Published in final edited form as:

Toxicol Appl Pharmacol. 2013 November 15; 273(1): 189–195. doi:10.1016/j.taap.2013.08.020.

N-Acetylcysteine protects against trichloroethene-mediated autoimmunity by attenuating oxidative stress

Gangduo Wang, Jianling Wang, Huaxian Ma, G.A.S. Ansari, and M. Firoze Khan*

Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA

Abstract

Exposure to trichloroethene (TCE), a ubiquitous environmental contaminant, is known to induce autoimmunity both in humans and animal models. However, mechanisms underlying TCE-mediated autoimmunity remain largely unknown. Previous studies from our laboratory in MRL+/+ mice suggest that oxidative stress may contribute to TCE-induced autoimmune response. The current study was undertaken to further assess the role of oxidative stress in TCE-induced autoimmunity by supplementing with an antioxidant N-acetylcysteine (NAC). Groups of female MRL+/+ mice were given TCE, NAC or TCE + NAC for 6 weeks (TCE, 10 mmol/kg, i.p., every 4th day; NAC, 250 mg/kg/day through drinking water). TCE exposure led to significant increases in serum levels of anti-nuclear, anti-dsDNA and anti-Sm antibodies. TCE exposure also led to significant induction of anti-malondialdehyde (MDA)- and anti-hydroxynonenal (HNE)-protein adduct antibodies which were associated with increased ANA in the sera along with increased MDA-/HNE-protein adducts in the livers and kidneys, and increases in protein oxidation (carbonylation) in the sera, livers and kidneys, suggesting an overall increase in oxidative stress. Moreover, TCE exposure also resulted in increased release of IL-17 from splenocytes and increases in IL-17 mRNA expression. Remarkably, NAC supplementation attenuated not only the TCE-induced oxidative stress, IL-17 release and mRNA expression, but also the markers of autoimmunity, as evident from decreased levels of ANA, anti-dsDNA and anti-Sm antibodies in the sera. These results provide further support to a role of oxidative stress in TCE-induced autoimmune response. Attenuation of TCE-induced autoimmunity in mice by NAC provides an approach for preventive and/or therapeutic strategies.

Keywords

Trichloroethene; N-Acetylcysteine; Oxidative stress; Carbonylation; Autoimmune diseases

Introduction

Autoimmune diseases (ADs) such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are chronic and life-threatening disorders. The etiology of these diseases is largely unknown, but increasing epidemiologic and experimental studies support a potential

*Corresponding author at: Department of Pathology, University of Texas Medical Branch, 2.319 Mary Moody Northen, Galveston, TX 77555-0438, USA. Fax: +1 409 765 5938. mfkhan@utmb.edu (M.F. Khan).

Conflict of interest

The authors declare no competing financial interests.

role of environmental factors including chemical exposure in the pathogenesis of such diseases (Cooper et al., 2009; Farhat et al., 2011; Gilbert et al., 2009; Khan et al., 1995; Kilburn and Warshaw, 1992; Parks and Cooper, 2006). Trichloroethene (TCE), a widely used industrial solvent, especially in metal degreasing operation, is a ubiquitous environmental pollutant. The involvement of TCE in the development of autoimmune disorders including SLE, systemic sclerosis and fasciitis has been well documented in both human and animal studies (Cai et al., 2008; Cooper et al., 2009; Flindt-Hansen and Isager, 1987; Griffin et al., 2000a; Khan et al., 1995, 2001; Kilburn and Warshaw, 1992; Wang et al., 2007a, 2007b, 2008b, 2012). However, mechanisms by which TCE induces/accelerates an autoimmune response remain largely unknown.

Reactive oxygen species (ROS), such as the superoxide anion and hydroxyl radicals, have been implicated in the pathogenesis of ADs via dysregulation of immune function, autoantigen production through oxidative modification and induction of autoantibody formation (Khan et al., 2001; Kurien and Scofield, 2008; Oates, 2010). Proteins perform vital functions within living cells, but even a relatively minor structural modification of proteins often leads to a marked change (generally lowering) in their activities (Orengo et al., 1999). A variety of ROS-mediated modifications of proteins have been reported in various diseases (Ben Mansour et al., 2010; Kurien and Scofield, 2008; Morgan et al., 2005). Increasing evidence suggests that those ROS-modified proteins such as protein carbonyls and lipid peroxidation-derived aldehydes [LPDAs, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE)]-protein adducts may elicit an autoimmune response and contribute to disease pathogenesis (Ben Mansour et al., 2010; Januszewski et al., 2005; Wang et al., 2010). Indeed higher levels of MDA-/HNE-modified proteins and protein carbonyls have been observed in AD patients (Ben Mansour et al., 2010; Frostegard et al., 2005; Grune et al., 1997; Kurien and Scofield, 2008; Wang et al., 2010), suggesting a potential role for these oxidatively modified proteins in ADs.

Even though TCE is known to generate free radicals, causes increased oxidative stress and induces autoimmune response (Channel et al., 1998; Khan et al., 2001; Wang et al., 2007a, 2007b, 2008b, 2012; Zhu et al., 2005), potential mechanisms by which TCE induced ROS generation lead to an autoimmune response and their contribution to disease pathogenesis remains largely unknown. To further establish the role of oxidative stress in the pathogenesis of TCE-induced ADs, we assessed the autoimmune response along with oxidative stress alterations in an animal model by supplementing N-acetylcysteine (NAC), a precursor of intracellular glutathione which provides a major cellular defense against oxidative stress. Groups of female MRL+/+ mice were treated with TCE or TCE along with NAC, and the markers of oxidative stress and their association with autoimmune response were evaluated in this study. Our data show that NAC supplementation leads to attenuation of TCE-induced autoimmune response in mice, thus, providing further evidence for a role of oxidative stress in TCE-induced autoimmunity.

Materials and methods

Animals and treatments

Five-week old female MRL+/+ mice (23–26 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in plastic cages on a bedding of wood chips at the UTMB animal house facility maintained at ~22 °C, 50–60% relative humidity, and a 12 h light/dark cycle. The animals were provided with standard lab chow and drinking water ad libitum and were acclimated for 1 week prior to the treatment. The experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. The mice, divided into 4 groups of 6 animals each, were treated with TCE, NAC or TCE + NAC [TCE, 10 mmol/kg in corn oil, i.p., every 4th day (Khan et al., 1995, 2001; Wang et al., 2007b, 2009); NAC, 250 mg/kg/day through drinking water (Suwannaroj et al., 2001)]. The control mice received an equal volume of corn oil only. After 6 weeks of TCE, NAC or TCE + NAC treatments, the animals were euthanized under nembutal (sodium pentobarbital) anesthesia, and blood was withdrawn from the inferior vena cava. Sera from mice, obtained following blood clotting and centrifugation, were stored in small aliquots at –80 °C until further analysis. At the same time, major organs were immediately removed and weighed. Portions of livers and kidneys from control and TCE-treated mice were snap-frozen in liquid nitrogen and stored at –80 °C for the further analysis.

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., 2008, 2010, 2012). 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).

Quantitation of MDA- and HNE-protein adducts in the livers and kidneys

Quantitative competitive ELISAs for MDA- and HNE-protein adducts in the liver and kidney homogenates of control and TCE-treated mice were performed according to Wang et al. (2007b, 2012). 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 (1:2000 diluted anti-MDA or 1:3000 diluted anti-HNE) were incubated with test samples (standards or unknown) at 4 °C overnight. The coated plates were blocked with a blocking buffer (50 mM Tris-buffered saline with 1% BSA, pH 8.0; Bio-Rad Laboratories) 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-HRP (1:2000 dilution, 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).

Determination of protein carbonyls in the sera, livers and kidneys

Carbonyl content in the mouse serum was quantitated by the protein carbonyl assay (Lushchak et al., 2005; Wang et al., 2009) with slight modification. Briefly, 100 μ l of mouse serum was incubated with 400 μ l of 10 mM 2,4-dinitrophenylhydrazine (DNPH) or 400 μ l of 2.5 M HCl. After 1 h incubation at RT in dark, 0.5 ml of 20% trichloroacetic acid (TCA) was added, and then washed with 0.5 ml 10% TCA, followed by 0.5 ml mixture of ethanol/ethyl acetate (1:1). The protein pellets were dissolved in 250 μ l of 6 M guanidine hydrochloride and centrifuged at 10,000 g for 10 min at 4 °C. The OD of the supernatants was read at 370 nm on a Bio-Rad Benchmark Plus microplate spectrophotometer. Protein carbonyls were calculated using a molar extinction coefficient of 22,000 M⁻¹ cm⁻¹. The liver and kidney homogenates (10%, w/v) were made in phosphate-buffered saline (PBS, pH 7.4) containing protease inhibitor cocktail (Sigma). The homogenates were centrifuged at 10,000 g at 4 °C for 15 min and the protein carbonyls in the supernatants analyzed by the method described above for serum.

Anti-nuclear, anti-ssDNA and anti-Sm antibodies in the serum

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

Isolation of splenocytes and determination of IL-17 release in splenocyte cultures

Splenocytes were prepared as described in earlier studies (Wang et al., 2009, 2012). The cell pellet was suspended in RPMI 1640 medium supplemented with 2 mM glutamine, 50 μ g/ml gentamycin and 10% heat-inactivated FBS, and splenocytes were counted. The splenocytes were plated in 24-well plates at a density of 2×10^6 /ml/well. ConA (5 μ g/ml, Sigma) or anti-mouse CD3/CD28 (2.5 μ g/ml/1 μ g/ml, BD Biosciences) was added to stimulate lymphocytes in the culture and incubated at 37 °C with 5% CO₂. After 72 h, culture supernatants from each well were harvested and the release of IL-17 into the cultures was quantitated using specific ELISA kits (Biosource, Camarillo, CA; Wang et al., 2012).

RNA isolation and real-time PCR analysis for IL-17 gene expression in spleen

RNA was isolated from the spleen tissue using RiboPure kit (Ambion, Austin, TX) as described in our earlier studies (Wang et al., 2009). The real-time PCR was performed as described earlier (Wang et al., 2009). Briefly, cDNA was prepared from isolated RNA by using SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) described earlier (Wang et al., 2009). Quantitative real-time PCR employing a two-step cycling protocol (denaturation and annealing/extension) was carried out using the primers (forward 5'-CCCTCT GTGATCTGGGAAGC and reverse 5'-TCTCAGGCTCCCTCTTCAGG) by the Smart Cycler System. For each cDNA sample, parallel reactions were performed in triplicate for the detection of mouse IL-17 and 18S. Amplification conditions were identical for all reactions: 95 °C for 2 min for template denaturation and hot start prior to PCR cycling. A typical cycling protocol consisted of three stages: 15 s at 95 °C for denaturation, 30 s at 65 °C for annealing, 30 s at 72 °C for extension, and an additional 6 s hold for fluorescent

signal acquisition. To avoid the non-specific signal from primer-dimers, the fluorescence signal was detected 2 °C below the melting temperature (T_m) of individual amplicon and above the T_m of the primer-dimers (Wang et al., 2009). A total of 45 cycles were performed for the studies.

Quantitation of PCR was done using the comparative C_T method as described in User Bulletin No. 2 of Applied Biosystems (Foster City, CA), and reported as fold difference relative to the calibrator cDNA (QuantumRNA Universal 18S Standards, Ambion). The fold change in iNOS cDNA (target gene) relative to the 18S endogenous control was determined by:

$$\text{Fold change} = 2^{-\Delta\Delta C_T}, \text{ where } \Delta\Delta C_T = (C_{T_{TCE}} - C_{T_{18S}}) - (C_{T_{CONTROL}} - C_{T_{18S}}).$$

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 statistical analysis. Spearman's rank correlation was used to calculate correlation coefficients between serum anti-MDA/HNE-protein adduct antibodies and ANA. A p value $\leq$ 0.05 was considered as statistically significant.

Results

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

TCE has been shown to generate free radicals and induce oxidative stress both in vivo and in vitro (Channel et al., 1998; Khan et al., 2001; Wang et al., 2007a, 2007b, 2008b, 2009b, 2012; Zhu et al., 2005). To further assess the role of TCE-induced lipid peroxidation in the induction/exacerbation of an autoimmune response, the serum levels of anti-MDA/HNE-protein adduct antibodies in mice treated with TCE, NAC or TCE + NAC were determined (Fig. 1). As evident from Fig. 1A, the levels of anti-MDA-protein adduct antibodies in the mice treated with TCE for 6 weeks were significantly increased compared to those of the controls. Moreover, the number and percentage of samples positive (+), highly positive (++) and strongly positive (+++) for these antibodies were also higher in the TCE-treated mice compared to those of the controls or TCE + NAC treated mice (data not shown). Interestingly, NAC supplementation significantly attenuated the increases in these antibodies as evident from significantly reduced level of anti-MDA-protein adduct antibodies in mice treated with TCE + NAC as compared to that of TCE only group.

Serum levels of anti-HNE-protein adduct antibodies, and the number and percentage of serum samples positive for these antibodies in TCE-treated mice showed a pattern similar to those of anti-MDA-protein adduct antibodies. The increases in these antibodies in TCE-treated mice were also attenuated by NAC supplementation (Fig. 1B).

MDA- and HNE-protein adducts in the livers and kidneys

Since NAC attenuated the TCE-induced increases in anti-MDA/HNE-protein adduct antibodies in the sera of MRL+/+ mice, it was of interest to obtain further evidence for the

involvement of LPDA-protein adducts in TCE-mediated autoimmunity. Therefore, formation of these adducts in the liver and kidney homogenates of control, TCE, NAC and TCE + NAC-treated mice was also quantitated. TCE treatment in MRL^{+/+} mice led to significantly increased formation of MDA-protein adducts in the livers and kidneys in comparison to the controls. The increases in these adducts were also attenuated by NAC treatment (Fig. 2). Similarly, the HNE-protein adducts were also significantly higher in the livers and kidneys of TCE-treated mice in comparison to the controls, and their formation was attenuated by NAC supplementation.

Protein carbonyl content in the sera, livers and kidneys of MRL^{+/+} mice

Protein carbonyl content is not only a biomarker of oxidative stress, but also provides evidence of oxidative protein damage (Morgan et al., 2005; Renke et al., 2000). To assess the status of protein oxidation (protein carbonyl) in TCE-induced autoimmune response and to determine if NAC supplementation also provides protection, serum level of protein carbonyls in the controls, TCE alone, NAC alone and TCE + NAC-treated mice was determined. Our data show that carbonyl content in the sera was significantly increased in TCE-treated mice compared to that in controls, and the increase was attenuated by NAC supplementation, as shown in Fig. 3A. To evaluate the extent of protein oxidation in livers and kidneys, protein carbonyls in these major organs, which are targets of TCE, were also analyzed. As evident from Figs. 3B and C, similar pattern of alteration in protein carbonyl content, as seen in the sera, was also observed in both livers and kidneys of mice treated with TCE or TCE + NAC, suggesting increased protein oxidation (carbonylation) as a result of TCE exposure and the protective effect of NAC against TCE-induced protein carbonylation.

Serum autoantibodies in MRL^{+/+} mice

Autoantibodies, such as ANA, anti-dsDNA and anti-SM antibodies, are considered important laboratory evaluation indices and biomarkers of ADs (Egner, 2000; Ippolito et al., 2011; Reveille, 2004). These autoantibodies were analyzed in the serum of MRL^{+/+} mice treated with TCE alone, NAC alone and TCE + NAC for 6 weeks (Fig. 4). In comparison to controls, there were significant increases in serum ANA levels in mice treated with TCE, which were significantly attenuated following NAC supplementation, evidenced from data in mice treated with TCE + NAC (Fig. 4A). Similarly, serum levels of anti-dsDNA and anti-SM antibodies also increased with TCE exposure, and the increases were attenuated by NAC supplementation (Figs. 4B, C), suggesting that NAC supplementation protects against TCE-induced autoimmunity in these mice. To further assess the role of oxidative stress in TCE-induced autoimmune response, we analyzed the association between lipid peroxidation and autoimmunity. As evident in Fig. 5, both serum anti-MDA-protein antibodies (Fig. 5A) and anti-HNE-protein antibodies (Fig. 5B) showed a good correlation with ANA, suggesting that improved TCE-mediated autoimmune response following NAC supplementation could be via averting the oxidative stress.

Effect of NAC on TCE-induced release of IL-17 into splenocyte cultures

IL-17 is a potent pro-inflammatory 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 RA (Miossec and Kolls, 2012; Nalbandian et al., 2009; Rodeghero et al., 2013). To investigate the impact of NAC supplementation on TCE-mediated IL-17 release, splenocytes isolated from control, TCE alone, NAC alone or TCE + NAC-treated mice were cultured with or without ConA or anti-mouse CD3/CD28, and the release of IL-17 into the culture supernatants was determined. The levels of IL-17 secreted by the splenocytes from TCE-treated mice were not only significantly higher than those from untreated control mice, but also remarkably higher than those from NAC alone or TCE + NAC-treated mice following 72 h stimulation with ConA or CD3/CD28 (Fig. 6). Interestingly, the splenocytes from TCE-treated mice even without stimulation with ConA or CD3/CD28 secreted more IL-17 compared to the unstimulated splenocytes from control, NAC alone and TCE + NAC-treated mice (Fig. 6).

IL-17 mRNA expression in the spleens

To further evaluate the impact of TCE and TCE + NAC exposure on IL-17 expression and thus, contribution to autoimmunity, the IL-17 mRNA was analyzed in the spleens of various treatment groups using real-time PCR and the results are shown in Fig. 7. TCE treatment led to significantly increased (1.9 fold) IL-17 mRNA levels, but the increases were attenuated by the NAC supplementation. The changes in mRNA expression, followed a pattern similar to IL-17 protein pattern evaluated in the splenocyte culture supernatants as determined by IL-17-specific ELISA kit (Fig. 6).

Discussion

TCE, a widely used industrial agent and a ubiquitous environmental contaminant, has been implicated in the development of autoimmune disorders in humans (Cooper et al., 2009; Flindt-Hansen and Isager, 1987; Kilburn and Warshaw, 1992) and induces autoimmune response in experimental animals (Cooper et al., 2009; Griffin et al., 2000a; Khan et al., 1995, 2001; Wang et al., 2007a, 2007b, 2009b, 2012). However, the mechanisms by which TCE induces/accelerates the pathogenesis of ADs have not been clearly elucidated. In recent years, free radical-mediated reactions as the potential mechanism in the pathogenesis of ADs have drawn increasing attention (Khan et al., 2001; Kurien and Scofield, 2008; Oates, 2010). TCE is known to generate free radicals, and causes increased lipid peroxidation both in vivo and in vitro (Channel et al., 1998; Khan et al., 2001; Wang et al., 2007a, 2007b, 2008b, 2009b, 2012; Zhu et al., 2005). Several lines of evidence in lupus-prone mice showed that increased ROS generation was associated with increased formation of autoantibodies, suggesting a potential role of oxidative stress in TCE-induced autoimmune response (Khan et al., 2001; Wang et al., 2007a, 2007b, 2008b, 2009b, 2012). To support these findings and provide new mechanistic evidence for the role of oxidative stress in the pathogenesis of ADs, especially TCE-mediated autoimmune response, we treated groups of female MRL^{+/+} mice with TCE alone, NAC alone or TCE along with NAC, and evaluated the markers of oxidative stress for their association with the markers of autoimmune response.

NAC is a cell-permeable antioxidant that serves as glutathione precursor through the elevation of intracellular cysteine stores (Amrouche-Mekkioui and Djerdjouri, 2012; Lai et al., 2012; Zafarullah et al., 2003). It improves oxidative stress by directly scavenging ROS

(Amrouche-Mekkioui and Djerdjouri, 2012; Benrahmoune et al., 2000; Samuni et al., 2013; Zafarullah et al., 2003) and modulates inflammatory responses through signaling pathways that control pro-inflammatory NF- κ B activation (Amrouche-Mekkioui and Djerdjouri, 2012; Andresen et al., 2005; Hutter and Greene, 2000). Previous studies suggest that NAC improves redox status via decreasing oxidative stress both in humans and animals, and can even reduce disease activity in SLE patients (Amrouche-Mekkioui and Djerdjouri, 2012; Lai et al., 2012; Niwano et al., 2011; Nur et al., 2012). Therefore, to further assess the role of oxidative stress in TCE-induced autoimmune response, we first examined the markers of oxidative stress in TCE-treated mice with or without NAC supplementation. MRL+/+ mice treated with TCE for 6 weeks in this study, showed greater formation of anti-MDA- and anti-HNE-protein adduct antibodies in their sera in comparison to untreated controls. TCE treatment also led to increased formation of MDA- and HNE-protein adducts in both livers and kidneys, two major organs where TCE is known to generate free radicals and induce autoimmune disorders (Atkinson et al., 1993; Cai et al., 2008; Channel et al., 1998; Cojocel et al., 1989; Griffin et al., 2000b), further confirming earlier findings of the potential of TCE in inducing increased lipid peroxidation. Interestingly, NAC supplementation attenuated the formation of these MDA-/HNE-protein adducts and their respective antibodies induced by TCE exposure, suggesting that NAC has the potential to improve or avert TCE-induced oxidative stress.

Protein carbonyl content is one of the most widely used biomarkers of protein oxidation, and increased protein carbonylation is also associated with SLE disease activity and other ADs (Morgan et al., 2005; Renke et al., 2000, 2007). To further evaluate that oxidative modification of proteins resulting in generation of neoantigens might be a potential mechanism in TCE-induced autoimmunity, protein carbonyl levels were measured in the sera, livers and kidneys of TCE-treated MRL+/+ mice. It was interesting to note that TCE treatment led to significant increases in protein carbonyl levels in the sera, livers and kidneys, and these increases were also attenuated following NAC treatment. These results provide further support to our earlier findings that TCE is capable of inducing carbonylation of proteins in MRL+/+ mice (Khan et al., 2001; Wang et al., 2007a). More importantly, this study also provides evidence that increased formation of ROS-modified proteins could be averted by antioxidants, such as NAC supplementation.

Autoantibodies, such as ANA, anti-dsDNA and anti-SM antibodies, serve as indices and biomarkers of ADs (Egner, 2000; Reveille, 2004). It was especially important to assess whether NAC, which attenuated TCE-generated oxidative stress, also protected and/or averted TCE-induced autoimmune response. Our results show that TCE exposure led to remarkably increased levels of ANA, anti-dsDNA and anti-SM antibodies in the serum of MRL+/+ mice, and NAC supplementation indeed attenuated the formation of these autoantibodies. Furthermore, our data showed a good relationship between both serum anti-MDA- and anti-HNE-protein adduct antibodies and ANA. These findings not only support conclusions of previous studies that TCE exposure induces autoimmune response (Cai et al., 2008; Griffin et al., 2000a, 2000b; Khan et al., 1995; Wang et al., 2007a, 2007b, 2009b, 2012), but also support contribution of oxidative stress in TCE-induced autoimmunity.

It is well-known that T cells contribute to the initiation and perpetuation of ADs and seem to be directly involved in the development of related organ pathology (Chen et al., 2012; Miossec and Kolls, 2012; Nalbandian et al., 2009; Rodeghero et al., 2013). Th17 cells, a novel distinct subset of Th cell, have been implicated in the pathogenesis of ADs, including SLE and RA (Chen et al., 2012; Miossec and Kolls, 2012; Rodeghero et al., 2013). IL-17, a potential pro-inflammatory cytokine mainly produced by Th17 cells, plays important role in disease progression and pathogenesis of various ADs (Chen et al., 2012; Miossec and Kolls, 2012; Nalbandian et al., 2009). To provide mechanistic evaluation of the autoimmune response following TCE exposure and potential involvement of Th17 cells, the IL-17 release and IL-17 mRNA expression from TCE or TCE + NAC-treated mice were determined. TCE treatment significantly increased both IL-17 release and IL-17 mRNA expression. Interestingly, NAC supplementation remarkably attenuated these increases. These findings further support the previous idea that TCE-mediated autoimmune response might involve Th17 cell activation (Wang et al., 2012), and also provide evidence for an association among oxidative stress, Th17 cell activation and TCE-induced autoimmunity. However, more studies are needed to evaluate the contribution of specific cell types and the role of IL-17 in TCE-mediated autoimmunity.

Taken together, the results of this study show that TCE exposure leads to significant induction of anti-MDA- and anti-HNE-protein adduct antibodies, increased formation of MDA-/HNE-protein adducts in the livers and kidneys, and increases in protein oxidation (carbonylation) in the sera, livers and kidneys, suggesting the potential of TCE to induce an overall increase in oxidative stress. The responses to oxidative modifications, especially formation of anti-MDA and anti-HNE-protein antibodies were associated with increases in the serum levels of ANA, anti-dsDNA- and anti-SM-antibodies. Moreover, TCE exposure also resulted in an increased release of IL-17 from splenocytes and increases in IL-17 mRNA expression. Interestingly, NAC supplementation attenuated not only the TCE-induced oxidative stress, IL-17 release and its mRNA expression, but also the markers of autoimmune response, as evident from decreased levels of autoantibodies in the sera. These findings have led us to outline the potential pathways of TCE-induced autoimmunity and its attenuation by NAC supplementation (Fig. 8). Our results clearly support the role of oxidative stress in TCE-induced autoimmune response. Attenuation of TCE-induced autoimmune response in mice by NAC could be important in developing preventive and/or therapeutic strategies.

Acknowledgments

This work was supported by Grant ES016302 from the National Institute of Environmental Health Sciences (NIEHS), NIH, and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

References

- Amrouche-Mekkioui I, Djerdjouri B. N-acetylcysteine improves redox status, mitochondrial dysfunction, mucin-depleted crypts and epithelial hyperplasia in dextran sulfate sodium-induced oxidative colitis in mice. *Eur J Pharmacol.* 2012; 691:209–217. [PubMed: 22732651]

- Andresen L, Jørgensen VL, Perner A, Hansen A, Eugen-Olsen J, Rask-Madsen J. Activation of nuclear factor kappaB in colonic mucosa from patients with collagenous and ulcerative colitis. *Gut*. 2005; 54:503–509. [PubMed: 15753535]
- Atkinson A, Meeks RG, Roy D. Increased oxidative stress in the liver of mice treated with trichloroethylene. *Biochem Mol Biol Int*. 1993; 31:297–304. [PubMed: 8275017]
- Ben Mansour R, Lassoued S, Elgaied A, Haddouk S, Marzouk S, Bahloul Z, Masmoudi H, Attia H, Aïfa MS, Fakhfakh F. Enhanced reactivity to malondialdehyde-modified proteins by systemic lupus erythematosus autoantibodies. *Scand J Rheumatol*. 2010; 39:247–253. [PubMed: 20429675]
- Benrahmoune M, Therond P, Abedinzadeh Z. The reaction of superoxide radical with N-acetylcysteine. *Free Radic Biol Med*. 2000; 29:775–782. [PubMed: 11053779]
- Cai P, König R, Boor PJ, Kondraganti S, Kaphalia BS, Khan MF, Ansari GAS. Chronic exposure to trichloroethene causes early onset of SLE-like disease in female MRL+/+ mice. *Toxicol Appl Pharmacol*. 2008; 228:68–75. [PubMed: 18234256]
- Channel SR, Latendresse JR, Kidney JK, Grabau JH, Lane JW, Steel-Goodwin L, Gothaus MC. A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male B6C3F1 mouse liver. *Toxicol Sci*. 1998; 43:145–154. [PubMed: 9710956]
- Chen DY, Chen YM, Wen MC, Hsieh TY, Hung WT, Lan JL. The potential role of Th17 cells and Th17-related cytokines in the pathogenesis of lupus nephritis. *Lupus*. 2012; 21:1385–1396. [PubMed: 22892208]
- Cojocel C, Beuter W, Müller W, Mayer D. Lipid peroxidation: a possible mechanism of trichloroethylene-induced nephrotoxicity. *Toxicology*. 1989; 55:131–141. [PubMed: 2711399]
- Cooper GS, Makris SL, Nietert PJ, Jinot J. Evidence of autoimmune-related effects of trichloroethylene exposure from studies in mice and humans. *Environ Health Perspect*. 2009; 117:696–702. [PubMed: 19479009]
- Egner W. The use of laboratory tests in the diagnosis of SLE. *J Clin Pathol*. 2000; 53:424–432. [PubMed: 10911799]
- Farhat SC, Silva CA, Orione MA, Campos LM, Sallum AM, Braga AL. Air pollution in autoimmune rheumatic diseases: a review. *Autoimmun Rev*. 2011; 11:14–21. [PubMed: 21763467]
- Flindt-Hansen H, Isager H. Scleroderma after occupational exposure to trichloroethylene and trichloroethane. *Acta Derm Venereol*. 1987; 67:263–264. [PubMed: 2442944]
- Frostegard J, Svenungsson E, Wu R, Gunnarsson I, Lundberg IE, Klareskog L, Horkko S, Witztum JL. Lipid peroxidation is enhanced in patients with systemic lupus erythematosus and is associated with arterial and renal disease manifestations. *Arthritis Rheum*. 2005; 52:192–200. [PubMed: 15641060]
- Gilbert KM, Przybyla B, Pumford NR, Han T, Fuscoe J, Schnackenberg LK, Holland RD, Doss JC, Macmillan-Crow LA, Blossom SJ. Delineating liver events in trichloroethylene-induced autoimmune hepatitis. *Chem Res Toxicol*. 2009; 22:626–632. [PubMed: 19254012]
- Griffin JM, Blossom SJ, Jackson SK, Gilbert KM, Pumford NR. Trichloroethylene accelerates an autoimmune response by Th1 T cell activation in MRL+/+ mice. *Immunopharmacology*. 2000a; 46:123–137. [PubMed: 10647871]
- Griffin JM, Gilbert KM, Lamps LW, Pumford NR. CD4(+) T-cell activation and induction of autoimmune hepatitis following trichloroethylene treatment in MRL+/+ mice. *Toxicol Sci*. 2000b; 57:345–352. [PubMed: 11006364]
- Grune T, Michel P, Sitte N, Eggert W, Albrecht-Nebe H, Esterbauer H, Siems WG. Increased levels of 4-hydroxynonenal modified proteins in plasma of children with autoimmune diseases. *Free Radic Biol Med*. 1997; 23:357–360. [PubMed: 9214570]
- Hutter D, Greene JJ. Influence of the cellular redox state on NF-kappaB-regulated gene expression. *J Cell Physiol*. 2000; 183:45–52. [PubMed: 10699965]
- Ippolito A, Wallace DJ, Gladman D, Fortin PR, Urowitz M, Werth V, Costner M, Gordon C, Alarcón GS, Ramsey-Goldman R, Maddison P, Clarke A, Bernatsky S, Manzi S, Bae SC, Merrill JT, Ginzler E, Hanly JG, Nived O, Sturfelt G, Sanchez-Guerrero J, Bruce I, Aranow C, Isenberg D, Zoma A, Magder LS, Buyon J, Kalunian K, Dooley MA, Steinsson K, van Vollenhoven RF, Stoll T, Weisman M, Petri M. Autoantibodies in systemic lupus erythematosus: comparison of historical and current assessment of seropositivity. *Lupus*. 2011; 20:250–255. [PubMed: 21362750]

- Januszewski AS, Alderson NL, Jenkins AJ, Thorpe SR, Baynes JW. Chemical modification of proteins during peroxidation of phospholipids. *J Lipid Res.* 2005; 46:1440–1449. [PubMed: 15805546]
- Khan MF, Kaphalia BS, Prabhakar BS, Kanz MF, Ansari GA. Trichloroethene-induced autoimmune response in female MRL+/+ mice. *Toxicol Appl Pharmacol.* 1995; 134:155–160. [PubMed: 7676450]
- Khan MF, Wu X, Ansari GA. Anti-malondialdehyde antibodies in MRL+/+ mice treated with trichloroethene and dichloroacetyl chloride: possible role of lipid peroxidation in autoimmunity. *Toxicol Appl Pharmacol.* 2001; 170:88–92. [PubMed: 11162772]
- Kilburn KH, Warshaw RH. Prevalence of symptoms of systemic lupus erythematosus (SLE) and of fluorescent antinuclear antibodies associated with chronic exposure to trichloroethylene and other chemicals in well water. *Environ Res.* 1992; 57:1–9. [PubMed: 1740091]
- Kurien BT, Scofield RH. Autoimmunity and oxidatively modified autoantigens. *Autoimmun Rev.* 2008; 7:567–573. [PubMed: 18625446]
- Lai ZW, Hanczko R, Bonilla E, Caza TN, Clair B, Bartos A, Miklossy G, Jimah J, Doherty E, Tily H, Francis L, Garcia R, Dawood M, Yu J, Ramos I, Coman I, Faraone SV, Phillips PE, Perl A. N-acetylcysteine reduces disease activity by blocking mammalian target of rapamycin in T cells from systemic lupus erythematosus patients: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 2012; 64:2937–2946. [PubMed: 22549432]
- Lushchak VI, Bagnyukova TV, Husak VV, Luzhna LI, Lushchak OV, Storey KB. Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. *Int J Biochem Cell Biol.* 2005; 37:1670–1680. [PubMed: 15896673]
- Miossec P, Kolls JK. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov.* 2012; 11:763–776. [PubMed: 23023676]
- Morgan PE, Sturges AD, Davies MJ. Increased levels of serum protein oxidation and correlation with disease activity in systemic lupus erythematosus. *Arthritis Rheum.* 2005; 52:2069–2079. [PubMed: 15986354]
- Nalbandian A, Crispín JC, Tsokos GC. Interleukin-17 and systemic lupus erythematosus: current concepts. *Clin Exp Immunol.* 2009; 157:209–215. [PubMed: 19604260]
- Niwano S, Niwano H, Sasaki S, Fukaya H, Yuge M, Imaki R, Machida Y, Izumi T. N-acetylcysteine suppresses the progression of ventricular remodeling in acute myocarditis: studies in an experimental autoimmune myocarditis (EAM) model. *Circ J.* 2011; 75:662–671. [PubMed: 21187656]
- Nur E, Brandjes DP, Teerlink T, Otten HM, Oude Elferink RP, Muskiet F, Evers LM, ten Cate H, Biemond BJ, Duits AJ, Schnog JJ, CURAMA study group. N-acetylcysteine reduces oxidative stress in sickle cell patients. *Ann Hematol.* 2012; 91:1097–1105. [PubMed: 22318468]
- Oates JC. The biology of reactive intermediates in systemic lupus erythematosus. *Autoimmunity.* 2010; 43:56–63. [PubMed: 20001422]
- Orengo CA, Todd AE, Thornton JM. From protein structure to function. *Curr Opin Struct Biol.* 1999; 9:374–382. [PubMed: 10361094]
- Parks CG, Cooper GS. Occupational exposures and risk of systemic lupus erythematosus: a review of the evidence and exposure assessment methods in population- and clinic-based studies. *Lupus.* 2006; 15:728–736. [PubMed: 17153843]
- Renke J, Popadiuk S, Korzon M, Bugajczyk B, Wozniak M. Protein carbonyl groups' content as a useful clinical marker of antioxidant barrier impairment in plasma of children with juvenile chronic arthritis. *Free Radic Biol Med.* 2000; 29:101–104. [PubMed: 10980398]
- Renke J, Szlagatyś A, Hansdorfer-Korzon R, Szumera M, Kamińska B, Knap N, Popadiuk S, Szarszewski A, Wozniak M. Persistence of protein oxidation products and plasma antioxidants in juvenile idiopathic arthritis. A one-year follow-up study. *Clin Exp Rheumatol.* 2007; 25:112–114. [PubMed: 17418001]
- Reveille JD. Predictive value of autoantibodies for activity of systemic lupus erythematosus. *Lupus.* 2004; 13:290–297. [PubMed: 15230281]
- Rodeghero R, Cao Y, Olalekan SA, Iwakura Y, Glant TT, Finnegan A. Location of CD4+ T cell priming regulates the differentiation of Th1 and Th17 cells and their contribution to arthritis. *J Immunol.* 2013; 190:5423–5435. [PubMed: 23630349]

- Samuni Y, Goldstein S, Dean OM, Berk M. The chemistry and biological activities of N-acetylcysteine. *Biochim Biophys Acta*. 2013; 1830:4117–4129. [PubMed: 23618697]
- Suwannaroj S, Lagoo A, Keisler D, McMurray RW. Antioxidants suppress mortality in the female NZB × NZW F1 mouse model of systemic lupus erythematosus (SLE). *Lupus*. 2001; 10:258–265. [PubMed: 11341102]
- Wang G, Cai P, Ansari GA, Khan MF. Oxidative and nitrosative stress in trichloroethene-mediated autoimmune response. *Toxicology*. 2007a; 229:186–193. [PubMed: 17123686]
- Wang G, Ansari GA, Khan MF. Involvement of lipid peroxidation-derived aldehyde-protein adducts in autoimmunity mediated by trichloroethene. *J Toxicol Environ Health A*. 2007b; 70:1977–1985. [PubMed: 17966069]
- Wang G, König R, Ansari GAS, Khan MF. Lipid peroxidation-derived aldehyde-protein adducts contribute to trichloroethene-mediated autoimmunity via activation of CD4+ T cells. *Free Radic Biol Med*. 2008; 44:1475–1482. [PubMed: 18267128]
- Wang G, Wang J, Ma H, Khan MF. Increased nitration and carbonylation of proteins in MRL+/+ mice exposed to trichloroethene: potential role of protein oxidation in autoimmunity. *Toxicol Appl Pharmacol*. 2009; 237:188–195. [PubMed: 19332086]
- Wang G, Pierangeli SS, Papalardo E, Ansari GA, Khan MF. Markers of oxidative and nitrosative stress in systemic lupus erythematosus: correlation with disease activity. *Arthritis Rheum*. 2010; 62:2064–2072. [PubMed: 20201076]
- Wang G, Wang J, Fan X, Ansari GA, Khan MF. 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. *Toxicology*. 2012; 292:113–122. [PubMed: 22178267]
- Zafarullah M, Li W, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. *Cell Mol Life Sci*. 2003; 60:6–20. [PubMed: 12613655]
- Zhu QX, Shen T, Ding R, Liang ZZ, Zhang XJ. Cytotoxicity of trichloroethylene and perchloroethylene on normal human epidermal keratinocytes and protective role of vitamin E. *Toxicology*. 2005; 209:55–67. [PubMed: 15725514]

Fig. 1.

Anti-MDA-protein adduct antibodies (A) and anti-HNE-protein adduct antibodies in the sera of MRL+/+ mice treated with TCE or TCE + NAC for 6 weeks. The values are means $\pm$ SD.

* $p < 0.05$ vs. controls; # $p < 0.05$ vs. TCE-treated mice.

Fig. 2. MDA-protein adducts (A) and HNE-protein adducts (B) in the livers and kidneys of MRL +/+ mice treated with TCE or TCE + NAC for 6 weeks. The values are means $\pm$ SD. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. TCE-treated mice.

Fig. 3. Protein carbonyl content in the sera (A), livers (B) or kidneys (C) of MRL+/+ mice treated with TCE or TCE + NAC for 6 weeks. The values are means $\pm$ SD. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. TCE-treated mice.

Fig. 4.

The ANA (A), anti-dsDNA antibodies (B) and anti-SM antibodies in the sera of MRL^{+/+} mice treated with TCE or TCE + NAC for 6 weeks. The values are means $\pm$ SD. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. TCE-treated mice.

Fig. 5. Correlation of (A) serum anti-MDA-protein adduct antibodies, and (B) serum anti-HNE-protein adduct antibodies with serum ANA. Spearman's rank correlation was used to calculate correlation coefficient.

Fig. 6.

Release of IL-17 into splenocyte cultures of MRL^{+/+} mice treated with TCE or TCE + NAC for 6 weeks. Splenocytes were incubated with medium alone, ConA (5 µg/ml) or anti-mouse CD3/CD28 antibodies (2.5 µg/ml/1 µg/ml) for 72 h, and the release of IL-17 into cultures was measured by specific ELISAs. The values are means ± SD of 6 animals in each group. US: unstimulated (medium alone) cells. *p < 0.05 vs. controls; #p < 0.05 vs. TCE-treated mice.

Fig. 7. Real-time PCR analysis of IL-17 mRNA expression in the spleens of MRL+/+ mice treated with TCE or TCE + NAC for 6 weeks. The fold changes of IL-17 mRNA (2^{-CT}) expression in spleens are shown. Values are means $\pm$ SD. *p < 0.05 vs. controls; #p < 0.05 vs. TCE-treated mice.

Fig. 8.
The plausible mechanisms of TCE-induced autoimmune response and its attenuation by NAC supplementation.