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Delineating Liver Events in Trichloroethylene-Induced Autoimmune Hepatitis

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Exposure to the environmental pollutant trichloroethylene (TCE) has been linked to autoimmune disease development in humans. Chronic (32-week) low-level exposure to TCE has been shown to promote autoimmune hepatitis in association with $CD4^+$ T cell activation in autoimmune-prone MRL+/+ mice. MRL+/+ mice are usually thought of as a model of systemic lupus rather than an organ-specific disease such as autoimmune hepatitis. Consequently, the present study examined gene expression and metabolites to delineate the liver events that skewed the autoimmune response toward that organ in TCE-treated mice. Female MRL+/+ mice were treated with 0.5 mg/mL TCE in their drinking water. The results showed that TCE-induced autoimmune hepatitis could be detected in as little as 26 weeks. TCE exposure also generated a time-dependent increase in the number of antibodies specific for liver proteins. The gene expression correlated with the metabolite analysis to show that TCE upregulated the methionine/homocysteine pathway in the liver after 26 weeks of exposure. The results also showed that TCE exposure altered the expression of selective hepatic genes associated with immunity and inflammation. On the basis of these results, future mechanistic studies will focus on how alterations in genes associated with immunity and inflammation, in conjunction with protein alterations in the liver, promote liver immunogenicity in TCE-treated MRL+/+ mice.

Introduction

Trichloroethylene (TCE[1]) is an organic solvent that has been used to degrease metal parts, as a paint stripper, and dry cleaning solvent. Although TCE has largely been replaced by other less toxic solvents in this country, its improper disposal has led to its becoming a major environmental pollutant found in air emissions, water supplies, and soil. Exposure to TCE is relatively common, but rarely occurs at levels thought to be directly toxic. However, a number of reports have linked chronic low-level TCE exposure to several autoimmune and other types of chronic diseases in humans including systemic lupus erythematosus, scleroderma, hepatitis, and diabetes (*1*−*4*).

A model using female MRL+/+ mice was developed to more directly examine the link between TCE and autoimmunity (*5*). Through an unknown mechanism, MRL+/+ mice spontaneously develop a form of systemic lupus erythematosus late in life (50% mortality at 17 months). MRL+/+ mice were treated with TCE with the expectation that this would accelerate the development of lupus nephritis. However, the mice given TCE in their drinking water (0.5 or 2.5 mg/mL) for 32 weeks developed histopathology commensurate with autoimmune hepatitis rather than lupus nephritis (*6*). The TCE treatment also increased the percentage of activated $CD4^+$ T cells that secreted IFN-γ and were resistant to activation-induced apoptosis (*7*−*9*).

Although the TCE-induced alterations in $CD4^+$ T cell function may have contributed to autoimmunity, it was not obvious why the autoimmunity was targeted toward the liver. Other examinations of TCE on liver function have used liver cells, rats, or nonautoimmune-prone mice (e.g., B6C3F1 mice) rather than autoimmune-prone MRL+/+ mice, and have used higher doses (e.g., 1500 mg/kg/day) and shorter (e.g., 7 days) exposures (*10, 11*). The current study was novel in its attempt to examine the effects of TCE on the liver using a dose that does not cause overt toxicity [e.g., increase in alanine aminotransferase (*7*)] but at a time point at which liver-specific immune system alterations can be demonstrated.

Thus far, our evaluation of a TCE-induced liver-specific immune response has been limited to examining infiltration of lymphocytes including CD3 T cells, the hallmark of autoimmune hepatitis in both humans and animal models. However, idiopathic autoimmune hepatitis (AIH) in humans is also associated with autoantibody production (*12*). Best characterized is the production of antibodies against liver-kidney microsome 1 and/or antiliver cytosol type 1 autoantibodies in patients with type 2 AIH (*13*). Although their role in pathology is still unclear, these autoantibodies are useful for diagnostic purposes and in some cases as a way to determine which liver antigens may be

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[1] Abbreviations: TCE, trichloroethylene; AIH, autoimmune hepatitis; RT-PCR, real-time polymerase chain reaction; IFN-γ, interferon gamma; PCA, principal component analysis; RXRα, retinoid X receptor α; SOD, superoxide dismutase.

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targeted by the immune response. Consequently, the generation of liver-specific antibodies was examined in the current study.

The liver normally demonstrates little inflammation and is relatively easy to transplant. Presumably, one or more of the mechanisms that discourage a liver-specific immune response must be circumvented during the development of idiopathic or toxicant-induced AIH. Most animal models of AIH induce acute liver injury with immune-mediated events measurable in hours and require exposure to potent exotoxins or other mitogens such as Concanavalin A (*14*−*16*). Although it is not clear how these mitogens promote AIH, it is believed to involve the production of inflammatory cytokines (*17*). In contrast, the TCE-induced AIH found in MRL+/+ mice occurred in the absence of overt liver injury and was associated with only early stages of fibrosis after 32 weeks of exposure. Thus, the TCE model more closely mimics the slow-developing idiopathic AIH in humans. In an attempt to identify some of the events associated with this more subtle disease development, we evaluated gene expression in the livers of female MRL+/+ mice exposed to TCE (0.5 mg/mL in drinking water) for 26 weeks. An additional metabolite analysis was conducted with the expectation that it would confirm the results of the gene array and/or to point out new TCE-induced pathways not revealed by examining gene expression.

This analysis of the liver following chronic rather than acute toxicant TCE exposure demonstrated the production of liver-specific antibodies in association with inflammatory gene expression. The results emphasized the efficacy of a multiparameter approach focused on a single target organ.

Experimental Procedures

Mice. Six to eight week old female MRL+/+ mice (Jackson Laboratories; Bar Harbor, ME) were housed in polycarbonate ventilated cages and provided with laboratory chow and drinking water ad libitum. TCE (purity 99+ %; Aldrich Chemical Co. Inc.; Milwaukee, WI) was suspended in drinking water with 1% emulsifier Alkamuls EL-620 from Rhone-Poulenc (Cranbury, NJ). Female mice were used since this sex, in both humans and mice, is generally more susceptible to the development of autoimmunity. The mice (6 mice/group) received either 0 or 0.5 mg/mL TCE in their drinking water for 26 weeks. Freshly made TCE-containing drinking water was provided every 3−4 days. TCE degradation in the drinking water was measured over the 3−4 day time period using gas chromatography separation in line with an ion trap mass spectrometer. All studies were approved by the Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Histopathology. At the time of sacrifice, the right kidneys and the caudal lobes of the livers were fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Liver and kidney sections were examined microscopically and scored in a blind manner by a veterinary pathologist for the severity of inflammation and fibrosis based on a four point scale.

Antibody Production. Microsomal liver protein (30 μg) obtained from untreated C3H/HeJ mice as described (*18*) was separated on 12% SDS−PAGE, electrotransferred onto nitrocellulose, and subsequently probed with IgG (1:1,000) purified from pooled sera obtained from control or TCE-treated MRL+/+ mice at different time points followed by HRP-conjugated goat antimouse IgG (1:10,000). Detection was performed using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Gene Arrays. Liver samples from 5 control and 5 TCE-treated mice were removed from all of the mice within the space of 1 h and were frozen at $^{-}$80 °C for subsequent analysis. Total RNA was extracted from the mouse liver tissues using a Qiagen RNeasy Kit (Qiagen, Valencia, CA) with on-column DNase digestion. The concentrations and A260/A280 ratios of the total RNA samples were measured using a NanoDrop ND-1000 spectrophotometer. The quality of the total RNA was further evaluated using an Agilent 2100 Bioanalyzer. All RNA samples used for microarray analysis had RNA Integrity Numbers >8.0. All of the liver tissue RNA samples from this study were labeled with Cy5 dye, and Rat Universal Reference RNA (Agilent Technologies, Inc. Palo Alto, CA) was labeled with Cy3 dye. The total RNA was reverse transcribed using amino-allyl dUTP, and cDNA were labeled. Labeled cDNA from liver tissue RNA and Universal Reference RNA were paired and hybridized with in-house printed mouse microarrays, which consisted of 20,000 50-mer oligonucleotide probes (MWG, Inc.). The hybridized slides were scanned with a GenePix 4000B scanner. The resulting images were analyzed using GenePix Pro Software (V6.0). The median fluorescence intensity of all the pixels within each feature was taken as the intensity value for that feature. All of the raw data were imported into ArrayTrack (NCTR/FDA) (*19*) and were normalized using LOWESS normalization. ArrayTrack and Ingenuity Pathway Analysis software (Redwood City, CA) were used for statistical and pathway analysis, respectively.

RT-PCR. For each sample, cDNA was synthesized with random hexamers and 2 ug of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). PCR primer sequences were designed using the NCBI gene databases and the Taqman Probe and Primer Design function, and synthesized by IDT, Inc. (Coralville, IA). All RT-PCR reactions were carried out using 2xSYBR Green Master Mix (iTaq SYBR Green Supermix with ROX, Bio-Rad Laboratories, Inc., Hercules, CA) and the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) according to manufacturer protocols. No-template controls were included to detect primer−dimer artifacts. DNA melting curve analysis was used as another control against primer−dimer formation and to ensure the lack of contaminating DNA in the RNA preparations. The relative gene expression was calculated using standard curves (log of the ng RNA-equivalents of cDNA versus cycle number) generated using 5-fold serial dilutions of cDNA. Data were normalized to Actin B RNA and reported as mean ± SD fold change in liver tissue from individual TCE-treated compared to control mice. Statistically significant differences in gene expression between TCE-treated and control mice were determined by paired Student's t test with p-value ≤ 0.05.

Metabolite Analysis. Liver samples were weighed and homogenized in distilled water. Half the sample was stored at $^{-}$80 °C for HPLC-ESI-MS/MS analysis of bile acids. The protein and lipid components were precipitated with acetonitrile. The supernatant was collected and the solvent evaporated under vacuum. Aqueous liver extracts were reconstituted in 180 μL of sodium phosphate buffer (pH 7.4) and 20 μL of a mixture of 10 mM imidazole and 1 mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TMSP) in D_2O. Spectra were acquired and processed as described (*35*).

Principal component analysis (PCA) based on covariance of the data was applied to the bucketed intensities using Statistica version 6.0 (Statsoft, Tulsa, OK). Metabolite identification and quantification was accomplished using the Chenomx NMR Suite (Chenomx, Calgary, Canada).

Table 1. Liver Pathology Induced by TCE[a]

trichloroethylene dose (mg/mL)	
0	0.5
Perivascular Lymphoid Infiltrates	
1	2
0	1
0	1
1	1
1	2

[a] Liver sections from control and TCE-treated mice were pathologically scored (from 0−4, ranging from no change to severe) for severity of perivascular infiltrates and fibrosis. The data from the two groups were analyzed by Wilcoxon rank sum test and determined to be significantly different ($p < 0.05$).

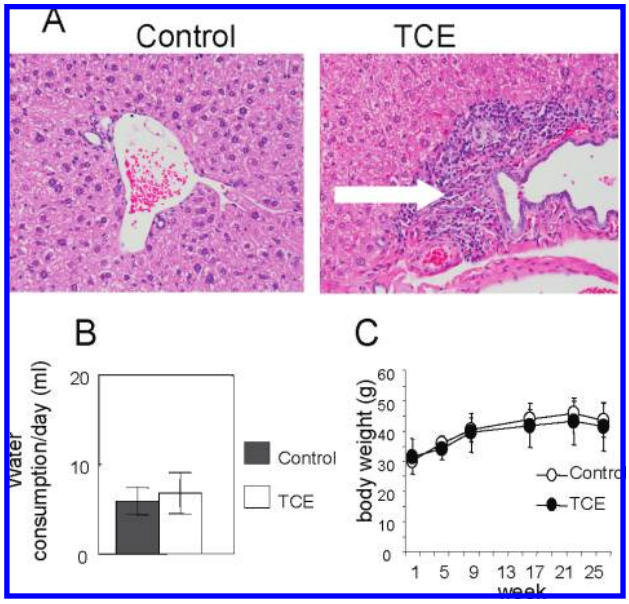

Figure 1. Gross pathology in MRL+/+ mice exposed to TCE. Female MRL+/+ mice were given water alone or water with TCE (0.5 mg/mL) for 26 weeks. A: Liver sections were fixed in formalin and stained with H&E. Shown are representative liver sections from a control and TCE-treated mouse. The arrow indicates mononuclear infiltration. B: Water consumption is presented as total per mouse per day for the entire 26-week time period. C: Body weight was monitored over time. The data are plotted as mean ± SD.

Oxidative Stress. TCE exposure has been shown to induce several markers of oxidative stress in mouse liver including alterations in superoxide dismutase (SOD) and glutathione (*20*). SOD activity (both SOD2; MnSOD and SOD1; Cu, Zn SOD) was measured in liver extracts using the nitroblue tetrazolium native gel assay (*21*). Protein oxidation associated with hydroxyl radical catalyzed oxidation of amine groups can be detected (*22*). This assay has been shown to detect oxidative stress in the livers of mice treated with higher concentrations of TCE (data not shown). The presence of oxidized proteins in the liver was detected as described using antidinitrophenyl antisera (*22*).

Results

Liver Pathology. As described in Table 1 and Figure 1A, the MRL+/+ mice treated with TCE for 26 weeks developed significant perivascular lymphoid infiltrates. These consisted of lymphocytes and some plasma cells that congregated within portal triads or near centrolobular veins. In contrast to the study with a longer exposure time, the livers from mice treated for 26 weeks with TCE did not yet demonstrate hepatocellular changes. Similar to the earlier study, TCE exposure did not induce measurable kidney histopathology at this time point (data not shown). Thus, even after a shorter exposure time, TCE induced liver pathology commensurate with an early stage of AIH in female MRL+/+ mice. The effects of TCE on liver pathology could not be attributed to alterations in water intake (Figure 1B) or differences in body weight (Figure 1C).

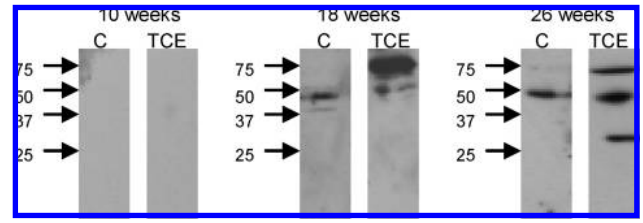

Figure 2. Detecting TCE-induced liver-specific antibodies. Microsomal liver protein from an untreated C3H/HeJ mouse was separated by SDS−PAGE and immunoblotted using IgG isolated from pooled sera from control or TCE-treated MRL+/+ mice collected at the 10, 18, or 26-week time points.

Liver-Specific Antibody Production. The production of liver-specific autoantibodies were examined using as target antigens liver microsomal proteins from untreated C3H/HeJ mice. The proteins were separated by SDS−PAGE and probed with IgG purified from sera obtained from control or TCE-treated mice at the 10, 18, or 26-week time points. As shown in Figure 2, IgG from the MRL+/+ mice at the 10 week time point, regardless of treatment, did not detect liver proteins. However, at the 18-week time point IgG from the TCE-treated MRL+/+ mice reacted with liver proteins of approximately 70−75 kDa and around 50 kDa. The IgG from control MRL+/+ mice also recognized a 50 kDa protein, but did not react with the 70−75 kDa protein. By 26-weeks of TCE exposure, the antibody repertoire had expanded to include liver proteins of approximately 30 kDa. In contrast, IgG from control mice remained restricted to the 50 kDa microsomal liver protein. It was the source of the antibody rather than the source of the antigen that determined specificity; the results did not differ if the source of the liver antigen was instead untreated or TCE-treated MRL+/+ mice (data not shown). These results showed that exposure to TCE induced a time-dependent increase in antibodies specific for liver microsomal proteins.

Hepatic Gene Expression. In order to examine the liver events that promoted lymphocyte infiltration and autoantibody production, the gene expression profiles in the livers of mice exposed to TCE were compared to those in mice given water alone. RNA from the livers of control and TCE-treated mice were profiled on microarrays fabricated by the FDA's National Center for Toxicological Research. After normalization and filtering of low intensity spots, *t* tests were performed, and these data were plotted against fold-change measurements. At a cutoff of $p < 0.05$ and fold-change >1.5, the expressions of over 200 genes were found to be significantly altered in the livers of mice exposed to TCE. More than 85% of the gene changes observed represented increased expression in the TCE-exposed mice. The genes whose expression was increased by TCE exposure at an arbitrary fold-change >1.8 are listed in Table 2.

TCE altered the expression of a number of hepatic genes involved in metabolism/detoxification including the phase I metabolizing enzymes such as CYP2A12 and CYP2C39, and phase II enzymes such as glutathione *S*-transferase (*23*). Expression of genes for aldehyde dehydrogenases was also upregulated. Similarly, TCE increased gene expression of betaine-homocysteine methyltransferase, a zinc metalloenzyme that catalyzes the transfer of methyl groups from betaine to homocysteine to produce dimethylglycine and methionine.

Table 2. Hepatic Genes Increased by TCE Exposure at a Fold-Change >1.8[a]

genebank accession number	gene symbol	gene name
		Metabolism
NM_009993	Cyp1a2	cytochrome p450, 1a2
NM_133657	**Cyp2a12**	**cytochrome P450, 2a12**
NM_010003	**Cyp2c39**	**cytochrome P450, 2c39**
NM_007824	Cyp7a1	cytochrome P450, 7a1
BC003903	Gstt3	similar to glutathione *S*-transferase theta 1
NM_010356	Gsta3	glutathione-*S*-transferase, α 3
NM_016668	**Bhmt**	**betaine-homocysteine methyltransferase**
NM_013467	**Aldh1a1**	**aldehyde dehydrogenase family 1, subfamily a1**
AK012213	Aldh1b1	homologue to aldehyde dehydrogenase, mitochondrial X precursor
BC026561	Ugt1a1	similar to UDP-glucuronosyltransferase 1 family, member 1
NM_011864	**Papss2**	**3′-phosphoadenosine 5′-phosphosulfate synthase 2; ATP sulfurylase**
BC025939	**Fthfd**	**similar to 10-formyltetrahydrofolate dehydrogenase**
		Immune Response
NM_010559	**IL6ra**	**interleukin 6 receptor, α**
D44592	**Azgp1**	**zinc α 2 glycoprotein; nonconventional MHC molecule**
NM_009984	Ctsl	cathepsin l
		Inflammation
NM_008648	**Mup4**	**major urinary protein 4**
NM_011034	Prdx1	proliferation-associated gene a; peroxiredoxin 1
NM_011305	**Rxra**	**retinoid X receptor α**
		Glucose-Fatty Acid
NM_026384	Dgat2	diacylglycerol *o*-acyltransferase 2; dgat2
NM_007981	Acsl1	fatty acid coenzyme a ligase, long chain 2
U89406	Fasn	fatty acid synthase
NM_023119	Eno1	enolase 1, α non-neuron; eno1
		Protein Synthesis/Degradation/Transport
NM_019775	Cpb2	carboxypeptidase b2 (plasma)
NM_011292	Rpl9	ribosomal protein l9
NM_008512	Lrp1	low density lipoprotein receptor-related protein 1
		Electron Transport
NM_011017	**Slc25a15**	**solute carrier family 25, member 15; ornithine transporter**
NM_025797	**Cyb5**	**cytochrome B5**
		Cytoskeleton
NM_007393	Actb	Actin, *β*, cytoplasmic
		Transcription Regulator
AK011545	**Basp1**	**brain abundant membrane attached signal protein**
		Other
AK002546	TMEM176A	Transmembrane protein 176A; hepatocellular carcinoma-associated antigen 112
NM_016702	Agxt	alanine-glyoxylate aminotransferase
NM_008777	Pah	phenylalanine hydroxylase
NM_007494	**Ass1**	**arginosuccinate synthetase 1**

[a] Genes in bold text represent >2-fold change.

A canonical pathway evaluation suggested that TCE altered the inflammation-related retinoid X receptor α (RXRα) pathway in the liver (data not shown). Consistent with this pathway involvement was the TCE-induced upregulation of *RXR*α as well as *Aldh1a1* and *Aldh1b1*, aldehyde dehydrogenases that help synthesize 9-*cis*-retinoic acid, one of the ligands for RXR (*24, 25*). Other hepatic genes involved in immunity or inflammation that were upregulated by TCE included *Azgp1*, a gene encoding a nonconventional MHC molecule, and *Cts1*, a cathepsin enzyme involved in antigen processing. Although TCE exposure promoted antibody production to liver proteins, it did not induce a detectable increase in the expression of other genes associated with antigen presentation such as *CD80* or *CD86* (data not shown).

A subsequent RT-PCR analysis of the liver tissue examined the expression of selected genes flagged in the high density gene arrays as well as some not included in the arrays (Figure 3). This analysis confirmed that TCE increased the expression of genes involved in metabolism/detoxification (*aldh1a1*, *Gstt3*) and the RXRα canonical pathway (*RXR*α, *Cyp7a1*). Expression of *Azg1* was not increased significantly, and other genes associated with antigen presentation (*CIITA*, *H2-IA-b*, and *H2-T10*) were unchanged in the livers of TCE-treated mice.

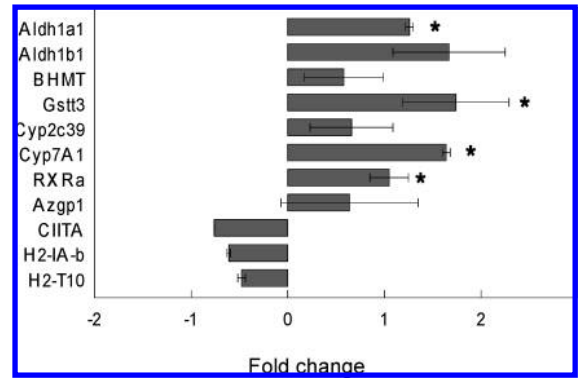

Figure 3. RT-PCR examination of TCE-induced gene expression. Quantitative real-time PCR was performed using total RNA from the livers of individual control or TCE-treated mice. The values represent the mean ± SD of relative fold change comparing gene expression in TCE-treated to control mice. *Statistically significant ($p > 0.05$) difference compared to controls.

Metabolite Production. Select metabolites were also analyzed in both aqueous and lipophilic liver extracts from the control and TCE-treated mice. The lipid extract samples did not show any clustering of the control versus the treated samples

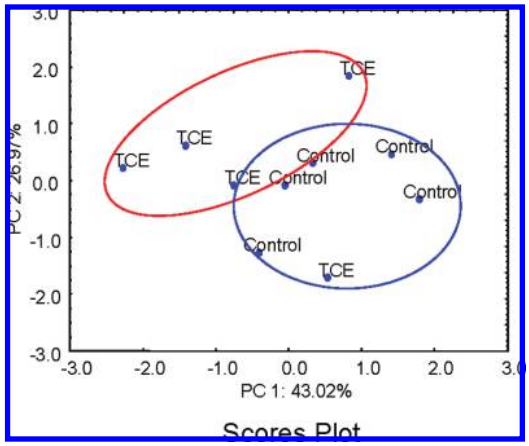

Figure 4. Principal component analysis of aqueous liver extracts. The metabolomic data obtained from the aqueous liver extracts was examined for clustering using a principal component analysis. PC1 versus PC2 loading plot from integrated intensities of liver spectra is shown.

in the principal component analysis (data not shown). In contrast, 4 out of 5 samples from the aqueous liver extract from the TCE-treated mice formed a cluster separate from that of controls (Figure 4). Analysis indicated bins related to glucose, alanine, lactate, and acetate as contributors to the separation (data not shown). This result showed that metabolites could be used to delineate differences between control and TCE-treated liver tissue.

An additional analysis of the aqueous liver extract was made of several metabolites and normalized to the mass of each individual liver sample (Table 3). There were significant differences between the groups in levels of cystathionine, *N*,*N*-dimethylglycine, and *S*-adenosylhomocysteine (SAM). Levels of glutathione and homocystine were not significantly altered in the livers of the TCE-treated mice. Taken together, these results supported a TCE-induced alteration in the methionine/homocysteine pathway in the liver.

Oxidative Stress. Short-term exposure to toxicants known to induce oxidative stress in the liver can alter the expression of genes such as metallothionein I and II, epoxide hydrolase, heme oxygenase, SOD 3, glutathione *S*-transferase a2, UDP-glucose dehydrogenase, NAD(P) dehydrogenase quinine 1, and betaine homocysteine methyltransferase (*23, 26*). With the exception of betaine homocysteine methyltransferase, the expression of these genes was not altered in the livers of the TCE-treated mice. Increased carbonyl content in proteins is an indication of oxidative stress. Immunochemical detection of carbonyl-modified proteins by Western blotting revealed no difference in the cumulative levels of oxidized liver proteins between control and TCE-treated mice (Figure 5A). In addition, no specific bands were noticeably differentially oxidized (data not shown). Similarly, TCE did not alter cytoplasmic copper/zinc SOD (SOD1) or mitochondrial manganese SOD (SOD2) activity in the liver (Figure 5B). Glutathione levels were also not decreased (Table 3). Thus, although the evaluation of oxidative stress was not comprehensive, the assays used failed to detect TCE-induced oxidative stress in livers at the 26-week time point of disease development.

Discussion

The current study found TCE-induced autoimmune liver pathology after only 26 weeks of exposure. On the basis of water intake, body weight, and measured TCE degradation in the water bottles, the mice were exposed to an average of 50 mg/kg/day TCE. This dose is occupationally relevant based on the current 8-h permissible exposure limit [established by the Occupational Safety and Health Administration (OSHA)] for TCE of 100 ppm or approximately 76 mg/kg/day. In terms of environmental exposure, federal and state surveys have determined that TCE is the most frequently detected organic solvent in groundwater supplies, and up to 34% of drinking water sources in the US are contaminated with TCE, albeit at low levels (*27*). The USEPA has established a maximum contaminant level (MCL) of 5 ppb of TCE in drinking water. However, the USEPA estimates that approximately 4 million people in the USA are served by water systems with TCE levels greater than the MCL (*28*). The amount of TCE ingested through drinking water is dwarfed by exposure through other water-related activities such as showering and dishwashing (*29*). Total TCE exposure must also take into account food sources, which have been shown to range from 16 ppb in meat to 3,6000 ppb in margarine (ATSDR, 1997) and a small level of dermal exposure from consumer products (e.g., cleaning supplies). Thus, total human exposure to TCE is difficult to estimate but cannot be predicted solely on drinking water consumption. In any case, regardless whether TCE is ingested or inhaled it is quickly absorbed through the gastrointestinal tract or lungs, respectively, and subsequently distributed to the liver and other tissues via the circulatory system.

A relationship between TCE exposure and autoimmune hepatitis in humans was suggested when significant clusters of individuals listed for liver transplantation with a diagnosis of autoimmune hepatitis were recently identified in association with U.S. EPA monitoring sites recording mean daily levels of chlorinated hydrocarbons (primarily trichloroethylene) in the 90th percentile (*30*). Although the hepatitis observed in the current study was at an early stage, the results confirmed that the liver is the first organ to demonstrate autoimmune pathology in MRL+/+ mice treated with low levels of TCE.

The lymphocyte infiltration into the livers of the TCE-treated mice was accompanied by the generation of antibodies specific for liver microsomal proteins. This TCE-induced autoantibody production was similar to that found in patients with AIH, especially type 2, which is characterized by antibodies to liver-kidney microsome type 1 and/or liver cytosol type 1 (*31*). It is possible that antibody production and/or lymphocyte infiltration is linked to adduct formation in the liver. During the early CYP-mediated conversion step, TCE exists transiently as a highly reactive intermediate (TCE-O-CYP) that can form adducts with nearby liver proteins. Modified proteins of 50 and 75 kDa were found in the microsomal liver fractions of TCE-treated MRL+/+ mice (*18, 32*). Interestingly, the IgG in the sera of MRL+/+ mice exposed to TCE was shown here to recognize microsomal proteins of the same molecular weight. However, since the IgG from the TCE-treated mice recognized liver proteins from untreated mice this implies that even if adduct formation is needed to trigger an immune response, the resulting immunoreactivity eventually encompasses unmodified proteins.

Aside from direct chemical modification, toxicants can also modify self-proteins via the generation of reactive oxygen species. Along these lines, Wang et al. found antibodies against lipid peroxidation products 4-hydroxynonenal and malondialdehyde in the serum of mice following chronic (48 weeks) exposure to TCE (*33*). However, in contrast to expectations, none of the assays used in the current study detected signs of oxidative stress in the livers of the MRL+/+ mice treated with 0.5 mg/mL TCE in their drinking water for 26 weeks. It is

Table 3. TCE-Induced Changes in Liver Metabolites[a]

metabolite	control (μM/mg tissue)	TCE (μM/mg tissue)	metabolite	control (μM/mg tissue)	TCE (μM/mg tissue)
1-methylnicotinamide	58 ± 21	48 ± 20	isoleucine	290 ± 40	330 ± 110
2-oxoglutarate	78 ± 39	75 ± 22	lactate	17944 ± 2054	19859 ± 4933
5-aminolevulinate	50 ± 30	30 ± 10	leucine	450 ± 60	590 ± 230
acetamide	1090 ± 350	910 ± 270	lysine	410 ± 120	410 ± 80
acetate	9000 ± 2790	7770 ± 1160	methionine	97 ± 25	98 ± 57
alanine	4900 ± 660	5870 ± 2040	***N*,*N*-dimethylglycine**	**77 ± 20**	**122 ± 28**
adp	139 ± 93	175 ± 117	NAD+	47 ± 30	27 ± 3
amp	610 ± 196	916 ± 694	NADH	22 ± 9	18 ± 6
atp	318 ± 170	355 ± 124	malate	14 ± 6	16 ± 7
adenosine	28 ± 16	51 ± 25	niacinamide	1150 ± 30	1380 ± 360
aspartate	190 ± 100	310 ± 130	nicotinate	54 ± 28	31 ± 9
betaine	3448 ± 834	5211 ± 2813	*O*-phosphoethanolamine	300 ± 40	340 ± 70
choline	383 ± 58	463 ± 116	*O*-phosphocholine	1350 ± 420	1850 ± 260
carnitine	350 ± 70	470 ± 270	propylene glycol	6390 ± 2180	5930 ± 1610
citrate	392 ± 160	272 ± 78	niacinamide	1150 ± 30	1380 ± 360
creatine	114 ± 28	117 ± 42	nicotinamide	76 ± 16	60 ± 11
cystathionine	**248 ± 60**	**334 ± 40**	nicotinate	54 ± 28	31 ± 9
cysteine	156 ± 62	124 ± 40	phosphocholine	1350 ± 420	1850 ± 260
cystine	380 ± 110	400 ± 220	phosphoserine	430 ± 160	400 ± 100
glucose	35620 ± 7290	37500 ± 11730	pyruvate	121 ± 57	193 ± 115
glutamate	1690 ± 330	1550 ± 680	***S*-adenosylhomocysteine**	**29 ± 3**	**16 ± 6**
glutamine	2630 ± 420	3140 ± 1220	SAM	59 ± 31	53 ± 17
glutathione	4604 ± 462	6617 ± 2228	sarcosine	90 ± 49	92 ± 44
glycerate	360 ± 130	430 ± 130	serine	590 ± 220	720 ± 320
glycine	2601 ± 501	2904 ± 787	succinate	63 ± 17	55 ± 9
homocystine	235 ± 65	155 ± 30	taurine	17000 ± 4060	13660 ± 8650
homoserine	580 ± 230	630 ± 270	threonine	380 ± 80	480 ± 160
hypoxanthine	523 ± 274	491 ± 112	trimethylamine *N*-oxide	430 ± 70	580 ± 150
isocitrate	545 ± 91	598 ± 125	valine	420 ± 70	570 ± 170

[a] Metabolites in bold text represent significant differences ($p < 0.05$).

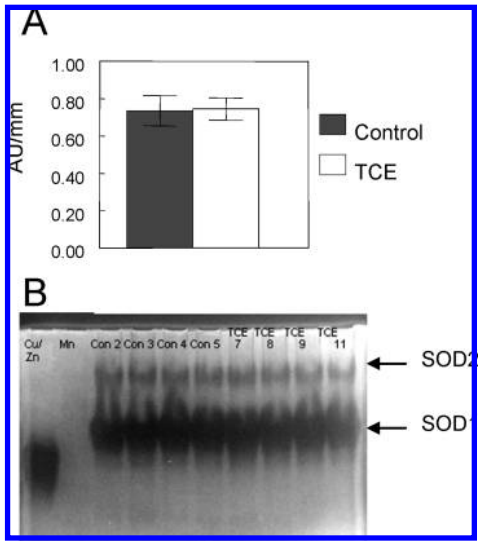

Figure 5. Measurement of oxidative stress in livers from TCE-treated mice. A: Oxidized proteins in the livers of control and TCE-treated mice were examined by Western blotting and subsequent densitometric analysis. The results are presented as mean ± SD AU (arbitrary units). B: Equal amounts of liver protein (50 μg) from 4 control and 4 TCE-treated mice were analyzed for SOD activity by the NBT gel assay.

possible that the 26-week exposure to TCE may represent a time point at which the compensatory antioxidant mechanisms are still working. Alternatively, assays to detect lipid peroxidate-induced autoantibodies may be more sensitive than those that detect the preceding oxidative events. In any case, TCE-induced oxidative stress could not be used to explain liver pathology in this study.

High density gene arrays and metabolite analysis were used to study why TCE targeted the liver instead of another tissue. Much of the work in toxicogenomics has focused on the response to short-term in vitro treatments or acute in vivo exposure (*34*−*37*). The current study confirmed that this approach can also be used to evaluate the effects of chronic toxicant exposure. One outcome was the finding that long-term TCE exposure upregulated the classic detoxification pathway regulated by GSH. The fact that the analysis of both the gene expression and metabolites pointed toward this pathway confirmed the validity of this conclusion and underscored the usefulness of a combined evaluation.

Perhaps more relevant to autoimmune disease etiology was the effect of TCE on genes associated with immunity and inflammation. It is possible that upregulation of inflammatory genes in the liver relates to the Schiff base-forming capacity of TCE metabolite trichloroacetaldehyde. Although not yet tested in liver cells, Schiff base-forming aldehydes similar to trichloroacetaldehyde have been shown to trigger activation pathways in other cell types (*38*). Another possible mechanism is based on the observation that TCE increases the percentage of activated $CD4^+$ T cells secreting increased levels of IFN-γ in MRL+/+ mice (*6*). The infiltration of the T cells secreting the pro-inflammatory IFN-γ may upregulate the expression of inflammatory genes in the livers of the TCE-treated mice. Future mechanistic studies will focus on how the changes in hepatic gene expression described here are induced and whether they, perhaps in conjunction with neoantigen formation, increase liver immunogenicity and thereby promote humoral and/or cellular liver-specific immune responses.

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Supporting Information Available: Loading plot data. This material is available free of charge via the Internet at http://pubs.acs.org.

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