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Protein adducts of malondialdehyde and 4-hydroxynonenal contribute to trichloroethene-mediated autoimmunity via activating Th17 cells: Dose- and time-response studies in female MRL+/+ mice

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Abstract

Trichloroethene (TCE), a common occupational and environmental toxicant, is known to induce autoimmunity. Previous studies in our laboratory showed increased oxidative stress in TCE-mediated autoimmunity. To further establish the role of oxidative stress and to investigate the mechanisms of TCE-mediated autoimmunity, dose- and time- response studies were conducted in MRL+/+ mice by treating them with TCE via drinking water at doses of 0.5, 1.0 or 2.0 mg/ml for 12, 24 or 36 weeks. TCE exposure led to dose-related increases in malondialdehyde (MDA)-/hydroxynonenal (HNE)-protein adducts and their corresponding antibodies in the sera and decreases in GSH and GSH/GSSG ratio in the kidneys at 24 and 36 weeks, with greater changes at 36 weeks. The increases in these protein adducts and decreases in GSH/GSSG ratio were associated with significant elevation in serum anti-nuclear- and anti-ssDNA-antibodies, suggesting an association between TCE-induced oxidative stress and autoimmune response. Interestingly, splenocytes from mice treated with TCE for 24 weeks secreted significantly higher levels of IL-17 and IL-21 than did splenocytes from controls after stimulation with MDA-mouse serum albumin (MSA) or HNE-MSA adducts. The increased release of these cytokines showed a dose-related response and was more pronounced in mice treated with TCE for 36 weeks. These studies provide evidence that MDA- and or HNE-protein adducts contribute to TCE-mediated autoimmunity, which may be via activation of Th17 cells.

Keywords

Trichloroethene; anti-MDA/HNE antibodies; oxidative stress; Th17 cells; autoantibodies; autoimmunity

1. Introduction

Trichloroethene (TCE) is a widely used organic solvent and a common occupational and environmental contaminant (Diot et al., 2002; Hardin et al., 2005; Bakke et al., 2007; Moran

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et al., 2007; ATSDR, 2010; Purdue et al., 2011). About 3.5 million people are occupationally exposed to TCE in the United States through its use as a degreasing agent for metals and as a solvent for various cleaning operations (Wu and Schaum, 2000; Bakke et al., 2007; ATSDR, 2010). Exposure to TCE also occurs through the air from waste disposal sites and contamination of the ground water. TCE has been identified in at least 861 of the 1428 hazardous waste sites, and is the most frequently reported organic contaminant in groundwater with up to 34% of the drinking water supplies in USA contaminated with TCE (IARC, 1995; Bourg et al., 1992; ATSDR, 2010). Because of its widespread commercial use and improper disposal, TCE has become a major occupational and environmental toxicant, and is one of the most abundant organic contaminants found in the Superfund sites (NTP, 1990; Bourg et al., 1992; Ashley et al., 1994; Hardin et al., 2005; Moran et al., 2007; ATSDR, 2010). Therefore, there is clearly a need to extensively study the potential adverse health affects of TCE.

Apart from diseases like cancer and heart defects (Boyer et al., 2000; Rhomberg, 2000; Caldwell and Keshava, 2006 ; Drake et al., 2006; Purdue et al., 2011), TCE exposure has also been implicated in the development of various autoimmune diseases (ADs), such as systemic lupus erythematosus (SLE), systemic sclerosis and fasciitis, both from occupational (Phoon et al., 1984; Flindt-Hansen and Isager, 1987; Lockey et al., 1987; Yanez Diaz et al., 1992; Waller et al., 1994; Nietert et al., 1998) and environmental exposures (Byers et al., 1988; Kilburn and Warshaw, 1992; Hayashi et al., 2000 ; Albert et al., 2005). An animal model, autoimmune-prone MRL+/+ mice, to provide direct evidence of an association between TCE exposure and autoimmunity was developed in our laboratory (Khan et al., 1995). This association was further substantiated by our recent studies and reports from other laboratories (Gilbert et al., 1999; Griffin et al., 2000; Khan et al., 2001; Wang et al., 2007, 2008, 2009; Cai et al., 2008). However, impact of dose and duration of exposure and the molecular mechanism(s) of TCE-induced autoimmunity remain largely unknown.

Increasing evidence suggests that free radical-mediated reactions play a potential role in the pathogenesis of ADs (Khan et al., 2001; Hadjigogos, 2003; Frostegard et al., 2005; Wang et al., 2008, 2010; Vasanthi et al., 2009; Iuchi et al., 2010) and increased oxidative stress is reported in ADs (Grune et al., 1997; Frostegard et al., 2005; Tam et al., 2005; Morgan et al., 2009; Vasanthi et al., 2009; Shah et al., 2010; Wang et al., 2010). Lipid peroxidation-derived aldehydes (LPDA) such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), bind covalently with proteins to form MDA- and HNE-modified protein adducts (Khan et al., 1997b, 1999; Januszewski et al., 2005; Wang et al., 2009; Reed et al., 2009; Ben Mansour et al., 2010), and it is not surprising that higher levels of MDA-/HNE-modified proteins have been observed in AD patients (Grune et al., 1997; Kurien and Scofield, 2003; Frostegard et al., 2005; D'souza et al., 2008; Ben Mansour et al., 2010; Wang et al., 2010), suggesting a potential role for oxidative stress in ADs. Even though human exposure to high levels of TCE in occupational setting or in some instances of environmental contamination are reported (Bruning et al., 1996; Lock and Reed, 2006; Bakke et al., 2007; Kamijima et al., 2008; ATSDR, 2010; Purdue et al., 2011), it is thought that low level TCE exposure is relatively common in general population (Hardin et al., 2005; Lock and Reed, 2006; Bakke et al., 2007; Moran et al., 2007; ATSDR, 2010). Previous studies in our laboratory showed that exposure to relatively high dose of TCE not only led to oxidative stress but also accelerated autoimmune responses in MRL+/+ mice (Khan et al., 1995, 2001; Wang et al., 2007, 2008). However, little is known about the impact of long-term low dose TCE exposure on the induction of an autoimmune response, especially at occupationally relevant concentrations (Griffin et al., 2000b; Gilbert et al., 2006, 2009; Wang et al., 2007a; Cai et al., 2008). This study, using our earlier established in vivo model (Khan et al., 1995, 2001; Wang et al., 2007, 2008), was aimed to evaluate the significance and role of LPDA-protein

adducts in TCE-induced/exacerbated autoimmunity and to determine if the responses are dependent on dose and duration of TCE exposure.

2. Materials and methods

2.1. Animals and treatments

Five-week old female MRL+/+ mice, purchased from The Jackson Laboratories (Bar Harbor, ME), were housed in plastic cages on a bedding of wood chips at the University of Texas Medical Branch (UTMB) animal house facility maintained at ~ 22 °C, 50-60% relative humidity, and a 12h light/dark cycle. The animals were provided standard lab chow and drinking water ad libitum and were acclimated for one week before the treatment. TCE (purity 99+ %, Sigma, St. Louis, MO) was dissolved in drinking water containing 1% Alkamuls EL-620 emulsifier (Rhone-Poulenc, Cranbury, NJ). The mice were divided into twelve groups of six each and received TCE (0.5, 1.0, 2.0 mg/ml) or drinking water containing 1% Alkamuls EL-620 emulsifier only (controls). These low doses of TCE were based on previous studies (Griffin et al., 2000b; Gilbert et al., 2006, 2009; Wang et al., 2007a; Cai et al., 2008) which showed an induction of autoimmune response. The consumption of TCE-containing drinking water was measured and the water was changed on alternate days, and the mice were weighed on a weekly basis to monitor weight changes. After 12, 24 and 36 weeks of TCE treatment, respectively, the animals were euthanized under Nembutal anesthesia (sodium pentobarbital, ip, 80 mg/kg), and blood was withdrawn from the inferior vena cava. Major organs were removed immediately and weighed. Portions of kidney from control and TCE-treated mice were frozen for glutathione (GSH) analysis. At the same time, spleens were removed immediately and splenocytes were isolated and suspended in RPMI 1640 medium supplemented with 2 mM glutamine, 50 µg/ml gentamycin and 10% fetal bovine serum (FBS; Khan et al., 2006; Wang et al., 2008). Individual sera, obtained following blood clotting and centrifugation, were stored in small aliquots at -80 °C till further analysis.

2.2. Quantitation of MDA- and HNE-protein adducts in the serum

For the quantitation of MDA- and HNE-protein adducts in the sera from TCE-treated mice and controls, competitive ELISAs were preformed as described earlier (Khan et al., 1997b, 2002; Wang et al., 2007b, 2010). Briefly, flat bottomed 96-well microtiter plates were coated with MDA-/HNE-ovalbumin adducts or ovalbumin (0.5 µg/well) overnight at 4 °C. For the competitive ELISA, rabbit antisera [anti-MDA (1:2000 diluted) or anti-HNE (1:3000 diluted); Alpha Diagnostics Int'l, San Antonio, TX] were incubated with test samples (standards or unknown) at 4 °C overnight. The coated plates were blocked with a blocking buffer (Sigma) for 2 h at room temperature (RT), then a 50 µl aliquot of each of the above mentioned incubation mixtures was added to duplicate wells and incubated for 2 h at RT. After washing, 50 µl of goat anti-rabbit IgG-horseradish peroxidase (HRP; 1:2000 diluted, Millipore, Billerica, MA) was added and incubated for 1 h at RT. After washing, 100 µl of TMB peroxidase substrate (KPL, Gaithersburg, MD) was added to each well. The reaction was stopped after 10 min by adding 100 µl 2 M H₂SO₄ and the absorbance was read at 450 nm on a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

2.3. ELISAs for anti-MDA- and anti-HNE-protein adduct specific antibodies in the serum

ELISAs to analyze anti-MDA- and -HNE-protein adduct-specific antibodies in the mouse serum were performed as described earlier (Khan et al., 2001; Wang et al., 2007, 2008). Briefly, flat-bottomed 96-well microtiter plates were coated with MDA-/HNE-ovalbumin adducts or ovalbumin (0.5 µg/well) overnight at 4 °C. The plates were washed with Tris-buffered saline-Tween 20 (TBST) and the non-specific binding sites were blocked with Tris-

buffered saline (TBS) containing 1% bovine serum albumin (BSA; Sigma) at RT for 1 h. After washing extensively with TBST, 50 μ l of 1:100 diluted mouse serum samples were added to duplicate wells of the coated plates and incubated at RT for 2 h. The plates were washed 5 times with TBST and then 50 μ l of rabbit anti-mouse IgG-HRP (1:2000 in TBS; Chemicon, Temecula, CA) was added and incubated at RT for 1 h. After washing, 100 μ l of TMB peroxidase substrate (KPL) was added to each well. The reaction was stopped after 10 min by adding 100 μ l 2 M H_2SO_4 and the OD was read at 450 nm on a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories). The samples with net OD values (the difference between OD for MDA-/HNE-ovalbumin and ovalbumin) greater than 0.2, 0.4, or 0.6 were graded as moderately positive (+), highly positive (++), or strongly positive (+++), respectively (Khan et al., 2001; Wang et al., 2007a, 2008).

2.4. Glutathione content in kidneys

The contents of reduced (GSH) and oxidized (GSSG) glutathione in the kidneys were analyzed by using Glutathione Assay kit (Cayman Chemical Co., Ann Arbor, MI). Briefly, pieces of each kidney were weighed and homogenates (20%) were made in ice-cold phosphate-buffered saline (PBS; pH 7.4) containing 1mM EDTA. A small amount of the supernatant after centrifugation (10,000 g for 15 min at 4°C) was used for protein assay (protein assay; Bio-Rad Laboratories). For deproteinization of the samples, an equal volume of 10% (w/v) of metaphosphoric acid was added to the supernatants. After centrifugation (2000 g for 5 min), the resulting supernatant was neutralized with 4 M of triethanolamine (50 μ l per ml supernatant) for the measurement of the GSH and GSSG levels, which were then determined according to the procedure described in the assay kit and were expressed as nmol mg^{-1} protein.

2.5. Anti-nuclear and anti-ssDNA antibodies in the serum

Serum levels of anti-nuclear antibodies (ANA) and anti-single stranded DNA (anti-ssDNA) antibodies were determined by using mouse-specific ELISA kits (Alpha Diagnostic Int'l, San Antonio, TX) as described earlier (Khan et al., 1995, 1997a; Wang et al., 2007, 2008).

2.6. Determination of IL-17 and IL-21 in splenocyte cultures

Splenocytes isolated from the spleens of control and TCE-treated MRL^{+/+} mice were plated into 24-well flat-bottom plates at 2×10^6 /well in a total volume of 1ml. Mouse serum albumin (MSA; 10 μ g/ml), MDA-MSA (10 μ g/ml), HNE-MSA (10 μ g/ml) or anti-mouse CD3 (2.5 μ g/ml, BD Biosciences) were added, respectively, to stimulate lymphocytes in the culture and incubated at 37 °C with 5% CO_2 . After 72 h, culture supernatants from each well were harvested and the release of IL-17 and IL-21 into the cultures was quantitated using specific ELISA kits (Biolegend Inc, San Diego, CA).

2.7. Statistical analysis

The values are means $\pm$ SD. One-way ANOVA followed by Tukey-Kramer multiple comparisons test (GraphPad Instat 3 software, La Jolla, CA) was performed for the statistical analysis. Spearman's rank correlation was used to calculate correlation coefficients between MDA-/HNE-protein adducts in sera or GSH/GSSG ratio in kidneys and serum ANA. A *p* value < 0.05 was considered to be statistically significant.

3. Results

3.1. MDA- and HNE-protein adducts in the sera of mice treated with TCE

TCE has been shown to generate free radicals and induce oxidative stress both in vivo and in vitro (Ogino et al., 1991; Channel et al., 1998; Khan et al., 2001; Zhu et al., 2005; Wang et

al., 2007, 2008). We attempted to investigate the significance and role of TCE-induced lipid peroxidation (LPO) in the induction/exacerbation of an autoimmune response by determining the serum levels of MDA-/HNE-protein adducts in the mice treated with various doses of TCE for 12, 24 or 36 weeks (Fig. 1). As evident from Fig. 1A, the levels of MDA-protein adducts in the mice treated with TCE for 12 weeks were only moderately higher at doses of 0.5 and 1.0 mg/ml, but significantly higher at the dose of 2.0 mg/ml (43% increase, $p < 0.05$) compared to the controls. The increases in these adducts following 24 weeks of TCE treatment were 12%, 38% and 74% at doses of 0.5, 1.0 and 2.0 mg/ml, respectively, and were statistically significant at doses of 1.0 and 2.0 mg/ml compared to the controls. The increases in these adducts at TCE dose of 2.0 mg/ml were also significant compared to the mice treated with the same dose of TCE for 12 weeks. After 36 weeks of TCE exposure, significant increases in the MDA-protein adducts (54%, 85% and 124%, respectively) were observed at all three dosages compared to controls. Furthermore, the increases in the mice treated with TCE for 36 weeks at all three dosages were significantly greater vs. the mice treated with TCE for 12 or 24 weeks at the corresponding dose groups (Fig. 1A). Similarly, dose- and time-related increases in the levels of HNE-protein adducts in the sera of TCE-treated mice were also observed (Fig. 1B). These results suggest that TCE exposure leads to increased formation of LPDA-protein adducts in a dose-and time-related pattern.

3.2. Anti-MDA- and anti-HNE-protein adduct antibodies in the serum

To assess if increased formation of LPDA could contribute to TCE-mediated autoimmunity, anti-MDA- and anti-HNE-protein adduct antibodies were also analyzed in the sera (Fig. 2 and Tables 1 and 2). There were moderate increases in serum anti-MDA-protein adduct antibodies in mice treated with TCE for 12 weeks, and the increases were 20%, 24% and 53% at the doses of 0.5, 1.0 and 2.0 mg/ml, respectively, compared to the respective controls (Fig. 2A). Relatively higher increases in these antibodies were observed in mice treated with TCE for 24 weeks (23%, 37% and 79% increases at 0.5, 1.0 and 2.0 mg/ml TCE dose, respectively), and the increase was statistically significant at the TCE dose of 2.0 mg/ml ($p < 0.05$). After 36 weeks of TCE exposure, there were significant increases in anti-MDA-protein adduct antibodies (53%, 60% and 81%, respectively) at all three dosages compared with the controls ($p < 0.05$). Moreover, the increases in mice treated with TCE for 36 weeks at 0.5, 1.0, 2.0 mg/ml were also significantly greater vs. the mice treated with TCE for 12 weeks at the corresponding dose groups (Fig. 2A). Interestingly, the number and percentage of samples positive (+), highly positive (++) and strongly positive (+++) for these antibodies showed dose- and time-related increases in the TCE-treated mice compared to the controls (Table 1).

Serum levels of anti-HNE-protein adduct antibodies, and the number and percentage of serum samples positive for anti-HNE-protein adduct antibodies also increased and showed a pattern similar to that of anti-MDA-protein adduct antibodies. However, serum anti-HNE-protein adduct antibodies in each dose group treated with TCE for 36 weeks were significantly higher than the respective TCE group exposed for 24 weeks (Fig. 2B & Table 2). The time- and dose-related increases in the levels of serum anti-MDA-/anti-HNE-protein adduct antibodies in TCE-treated mice not only further support that TCE induces increased formation of LPDA-modified protein adducts in the MRL +/+ mice in a dose-and time-related manner, but more importantly, suggest that the LPDA-protein adducts are immunogenic and could contribute to TCE-mediated autoimmunity.

3.3. Effect of TCE on GSH and GSH/GSSG ratio in the kidneys

Glutathione (GSH) is an antioxidant, which prevents damage to important cellular components caused by reactive oxygen species (Pompella et al., 2003; Ballatori et al., 2009).

Glutathione reduces disulfide bond formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form glutathione disulfide (GSSG). The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) within cells is a useful indicator of oxidative stress in cells and tissues (Rahman et al., 2005; Ballatori et al., 2009; Owen and Butterfield, 2010). Kidney is one of major TCE-targeted organs and oxidative stress may play role in TCE-induced nephrotoxicity and renal carcinoma (Cojocel et al., 1989; Lash et al., 2000; Cai et al., 2008; Tabrez and Ahmad, 2011). Autoimmune diseases, such as SLE, autoimmune nephritis, often target kidneys which may cause functional failure and possible mortality (Nieto et al., 2002; Charles et al., 2010; Lu et al., 2010; Zhang et al., 2011). To further assess the redox status following TCE exposure and evaluate the contribution of oxidative stress in TCE-induced autoimmunity, the glutathione level was analyzed in the kidneys. As evident from Table 3, TCE exposure led to significant decreases in kidney GSH at both 24 and 36 weeks in comparison to their respective controls, but the GSH level was even lower at 36 weeks and significantly lower than the respective TCE dose groups treated for 12 weeks. Remarkably, the observed decreases in the kidney GSH levels with increasing doses of TCE suggest a dose-dependent response. Similar pattern but more remarkable decreases in GSH/GSSG ratio were also observed (Table 3). Particularly, GSH/GSSG ratio in the mice treated with TCE for 24 weeks at the dose of 1.0 mg/ml was significantly lower not only compared to controls but also to the mice treated with the same dose of TCE for 12 weeks. Moreover, the GSH/GSSG ratio also significantly decreased following TCE exposure for 12 weeks at the dose of 2.0 mg/ml compared to both controls and mice treated with TCE at the dose of 0.5 mg/ml.

3.4. TCE potentiates autoantibody production in mice serum

Autoantibodies, such as ANA and anti-ssDNA, are considered important laboratory evaluation indices and biomarkers of ADs (Egner, 2000; Arbuckle et al., 2003; Reveille, 2004). These autoantibodies were analyzed in the serum of MRL^{+/+} mice treated with TCE for 12, 24 or 36 weeks (Fig. 3). In comparison to controls, there were mild increases in serum ANA levels in mice treated with TCE for 24 weeks (5%, 13% and 49% increases at 0.5, 1.0 and 2.0 mg/ml, respectively) but the increase was statistically significant in mice treated with 2.0 mg/ml of TCE ($p < 0.05$, Fig. 3A). After 36 weeks of TCE exposure, there were remarkable increases in the serum ANA levels in TCE-treated mice at all three dosages of 0.5, 1.0 and 2.0 mg/ml (46%, 55% and 100%, respectively) compared with respective controls ($p < 0.05$, Fig. 3A). Moreover, the ANA increases were significantly greater in mice treated with TCE for 36 weeks at 0.5, 1.0, 2.0 mg/ml vs. mice treated with TCE for 12 weeks in the respective dose groups. Furthermore, serum ANA levels in mice treated with 2.0 mg/ml of TCE for 24 or 36 weeks were significantly higher than the lower dose groups at the respective time points (Fig. 3A). Similarly, serum levels of anti-ssDNA antibodies also increased with the increasing dose and duration of TCE exposure, and anti-ssDNA antibodies in each group treated with TCE for 36 weeks were significantly higher than the respective TCE dose groups exposed for both 12 weeks and 24 weeks (Fig. 3B).

3.5. Correlation of MDA-/HNE-protein adducts, GSH/GSSG ratio and serum ANA

To further evaluate the significance of oxidative stress in TCE-induced autoimmunity, the correlations of the individual serum MDA-/HNE-protein adducts and GSH/GSSG ratio in kidneys with serum ANA were analyzed as shown in Fig. 4. A significant correlation was observed between serum MDA-protein adducts and ANA ($r = 0.774$, $p < 0.01$; Fig. 4A). Similarly, the increases in serum HNE-protein adducts and ANA were highly correlated ($r = 0.789$, $p < 0.01$; Fig. 4B). These results apart from showing an association between LPDA and TCE-induced autoimmunity, also suggest that the serum MDA- and HNE-protein adducts along with autoantibodies (ANA, anti-ssDNA antibodies) may also serve as a

predictive biomarker of severity of TCE-induced/accelerated autoimmunity. Interestingly, there was also a significant negative correlation between the GSH/GSSG ratio in kidneys and serum ANA ($r = -0.788$, $p < 0.01$; Fig. 4C), which is consistent with possible involvement of oxidative stress in the pathogenesis of TCE-mediated autoimmune response.

3.6. Release of IL-17 and IL-21 into splenocyte cultures following treatment with MDA-MSA or HNE-MSA

IL-17 is a potent proinflammatory cytokine produced by activated T cells, particularly Th17 cells and has been associated with pathogenesis of a wide range of inflammatory and autoimmune diseases including SLE and rheumatoid arthritis (RA) (Bettelli et al., 2007; Wong et al., 2008; Korn et al., 2009; Nalbandian et al., 2009). IL-21 is predominantly produced by Th17 cells, acts as an autocrine growth factor for Th17 cells and plays critical roles in autoimmune disease (Herber et al., 2007; Nurieva et al., 2007; Young et al., 2007). To further evaluate the role of LPDA-modified proteins in the pathogenesis of TCE-mediated autoimmunity and provide even more compelling support to our hypothesis that oxidative stress mediates an autoimmune response via activation of T lymphocytes, splenocytes isolated from TCE-treated and control mice were cultured with or without MDA-MSA, HNE-MSA or anti-mouse CD3 and the release of IL-17 and IL-21 in the culture supernatants was determined. Splenocytes from TCE-treated mice (exposed for 24 or 36 weeks) secreted significantly higher levels of IL-17 (Fig. 5) than did splenocytes from untreated control mice following 72h stimulation with MDA-MSA or HNE-MSA, and the response was dose- and time-related. As shown in Fig. 5A, after 12 weeks of TCE exposure, the increases in IL-17 release into cultures were 1.1-, 1.1-, 1.1 and 1.3-fold with HNE-MSA stimulation for 0.0 (TCE control group), 0.5, 1.0 and 2.0 mg/ml of TCE treatment, and 1.3-, 1.3-, 1.5 and 1.8-fold with MDA-MSA stimulation for 0.0, 0.5, 1.0 and 2.0 mg/ml of TCE-treated mice compared to their un-stimulated samples, respectively. The increases following 24 weeks of TCE exposure in IL-17 release into cultures were 1.3, 1.3-, 1.5- and 3.0-fold with HNE-MSA stimulation, and 1.4-, 1.6-, 3.5- and 3.8-fold with MDA-MSA stimulation for 0.0, 0.5, 1.0 and 2.0 mg/ml of TCE-treated mice compared to un-stimulated samples, respectively. More interestingly, after 36 weeks of TCE exposure, the increases in IL-17 release into cultures were 1.3-, 2.4, 2.9- and 3.2-fold with HNE-MSA stimulation, and 2.3-, 5.6-, 6.7- and 6.3-fold with MDA-MSA stimulation for 0.0, 0.5, 1.0 and 2.0 mg/ml of TCE-treated mice compared to un-stimulated samples, respectively. The increases in IL-21 release into the splenocyte cultures with the MDA-MSA or HNE-MSA stimulation also showed a similar pattern (Fig. 6). Especially, a clear dose-related response was observed following 36 week of TCE exposure as evident from increases in IL-21 release into the cultures which were 2.2-, 2.7, 2.8- and 4.5-fold with HNE-MSA stimulation, and 2.9-, 3.6-, 4.1- and 5.5-fold with MDA-MSA stimulation for 0.0, 0.5, 1.0 and 2.0 mg/ml of TCE-treated mice compared to un-stimulated samples, respectively (Fig. 6).

4. Discussion

Implication of TCE exposure in an autoimmune response was reported as early as in 1957 (Reinl, 1957). Since then an increasing number of reports, and epidemiologic as well as experimental studies have provided further evidence for the involvement of TCE in the development of various ADs (Phoon et al., 1984; Haustein and Ziegler, 1985; Lockey et al., 1987; Flindt-Hansen and Isager, 1987; Byers et al., 1988; Kilburn and Warshaw, 1992; Waller et al., 1994; Khan et al., 1995, 2001; Gilbert et al., 1999; Griffin et al., 2000; Wang et al., 2007, 2008, 2009). However, impact of TCE exposure at doses relevant to occupational exposure (Griffin et al., 2000b; Gilbert et al., 2006, 2009; Wang et al., 2007a; Cai et al., 2008) on immune responses and the mechanism(s) contributing to TCE-induced/accelerated autoimmunity remain largely unknown. This dose and time study was, therefore,

performed in female MRL^{+/+} mice by treating them with TCE via drinking water at doses of 0.5, 1.0 or 2.0 mg/ml for 12, 24 or 36 weeks. Our data show a dose-and time-related association among TCE exposure, oxidative stress and induction of autoimmunity. Our study also suggests that MDA- and/or HNE-modified proteins contribute to an autoimmune response by acting as an immunologic trigger through activation of Th17 cells.

The association of non-enzymatic oxidative modification of proteins with ADs has drawn increasing attention (Khan et al., 2001; Sander et al., 2004; Kurien and Scofield, 2003, 2008; Kurien et al., 2006; Wang et al., 2008, 2009, 2010). However, little is known about the potential of post-translational modification of proteins in the development of autoimmunity. Previously, we have shown an association of oxidative/nitrosative stress and SLE (Wang et al., 2010). We have also reported a correlation between the oxidative stress and TCE-induced autoimmune response at a relatively high dose of TCE (Khan et al., 2001; Wang et al., 2007, 2008, 2009). The current study was particularly focused on evaluating the progression of an immune response with increasing TCE doses and duration of exposure. Furthermore, we also evaluated if MDA- and HNE-protein adducts act as immunologic trigger in TCE-mediated autoimmunity. It is evident from our data that TCE exposure leads to increased formation of LPDA-modified protein adducts in a dose- and time-related manner. Interestingly, the serum levels of anti-MDA-/anti-HNE-protein adduct antibodies, and the number and percentage of serum samples positive for anti-MDA-/HNE-protein adduct antibodies also showed an increasing pattern with increasing doses or duration of TCE exposure. These findings provide support to our contention that LPDA-protein adducts are immunogenic and may act as neo-autoantigens in initiating an autoimmune response (Uchida, 2007; Kurien and Scofield, 2008; Otaki et al., 2010).

Glutathione is a ubiquitous molecule virtually produced in all organs and the principal non-protein thiol component of the antioxidant defence system in the living cells (Pastore et al., 2003; Pompella et al., 2003; Ballatori et al., 2009). The main function of glutathione is to protect the cellular constituents from the damaging effects of hydroperoxides formed during normal metabolism (Pastore et al., 2003; Jones, 2006; Kemp et al., 2008; Ballatori et al., 2009). Glutathione is also involved in the formation and maintenance of molecules, enzymes and proteins by catalyzing transhydrogenation of sulphhydryl groups. GSH/GSSG ratio also serves as an important indicator of redox status (Jones, 2006; Kemp et al., 2008; Ballatori et al., 2009; Owen and Butterfield, 2010). To further understand the redox state following TCE exposure, the levels of GSH were evaluated in the kidney, one of major TCE target organs (Cojocel et al., 1989; Lash et al., 2000; Tabrez and Ahmad, 2011). As evident from the results, TCE exposure led to dose- and time-related decreases in GSH and GSH/GSSG ratios in the kidneys. However, alterations in GSH/GSSG ratios were more remarkable, and even significant after 12 weeks of TCE exposure at the dose of 2.0 mg/ml. Thus, significant decreases in GSH, particularly the dose- and time-related decreases in GSH/GSSG ratio provide support to the likely cause of increased oxidative stress following TCE exposure and contribution to TCE-mediated autoimmunity.

Both human and experimental studies have documented that TCE exposure is associated with induction of various autoantibodies (Flindt-Hansen and Isager, 1987; Kilburn and Warshaw, 1992; Khan et al., 1995; Griffin et al., 2000a). The specific findings of the increases in serum ANA and anti-ssDNA antibodies following TCE exposure in this dose- and time-response study not only support our earlier findings that TCE is capable of inducing autoimmune response in MRL^{+/+} mice (Khan et al. 1995), but also suggest that dose and duration of TCE exposure could also influence the production of autoantibodies. It was interesting to note that TCE exposure induced oxidative stress in dose- and time-related manner and autoimmunity was triggered when TCE exposure yielded enough LPDAs to form neoantigens. Remarkably, patterns of serum autoantibodies appear to be similar to the

patterns of LPDA-protein adducts and their antibodies, suggesting a close association among them. Thus, LPDA-modified proteins may serve as an immunological trigger to promote the generation of autoantibodies (Toyoda et al., 2007; Kurien and Scofield, 2008; Otaki et al., 2010). Further evaluation of relationships of MDA/HNE-protein adducts in sera and GSH/GSSG ratio in kidneys with serum ANA levels showed a significant correlation, thus suggesting a strong association among oxidative stress, formation of LPDA-protein products and autoimmune response. These data thus provide evidence that LPO might contribute to TCE-mediated autoimmune response in MRL +/+ mice.

T cells contribute to the initiation and perpetuation of ADs and seem to be directly involved in the development of related organ pathology. T cells in patients with SLE display altered attributes and have an important role in disease pathogenesis (Stummvoll et al., 2008; Korn et al., 2009; Nalbandian et al., 2009). Activated CD4⁺ T cells differentiate into at least three subgroups, Th1, Th2 and Th17, according to their distinct cytokine secretions and functions. Th17 cells, which produce IL-17, IL-21 and IL-22, appear to be key effector T cells in a variety of human ADs, including SLE, RA, multiple sclerosis (Bettelli et al., 2007; Stummvoll et al., 2008; Korn et al., 2009; Nalbandian et al., 2009). IL-17 and IL-21 are potential proinflammatory cytokines produced by activated T cells, particularly Th17 cells and play important role in disease progression and pathogenesis of various ADs (Herber et al., 2007; Nurieva et al., 2007; Wong et al., 2008). Previous studies have demonstrated an association between oxidative stress and autoimmunity, and increasing evidence indicates the potential roles of LPDA modified endogenous proteins in the ADs including SLE, RA (Khan et al., 2001; Sander et al., 2004; Kurien and Scofield, 2003, 2008; Kurien et al., 2006; Wang et al., 2008, 2009, 2010). However, the mechanism by which LPDA-protein adducts affect the adoptive autoimmune response remains largely unknown. In the current study, we stimulated splenocytes obtained from TCE-treated and control mice in culture with LPDA-protein adducts, MDA-MSA or HNE-MSA, then quantitated the release of IL-17 and IL-21. The release of IL-17 and IL-21 from splenocytes isolated from TCE-treated mice showed a dose- and time-related response and was significantly higher than the release from splenocytes in control mice following 72 h stimulation with MDA-MSA or HNE-MSA. These findings, for the first time, provide an evidence that LPDA-protein adducts might contribute to autoimmunity via activating Th17 cells and suggest that TCE treatment promotes cytokine release (IL-17, IL-21) following activation of T cells by the LPDA-protein adducts. Thus, our findings strongly support the potential of oxidative stress in the pathogenesis of TCE-mediated autoimmunity and provide evidence that the LPDA-modified proteins contribute to autoimmunity via activating T lymphocytes (such as TH17 cells).

In conclusion, this study not only provides additional evidence for the existence of an association between oxidative stress and induction/acceleration of autoimmunity, but also strongly supports the concept that LPDA-modified proteins act as an immunologic trigger by activating T lymphocytes, especially Th17 cells, thus contributing to an autoimmune response. Moreover, our results also suggest that dose and duration of TCE exposure affects the oxidative stress response and autoantibody production. Further studies to unravel the distinct pathways by which oxidative stress contributes to autoimmunity, especially mapping of gene expression, analyzing proteome, blocking/inhibiting specific signal transduction pathways and knocking out/down target genes will also provide critical mechanisms in TCE-induced autoimmunity.

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Abbreviations

TCE	trichloroethene
MDA	malondialdehyde
HNE	4-hydroxynonenal
LPDA	lipid peroxidation-derived aldehydes
MSA	mouse serum albumin
ADs	autoimmune diseases
SLE	systemic lupus erythematosus
RA	rheumatoid arthritis
GSH	glutathione
FBS	fetal bovine serum
RT	room temperature
TBST	Tris-buffered saline Tween 20
BSA	bovine serum albumin
HRP	horseradish peroxidase

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Highlights

TCE exposure led to dose-and time-related increases in MDA-/HNE-protein adducts and their antibodies

Increased MDA-/HNE-adducts were associated with increases in serum autoantibodies

MDA-/HNE-albumin adducts trigger greater release of IL-17 and IL-21 from splenocytes of TCE-treated mice

Results support that MDA-/HNE-modified proteins could contribute to an autoimmune response

Fig. 1.

(A) MDA-protein adducts and (B) HNE-protein adducts in the sera of MRL^{+/+} mice treated with TCE for 12, 24 or 36 weeks. The values are means $\pm$ SD. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. respective TCE dose exposure for 12 or/and 24 weeks; @ $p < 0.05$ vs. lower TCE dose at the same exposure time.

Fig. 2.

(A) Anti-MDA-protein adduct antibodies and (B) anti-HNE-protein adduct antibodies in the sera of MRL^{+/+} mice treated with TCE for 12, 24 or 36 weeks. The values are means $\pm$ SD. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. respective TCE dose exposure for 12 weeks; \$ $p < 0.05$ vs. respective TCE dose exposure for 24 weeks.

Fig. 3. Serum levels of (A) anti-nuclear antibodies and (B) anti-ssDNA antibodies in MRL^{+/+} mice treated with TCE for 12, 24 or 36 weeks. The values are means $\pm$ SD. * $p < 0.05$ vs. controls; + $p < 0.05$ vs. mice treated with 0.5 mg/ml of TCE; # $p < 0.05$ vs. respective TCE dose exposure for 12 weeks; \$ $p < 0.05$ vs. respective TCE dose exposure for 24 weeks.

Fig. 4. Correlation of (A) serum MDA-protein adducts, (B) serum HNE-protein adducts or (C) kidney GSH/GSSG ratio with serum ANA. Spearman's rank correlation was used to calculate correlation coefficient.

Fig. 5. IL-17 release in the culture supernatants of splenocytes from control and TCE-treated mice [(A)12 weeks, (B) 24 weeks and (C) 36 weeks of TCE exposure]. Splenocytes were stimulated with MSA alone, HNE-MSA, MDA-MSA or anti-CD3 antibody for 72 h. US: un-stimulated cells. * $p < 0.05$ vs. US; # $p < 0.05$ vs. stimulated control group; + $p < 0.05$ vs. stimulated lower dose groups (0.5, 1 mg/ml).

Fig. 6. IL-21 release in the culture supernatants of splenocytes from control and TCE-treated mice [(A)12 weeks, (B) 24 weeks and (C) 36 weeks of TCE exposure]. Splenocytes were stimulated with MSA alone, HNE-MSA, MDA-MSA or anti-CD3 antibody for 72 h. US: un-stimulated cells. * $p < 0.05$ vs. US; # $p < 0.05$ vs. stimulated control group; + $p < 0.05$ vs. stimulated lower dose groups (0.5, 1 mg/ml).

Table 1

Number and percentage of anti-MDA-protein antibody positive sera in control and TCE-treated mice

Anti-MDA-protein antibody response*											
		Response		+		++		+++			
	Total No.	No.	%	No.	%	No.	%	No.	%	No.	%
12 weeks											
Controls	6	3	50.0	3	50.0	0	0.0	0	0.0	0	0.0
TCE 0.5 mg/ml	6	4	66.7	4	66.7	0	0.0	0	0.0	0	0.0
TCE 1.0 mg/ml	6	5	83.3	5	83.3	0	0.0	0	0.0	0	0.0
TCE 2.0 mg/ml	6	6	100.0	5	83.3	1	16.7	0	0.0		
24 weeks											
Controls	6	4	66.7	4	66.7	0	0.0	0	0.0	0	0.0
TCE 0.5 mg/ml	6	6	100.0	4	66.7	2	33.3	0	0.0		
TCE 1.0 mg/ml	6	6	100.0	4	66.7	2	33.3	0	0.0		
TCE 2.0 mg/ml	6	6	100.0	1	16.7	4	66.7	1	16.7		
36 weeks											
Controls	6	6	100.0	5	83.3	1	16.7	0	0.0		
TCE 0.5 mg/ml	6	6	100.0	1	16.7	4	66.7	1	16.7		
TCE 1.0 mg/ml	6	6	100.0	1	16.7	3	50.0	2	33.3		
TCE 2.0 mg/ml	6	6	100.0	1	16.7	2	33.3	3	50.0		

* Net OD value greater than 0.2 was considered positive.

+, moderately positive;

++, highly positive;

+++, strongly positive (Khan et al., 2001; Wang et al., 2008)

Table 2

Number and percentage of anti-HNE-protein antibody positive sera in control and TCE-treated mice

Anti-HNE-protein antibody response*											
Total No.		Response		+		++		+++			
		No.	%	No.	%	No.	%	No.	%		
12 weeks											
Controls	6	1	16.7	1	16.7	0	0.0	0	0.0	0	0.0
TCE 0.5 mg/ml	6	1	16.7	1	16.7	0	0.0	0	0.0	0	0.0
TCE 1.0 mg/ml	6	1	16.7	1	16.7	0	0.0	0	0.0	0	0.0
TCE 2.0 mg/ml	6	3	50.0	3	50.0	0	0.0	0	0.0	0	0.0
24 weeks											
Controls	6	2	33.3	2	33.3	0	0.0	0	0.0	0	0.0
TCE 0.5 mg/ml	6	4	66.7	4	66.7	0	0.0	0	0.0	0	0.0
TCE 1.0 mg/ml	6	5	83.3	5	83.3	0	0.0	0	0.0	0	0.0
TCE 2.0 mg/ml	6	5	83.3	4	66.7	1	16.7	0	0.0	0	0.0
36 weeks											
Controls	6	4	66.7	4	66.7	0	0.0	0	0.0	0	0.0
TCE 0.5 mg/ml	6	6	100.0	4	66.7	2	33.3	0	0.0	0	0.0
TCE 1.0 mg/ml	6	6	100.0	3	50.0	3	50.0	0	0.0	0	0.0
TCE 2.0 mg/ml	6	6	100.0	2	33.3	3	50.0	1	16.7	1	16.7

* Net OD value greater than 0.2 was considered positive.

+, moderately positive;

++, highly positive;

+++, strongly positive (Khan et al., 2001; Wang et al., 2008)

Table 3

GSH and GSSG in the kidneys from control and TCE-treated mice

	GSH	GSSG	GSH/GSSG
12 weeks			
Controls	2.068±0.106	0.124±0.009	16.67± 1.198
TCE 0.5 mg/ml	2.079±0.205	0.125±0.016	16.77± 1.549
TCE 1.0 mg/ml	2.024±0.124	0.132±0.014	15.45± 1.725
TCE 2.0 mg/ml	1.868±0.183	0.140±0.018	13.46± 1.522 ^{*+}
24 weeks			
Controls	2.061±0.111	0.130±0.012	16.00± 2.018
TCE 0.5 mg/ml	1.976±0.085	0.136±0.008	14.59± 0.653
TCE 1.0 mg/ml	1.803±0.117	0.143±0.010	12.65± 1.465 ^{*#}
TCE 2.0 mg/ml	1.582±0.178 ^{*+}	0.146±0.016	10.93± 1.536 ^{*+}
36 weeks			
Controls	1.856±0.137	0.129±0.015	12.44± 1.211
TCE 0.5 mg/ml	1.586±0.137 ^{*#}	0.139±0.015	11.39± 0.945 ^{*#}
TCE 1.0 mg/ml	1.214±0.149 ^{*#}	0.151±0.017	8.065± 0.691 ^{*+#}
TCE 2.0 mg/ml	1.033±0.099 ^{*+#}	0.168±0.031 [*]	6.373± 1.587 ^{*+#}

GSH and GSSG values are expressed as nmol/mg protein and means ± SD.

^{*} p < 0.05 vs. controls;⁺ p < 0.05 vs. 0.5 mg/ml of TCE-treated group;[#] p < 0.05 vs. respective group treated with TCE for 12 or/and 24 weeks.