conducted under the direction of the NTP (NCI, 1976; NTP, 1990), gavage was performed 5 days a week (but for only 8 weeks in our study), using 20-gauge feeding needles fitted to glass syringes. Concentrations of TCE in corn oil, formulated to deliver 0, 400, 800, and 1200 mg/kg/day in a total volume of 0.5 mL, were administered by gavage beginning at 0800 h each dosing day. Animal weights were recorded at the study start and weekly thereafter. The concentrations were adjusted weekly based on the mean body weight of each treatment group.

Liver harvest and sample preparation. Liver samples were obtained on study days 2, 3, 6, 10, 14, 21, 28, 35, 42, 49, and 56, starting at 1300 h each day. The harvest procedure was tightly controlled to standardize the time between last dosing and tissue collection. With the exception of the day 6 time point, all samples were scheduled to avoid sampling on a Monday following 2 days of off-dose time in order to minimize that source of variability. Mice from each treatment group were randomly selected and weighed. Thirty minutes prior to sacrifice, an intraperitoneal (ip) injection of N-N-buty1-α-phenylnitrone (PBN), 250 mg/kg, was administered to trap radical species for electron paramagnetic resonance spectroscopy. Preliminary work in this laboratory had determined that this dose of PBN is nontoxic and does not interfere with subsequent analyses. This was confirmed by including in the analysis three mice from a control group (treated with water) that were not injected with PBN. Euthanasia was accomplished by CO2 asphyxiation. The liver was quickly removed and washed in ice cold Dulbecco's phosphate-buffered saline (DPBS, Gibco BRL, Grand Island, NY) (pH 7.4), and a 2-mm-thick cross-section of the entire median lobe was placed in 10% neutral buffered formalin. The remaining liver from each animal was immediately snap-frozen in liquid nitrogen and apportioned for subsequent analyses. Liver tissue was not “pooled” since our process ensured that each animal contributed sufficient liver for all biochemical and histopathological assays. All reagents used in subsequent analyses were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Chicago, IL), unless otherwise noted, and were of the highest possible purity.

Thiobarbituric acid-reactive substances. Approximately 100 mg of frozen liver was placed in a microfuge tube and stored at −135°C. Within 24 h, the frozen samples were homogenized in 5× volume of ice-cold homogenization buffer containing 0.65 mM reduced glutathione (GSH), 1.34 mM ethylenediaminetetraacetic acid (EDTA), and trisodium salt, using a PowerGen 125 tissue homogenizer (Fisher Scientific). Homogenized samples were immediately returned to liquid nitrogen and stored at −135°C pending further analysis. Samples were analyzed for TBARS according to the method of Wasowicz et al. (1993). Briefly, an aliquot of homogenate was cleared with an equal volume (3% w/v) sodium dodecyl sulfate (SDS) in deionized H2O, reacted with 29 mM thiobarbituric acid in 8.75 mM acetic acid at 95–100°C for 60 min. Following extraction by n-butanol, fluorescence at 555 nm was read at excitation wave length of 529 nm using an LS 50 B luminescence spectrometer (Perkin Elmer Ltd., Beaconsfield, UK). Calibration against a 1,1,3,3-tetramethoxypropane standard curve returned values that are expressed as nanomoles of TBARS per milligram of protein.

8-Hydroxydeoxyguanosine (8OHdG). Nuclei were isolated from approximately 100 mg of liver tissue that had been stored at −140°C. Following homogenization in ice-cold buffer composed of mannitol (250 mM), sucrose (70 mM), Hepes (5 mM), and MgCl2 (5 mM), pH 7.4, using four to five strokes of a Potter-Elvehem homogenizer at 500–1000 rpm, the homogenate was centrifuged 10 min at 10000 g at 4°C; the supernate was discarded, and the nuclear pellet was resuspended in 200 μL phosphate-buffered saline (PBS). DNA was isolated from this suspension using an Easy DNA kit (Invitrogen, San Diego, CA). DNA was dissolved in 300 μL of 30 mM sodium acetate, pH 5.3, and frozen. High-performance liquid chromatographic (HPLC) analysis utilized 350-μL aliquots of DNA heated to 90°C in a water bath for 3 min and then cooled on ice for 10 min after which 20 μL P1 nuclease was added and
FIG. 4. Day 10. Electron photomicrographs of peroxisomes in hepatocytes from a corn oil control mouse (a) and a 1200 mg/kg TCE-exposed mouse (b). Note the numerous membrane-delimited electron dense peroxisomes (arrows) in the treatment specimen (b) compared to the control (a). BC, bile canaliculus; M, mitochondria. X 22,500.

TABLE 2

<table>
<thead>
<tr>
<th>Exposure day</th>
<th>Group</th>
<th>Percent peroxisomal areaa</th>
<th>Number of peroxisomesb</th>
<th>Peroxisomal sizecd</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1200 mg/kg TCE</td>
<td>2.87 ± 0.30</td>
<td>48.3 ± 6.9</td>
<td>6.05 ± 0.43</td>
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<tr>
<td></td>
<td>Corn oil</td>
<td>1.16 ± 0.15**</td>
<td>18.7 ± 2.0*</td>
<td>6.19 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>1.41 ± 0.22</td>
<td>17.1 ± 1.8*</td>
<td>8.69 ± 2.30</td>
</tr>
<tr>
<td>10</td>
<td>1200 mg/kg TCE</td>
<td>6.39 ± 0.60f</td>
<td>64.2 ± 5.2</td>
<td>10.04 ± 1.14</td>
</tr>
<tr>
<td></td>
<td>Corn oil</td>
<td>1.38 ± 0.21*</td>
<td>20.6 ± 1.9*</td>
<td>06.65 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>1.16 ± 0.39*</td>
<td>14.6 ± 4.1*</td>
<td>07.76 ± 1.90</td>
</tr>
<tr>
<td>14</td>
<td>1200 mg/kg TCE</td>
<td>4.69 ± 0.22g</td>
<td>48.2 ± 3.4</td>
<td>9.84 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>Corn oil</td>
<td>0.77 ± 0.08*</td>
<td>09.5 ± 0.9*</td>
<td>8.10 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>0.88 ± 0.03*</td>
<td>10.2 ± 1.9*</td>
<td>9.50 ± 2.04</td>
</tr>
</tbody>
</table>

a Data are given as means ± SE; n = 3 per group.

b Calculated using 100 X peroxisomal area/total cytoplasmic area examined.

c Number of peroxisomes per 10 mm² of cytoplasm.

d Calculated using total peroxisomal area/total number of peroxisomes; units are 10⁴ mm².

* Significantly (p < 0.05) different from 1200 mg/kg TCE.

* Significantly (p < 0.05) different from day 6.

* Significantly (p < 0.05) different from day 10.
the sample was incubated at 70°C for 30 min. Then 30 μl of 1 M Tris–HCl (pH 7.4) and 13 μl (1.3 U) *Escherichia coli* alkaline phosphatase were added and the sample was incubated for 60 min at 37°C, followed by 10,000 MW cutoff filtration at 3500g and 4°C. Aliquots of 100 μl were analyzed on a DX-300 Dionex liquid chromatography system (ESA, Chelmsford, MA) with a Supelcosil LC-18S 25 cm × 4.6 mm column and a 2-cm guard column with a mobile phase of 10% methanol and 50 mM NaH₂PO₄ (adjusted to pH 5.5) using UV (254 nM) and amperometric electrochemical (glassy carbon working electrode at 550 mV) detection. Samples were quantified using external 2′-deoxyguanosine and 8OH-deoxyguanosine standards and expressed as percentage of control. A Thermo Separations Products SpectraSystem AS 3000 autosampler maintained samples at 4°C and the HPLC column at 30°C. Chromatograms were integrated using Dionex AI-450 software.

**Free radical electron paramagnetic resonance (EPR).** The total radicals in the liver samples were measured using a Bruker EMS 104 EPR analyzer for initial quantification and screening and a Bruker EMS 300E spectrometer for measurement of radicals in 10-ng liver portions. A Varian E109 was used to measure radicals in the aqueous phase of homogenized liver extracts. The machine parameters for the EMS 104 EPR analyzer were microwave power, 25 mW; sweep width, 100 G; modulation amplitude, 4.02 G; sweep time, 10.49 s; filter time constant, 20.48 ms; receiver gain, 60. All samples were maintained at −135°C until prepared for EPR analysis. Twenty randomly selected samples were analyzed each day by EPR. Random sampling and analysis was repeated using new tissue samples to confirm the integrity of the initial results. The analytic method was identical for all samples. All spectra were normalized against control values to determine the radical levels induced by TCE treatment. The spin label 2,2,5,5-tetramethyl-1-pyrroldinonyl-3-carboxyamide (Aldrich Chemical Co.) was used as an internal standard to quantify the EPR signals. Data were expressed as number of radicals per milligram of protein.

**Peroxisome detection and analysis.** Liver slices from three mice each from the 1200 mg/kg TCE and control (oil and water) groups were diced into 1-mm cubes and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for at least 24 h. Tissues were rinsed four times in cacodylate buffer, postfixed in 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h and dehydrated in a graded ethanol series. After two changes in propylene oxide, tissues were embedded in Poly/Bed 812 (Polysciences, Warrington, PA) according to Glauert (1974). One-micrometer-thick sections were cut on a MT 7000 (RMC, Tucson, AZ) ultramicrotome, stained with toluidine blue. These specimens were examined by a pathologist without prior knowledge of the origin of the specimens in regard to experimental group (treatment or control). Only centrilobular regions of hepatic lobules, the hallmark location of peroxisome proliferation, were identified and selected for further processing. Thin sections (60–90 nm) from these selected regions were cut on a JEOL 1200 EX II TEM (JEOL, Peabody, MA) ultramicrotome, placed on glass slides, and stained with uranyl acetate followed by Reynold’s lead citrate, and viewed in a JEOL 1200 EX II TEM (JEOL, Peabody, MA) at 60 kV. Micrographs of randomly chosen hepatocytes immediately adjacent to the central vein were taken at 10,000X. Nucleus was excluded from fields photographed. From each experimental group, seven micrographs were randomly chosen from which to measure peroxisomes. Peroxisomes in these micrographs were manually demarcated
and then quantified using computer-based morphometric analysis (Quantimet 570c, Leica, Inc., Deerfield, IL). Peroxisomal proliferation end points quantified were peroxisomal area to cytoplasmic area, the number of peroxisomes per unit area of cytoplasm, and the average peroxisomal size.

Detection of proliferating cell nuclear antigen (PCNA). PCNA is present in all stages of the cell cycle except G0 and is a well-established marker of cell proliferation used extensively to demonstrate cell growth (Kurki et al., 1986; Bravo and MacDonald-Bravo, 1987; Garcia et al., 1989; Hall et al., 1990; Bolton et al., 1992; Sarli et al., 1995). The liver specimens were fixed in 10% neutral buffered formalin for 15 h, dehydrated in ethanol and Hemo-De (Fisherbrand, Fisher Scientific, Pittsburgh, PA), and paraffin embedded at less than 60°C using a Histo-Matic MVP (Fisher Scientific). Four-micrometer-thick sections were cut, mounted on ChemMate Plus slides (BioTek Solutions, Santa Barbara, CA), and air dried. Sections were deparaffinized in xylene and then rehydrated through a graded ethanol series to PBS buffer (pH 7.4).

Immunohistochemical processing of the sections was performed on a TechMate 1000 (BioTek Solutions, Santa Barbara, CA) automated immunostaining system using a modification of the method of Foley et al. (1991). This automated procedure allowed all the specimens to be processed and stained at one time using batch prepared reagents which minimized the inherent procedural variability of manual immunostaining processes. For antigen retrieval, sections were microwaved in citrate buffer (BioTek Solutions) and then reacted with hydrogen peroxide (3% for 20 min) and blocked with normal horse serum (BioTek Solutions). Slides were then incubated overnight at room temperature with mouse monoclonal antibody (PCNA-Ab-1, Oncogene Science, Cambridge, MA). Detection of PCNA-positive nuclei was performed using biotinylated horse anti-mouse (BioTek Solutions) secondary antibody and avidin-biotin complex (ABC kit, BioTek Solutions) (20 min incubation for each, at room temperature), followed by incubation in hydrogen peroxide/ diaminobenzidine (BioTek Solutions) for 21 min. Tissues were counterstained with hematoxylin. Positive and negative tissue controls (neonatal and adult mouse liver, respectively) as well as antibody binding controls were included in each procedure.

Quantitative image analysis was performed using a Quantimet 570c Image Analysis System (Leica, Inc., Deerfield, IL). Digital color images of liver sections immunohistochemically labeled for PCNA were analyzed to detect positive and negative hepatocyte nuclei. Detection criteria were feature color identification between brown (positive) and blue (negative) nuclei as well as nuclear morphological characteristics (area, fullness, aspect ratio, and roundness). Serial images were analyzed from each liver section by digitizing adjacent microscopic fields along a linear path that traversed the section along its greatest width. Data was collected in this manner for a minimum of 18 fields per liver section.

In situ detection of apoptosis. Apoptosis was detected using the Apoptag in situ apoptosis kit (Oncor, Gaithersburg, MD) which extends and labels fragmented DNA by incorporating deoxyribonucleotide triphosphates. Following deparaffinization in xylene and graded ethanol, slides were rinsed in distilled/deionized water and processed according to the kit manufacturer's protocol. This procedure was automated using a BioTek Tech Mate 1000 tissue processor equipped with an in situ oven (BioTek Solutions). Following apoptotic tagging, the specimens were counterstained with hematoxylin for 1 min, dehydrated in graded ethanol to xylene, and coverslipped using Permount. Estrous-phase rat uterus with significant endometrial gland involution was used as a positive control. Liver and uterus incubated without terminal deoxynucleotidyl transferase served as negative controls. Apoptosis was quantified using a single liver section from the median lobe, based on the number of positively labeled cells per 10 mm² of tissue in combination with the morphological criteria for apoptosis (Columbano et al., 1985). A single apoptotic body was considered to be one cell; two or more apoptotic bodies clustered closely together also were considered as derived from one cell. All positive staining cells meeting the morphological criteria were counted manually. Liver area was determined using a Quantimet 570c image analysis system (Leica, Inc., Deerfield, IL).

**Protein.** Protein from all samples was determined using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

**Statistics.** Unless otherwise stated, data were plotted and treatment differences determined by one-way analysis of variance (ANOVA) using SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA). Body weights were analyzed using a repeated measures (time) one factorial (treatment) ANOVA with missing values. If found to be significant, appropriate post hoc comparisons such as Dunnett's method or Student Neuman-Keuls were applied. For analysis of peroxisomal area to total cytoplasmic area and number of peroxisomes per unit area of cytoplasm, a two factorial analysis of variance (treatment and day) was used, applying Levene's criteria to test the assumption of equality of variances.

**RESULTS**

Body weight and gross pathology. Weight gain was not adversely affected by TCE dosing, at any concentration, over the time course of this study. Moreover, at necropsy, gross observation did not reveal any relative differences in body fat among treatment animals compared to water controls. No gross lesions were observed in any group.

Lipid peroxidation. Lipid peroxidation, as measured by TBARS, was significantly elevated above oil controls in the 1200 mg/kg dose group from day 6 to day 14 and from day 6 to day 28 in the 800 mg/kg group (Fig. 1). A dose–response trend was evident over the same period (Fig. 1, inset). The increase for only the two highest dose groups suggests that for this endpoint 400 mg/kg/day was a no-effect level. By day 35 all treatment groups had TBARS values not statistically different from the oil control mice. In addition, TBARS values in the control animals decreased by half rapidly from a peak at day 6 to reach and maintain relatively steady levels from day 10 to study end.

8OHdG. Elevations in 8OH-2'-deoxyguanosine levels were modest. The greatest increase was noted in the 1200 mg/kg/day treatment group, being 196% of oil controls on day 56 (Fig. 2). Levels fluctuated throughout the study, averaging 129% of control for the 1200 mg/kg/day group, with statistically significant elevation (p < 0.05) noted on days 2, 10, 28, 49, and 56 and depression on day 3. No statistically significant effects were observed at lower doses.

Free radical EPR. The TCE-induced radicals, measured by subtraction of the EPR signal of lyophilized liver of the controls from the TCE group, were significantly elevated only in the 1200 mg/kg dose group. The greatest elevation centered on day 6 (307% of controls) with modest increases seen on days 21, 42, and 56 (77, 30, and 11% of controls, respectively) (Fig. 3). The results of the initial assessment were confirmed by repeating the analysis on random samples of frozen liver from the same animals (data not shown).

Peroxisomal proliferation. Only the 1200 mg/kg/day dose group was evaluated for peroxisome proliferation. As summarized in Table 2, the ultrastructural data shows a
FIG. 5. Cell proliferation time course. TCE gavage caused a significant
(p < 0.05) burst of cell proliferation centered at day 10 of treatment in the 1200
mg/kg/day group. Each symbol in large graph represents mean of a minimum
of 18 microscopic fields examined by image analysis as described under
Materials and Methods. Inset graph shows mean ± SE for n = 6 independent
samples. A dose–response trend is evident at day 10.

The role of free radicals and oxidative stress in tumor
promotion has been well reviewed (Kozumbo et al., 1985;
Thrush and Kensler, 1991; Janssen et al., 1993). Studies have
demonstrated that reactive oxygen species may alter cellular
growth regulation by disrupting signal transduction molecules
and receptors without directly attacking nuclear DNA, thus
acting as “epigenetic” carcinogens when tumors result (re-
viewed in Van Der Vliet and Bast, 1992; Burdon, 1995; Byc-
zkowski and Channel, 1996; Corcoran et al., 1994). With the
sole exception of CH, TCE and its other principal metabolites,
TCA and DCA, are repeatedly nonmutagenic and thus are
classed as epigenetic carcinogens (ECETOC, 1994). Common
to this epigenetic class is a group of “cancer-promoter” com-
pounds that induce hepatocellular peroxisomal proliferation
(Ashby et al., 1994). Our findings confirm other reports of
TCE-induced peroxisomal proliferation (Elcombe et al, 1985).

Agreeing with its epigenetic classification, the modest, and
inconsistent, elevations in 8-OHdG we report suggest that
TCE-induced oxidative stress does not target nuclear material
directly. This is entirely concordant with TCE’s reported lack
of genotoxicity (ECETOC, 1994). We observed no time-course
pattern in this endpoint as we did with TBARS, peroxisome
and cell proliferation, and free radical load. In fact, high data

Repeated oral gavage with an appropriate dose of TCE
clearly precipitates several events that are temporally related.
The parameters suggesting a state of oxidative stress—lipid
peroxidation (Fig. 1), excess free radical production (Fig. 3),
and peroxisomal proliferation (Table 2)—all center around
treatment days 6–14. Interestingly, cell proliferation was ele-
vated in this same period (Fig. 5); however, apoptosis or cell
necrosis was absent. Cell proliferation resulting from chemical
exposures in the liver is often classified as either cytotoxic or
mitogenic (Goldsworthy et al., 1991; Butterworth et al., 1992).
Cytotoxicants can produce necrosis and regenerative growth.
Sustained cell proliferation resulting from regenerative growth
may increase the frequency of spontaneous mutations or fix
mutations prior to DNA repair (Goldsworthy et al., 1993). In
contrast, hepatic mitogens produce a transient increase in he-
patocyte proliferation in the absence of hepatocellular cytole-
thality (Butterworth et al., 1992). Because microscopic exam-
ination revealed no hepatic necrosis in this study and the
proliferative response was transient (Fig. 5), the data support
the conclusion that TCE in this study acted as a hepatocellular
mitogen. Mitogenic agents may provide a selective growth
advantage to certain cell populations by direct growth stimu-
lation and/or by suppression of “normal” hepatocyte prolifer-
ation (Columbano et al., 1991; Goldsworthy et al., 1993). The
absence of increased apoptosis over background, itself negli-
gible, with any treatment level in this study suggests that TCE
exposure provides some transitory growth advantage for cer-
tain cell populations within the liver.

Cell proliferation and apoptosis. Oral gavage of TCE in
corn oil caused a significant burst of cell proliferation in the
liver centered around day 10 on dose (Fig. 5). A dose–response
trend is evident (Fig. 5, inset); however, only the 1200 mg/kg
dose group showed a significant increase in cell proliferation
(p < 0.05). PCNA-positive cells, as well as mitotic figures,
were present in centrilobular, midzonal, and portal regions
with no apparent predilection for a particular lobular distribu-
tion (Fig. 6). Cytotoxicity manifested as hepatocellular necro-
sis was not observed in any dose group. There were no significant
differences in apoptosis between treatment and control
groups (data not shown).

treatment and time effect for percent peroxisomal area, a
“treatment only” effect for number of peroxisomes and no
effect for peroxisomal size. Hepatocytes examined from
corn oil control rats were no different from those from water
control rats for all the peroxisomal parameters, thus dis-
counting a vehicle effect. At the highest dose level in this
study, TCE gavage significantly increased the percent per-
oxosomal area of total cytoplasmic area compared to vehicle
(corn oil) controls at days 6, 10 (Fig. 4), and 14. Moreover,
total peroxisomal area per unit area of cytoplasm (percent-
age peroxisomal area) peaked at exposure day 10. For all
days (6, 10, and 14), TCE treatment resulted in a signifi-
cantly higher number of peroxisomes per unit area of cyto-
plasm than either corn oil or water treatment alone.
variability and minimal differences from control values suggest that nuclear DNA oxidative stress resulting from a clearly tumorigenic dose of TCE may be of questionable biological importance. This is consistent with reports of prolonged (71-day) drinking water exposures to the two metabolites TCA and DCA, which produced no significant elevation of 8-OHdG levels (Parrish et al., 1996). Interestingly, the same study reported significant increase in 8-OHdG levels in control B6C3F1 mice as a function of age. Additionally, while an acute single dose of TCA or DCA can elevate B6C3F1 mouse liver TBARS (Austin et al., 1996), a 14 day course 1 g/liter TCA or DCA in drinking water for 14 days does not (Austin et al., 1995). This is consistent with our findings of an "accommodation" to the effects of TCE dosing as measured by TBARS since clearly TBARS returned to control levels by the end of our study. The rapid decrease in control animal TBARS from day 6 to day 10 is not mirrored in the dosed animals. This is consistent with the suggestion that TCE generates an oxidative burden, albeit temporary, that may overwhelm cellular compensatory mechanisms.

Taken together, the time-course data suggest that the oxidative stress produced by TCE in the liver may be associated with events that could result in an initial "burst" of cell proliferation. After a 2- to 3-week period an accommodation process occurs, likely the result of alterations in enzyme levels - adequately demonstrated for TCA and DCA exposures within this time frame (Austin et al., 1995). For longer exposure times, as in our study, a "steady state" apparently develops wherein homeostatic mechanisms are able to compensate for the continued oxidative challenge. It is reasonable to speculate that under these conditions a selection process is occurring, wherein certain hepatocyte subpopulations eventually respond to the alterations in regulatory signal transduction pathways induced by the chronically altered tissue microenvironment. Given the general understanding of the role of radical species in tumor promotion (Kozumbo et al., 1985; Thrush and Kessler, 1991), this would be similar to the pleiotropic effects from carbon tetrachloride which produces lipid peroxidation (Slater, 1984), activates phospholipase A₂ (Glende and Pushpendran, 1986), and induces the transcription factor AP-1 (Kaplan and
Novak, 1994), depending on dose. With no compensatory increase in apoptosis (as we report here for TCE) the net effect would be tissue growth and a higher probability that subsequent chemical challenge would select for growth advantage from the phenotypically diverse expanded cell population.

The initial cellular response may be a characteristic of the B6C3F1 mouse liver that helps explain the marked species and strain susceptibility to TCE-induced hepatic tumorigenesis. There is mounting evidence that the unique genetic makeup of the B6C3F1 strain may predispose it to a high spontaneous rate of liver tumor formation (Drinkwater and Ginsler, 1989; Drinkwater et al., 1989; Pugh and Goldfarb, 1992) and that epigenetic chemicals may produce tumors by selection of intrinsically unique cellular phenotypes (Pitot et al., 1991). In the present study, we cannot determine whether the hepatocytes that result from the initial period of TCE-induced cell proliferation are the subpopulation that subsequently progress to neoplasia. What is certain is that such an early expansion of the total exposed cell population can only increase the probability of selection for “abnormal” phenotype. If indeed this is the case, our study suggests that the state of continued oxidative stress caused by TCE oral gavage may provide the hepatic microenvironment necessary for that selection to occur.

ACKNOWLEDGMENTS

This research was conducted under both the Strategic Environmental Research and Development Program (CU-115) and the Air Force Office of Scientific Research (Environmental Initiative). The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication No. 85-23, 1985, and the Animal Welfare Act of 1966, as amended. The authors would like to thank all the personnel from the testing and pathology branches for their technical assistance, and Ms. Susan Godfrey and Drs. Darrol Dodd, Jeffery English, and Janusz Byczkowski for their editorial and scientific reviews.

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