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Increased Nitration and Carbonylation of Proteins in MRL +/- Mice Exposed to Trichloroethene: Potential Role of Protein Oxidation in Autoimmunity

Gangduo Wang, Jianling Wang, Huaxian Ma, and M. Firoze Khan*

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

Abstract

Even though reactive oxygen and nitrogen species (RONS) are implicated as mediators of autoimmune diseases (ADs), little is known about contribution of protein oxidation (carbonylation and nitration) in the pathogenesis of such diseases. The focus of this study was, therefore, to establish a link between protein oxidation and induction and/or exacerbation of autoimmunity. To achieve this, female MRL +/- mice were treated with trichloroethene (TCE), an environmental contaminant known to induce autoimmune response, for 6 or 12 weeks (10 mmol/kg, i.p., every 4th day). TCE treatment resulted in significantly increased formation of nitrotyrosine (NT) and induction of iNOS in the serum at both 6 and 12 weeks of treatment, but the response was greater at 12 weeks. Likewise, TCE treatment led to greater NT formation, and iNOS protein and mRNA expression in the livers and kidneys. Moreover, TCE treatment also caused significant increases (~3 fold) in serum protein carbonyls (a marker of protein oxidation) at both 6 and 12 weeks. Significantly increased protein carbonyls were also observed in the livers and kidneys (2.1 and 1.3 fold, respectively) at 6 weeks, and to a greater extent at 12 weeks (3.5 and 2.1 fold, respectively) following TCE treatment. The increases in TCE-induced protein oxidation (carbonylation and nitration) were associated with significant increases in Th1 specific cytokine (IL-2, IFN- γ) release into splenocyte cultures. These results suggest an association between protein oxidation and induction/exacerbation of autoimmune response. The results present a potential mechanism by which oxidatively modified proteins could contribute to TCE-induced autoimmune response and necessitates further investigations for clearly establishing the role of protein oxidation in the pathogenesis of ADs.

Keywords

Trichloroethene; iNOS; Nitration; Carbonylation; Autoimmune Diseases

Introduction

An estimated 3% of the population in the United States is affected by autoimmune diseases (ADs) (Jacobson et al., 1997). ADs such as systemic lupus erythematosus (SLE) and rheumatoid arthritis, affect women more frequently and contribute disproportionately to morbidity and mortality among young to middle-aged women (Jacobson et al., 1997; Walsh

*Address for correspondence: M. Firoze Khan, Ph.D., Professor, Department of Pathology, University of Texas Medical Branch, 2.319 Mary Moody Northen, Galveston, TX 77555-0438, Tel: 409-747-6881, Fax: 409-765-5938, Email: mfkhan@utmb.edu.

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and Rau, 2000). The etiology of these diseases is largely unknown, but there is evidence that ADs are multifactorial and involve genetic, hormonal and environmental influences. Increasing epidemiologic and experimental studies support a potential role of environmental factors including chemical exposure in the development of ADs (Kilburn and Warshaw, 1992; Khan et al., 1995; Mayes, 1999; Parks et al., 2006). Trichloroethene (TCE) is a common environmental contaminant and a widely used industrial solvent. The involvement of TCE in the development of autoimmune disorders including SLE, systemic sclerosis and fascitis has been well documented, both in human and animal studies (Haustein and Ziegler, 1985; Flindt-Hansen and Isager, 1987; Kilburn and Warshaw, 1992; Khan *et al.*, 1995, 2001; Griffin et al., 2000a; Wang et al., 2007a, 2007b, 2008). However, mechanisms by which TCE-induces/accelerates the pathogenesis of ADs remain mostly unclear.

In recent years, free radical-mediated reactions as the potential mechanism in the pathogenesis of ADs has drawn increasing attention (Khan et al., 2001; Hadjigogos, 2003; Karpuzoglu et al., 2006; Kurien et al., 2006; Cuzzocrea, 2006; Nagy et al., 2007b). An essential part of the innate immune response in ADs is the production of reactive oxygen and nitrogen species (RONS) (Ohmori and Kanayama, 2005; Karpuzoglu et al., 2006; Kurien et al., 2006; Habib et al., 2006; Nagy et al., 2007a; Kurien and Scofield, 2008). One of the widely studied RONS, nitric oxide (NO), generated by the enzyme inducible nitric oxide synthase (iNOS), is overproduced during SLE disease activity (Weinberg et al., 1994; Dixit and Ali, 2004; Cuzzocrea, 2006; Nagy et al., 2007b). Its potential in the pathogenesis SLE and other ADs lies largely on the extent of its production and generation of superoxide anion, which react each other to form peroxynitrite (ONOO⁻), a much more reactive and potentially pathogenic molecule (Weinberg et al., 1994; Xia and Zweier, 1997). Several lines of evidence in murine lupus show that iNOS activity increases with the development and progression of ADs, and studies utilizing competitive inhibitors of iNOS suggest a role for iNOS in murine ADs (Weinberg et al., 1994; Xia and Zeier, 1997; Reilly et al., 2002; Karpuzoglu et al., 2006). In addition, ONOO⁻-mediated modifications of proteins and DNA may increase the immunogenicity of these self antigens, leading to a break in immune tolerance (Dixit and Ali, 2004; Ohmori and Kanayama, 2005; Habib et al., 2006; Kurien et al., 2006). Furthermore, data from human studies suggest that overexpression of iNOS and increased production of ONOO⁻ lead to glomerular and vascular pathology and other ADs (Belmont et al., 1997; Wanchu et al., 1998; Gilkeson et al., 1999; Nagy et al., 2007b).

Proteins constitute major components of living cells. A relatively minor structural modification often leads to a marked change (generally lowering) in their activities (Sánchez et al., 1994; Orengo et al., 1999). A variety of RONS-mediated modifications of proteins have been reported in aging and diseases (Stadtman and Berlett 1998; Oates et al., 1999; Beal, 2002; Morgan et al., 2005). For example, nitric oxide radical (NO[•]) can react with superoxide radical to form ONOO⁻, a potent nitrating and oxidizing agent that in turn can react with tyrosine to form nitrotyrosine (NT). Increased presence of nitrated proteins is found in many diseases including ADs (Oates et al., 1999; Morgan et al., 2005; Khan et al., 2006; Ohmori and Kanayama, 2006). Protein carbonyl content (PCC) formed on amino acid residues is a global marker of protein oxidation. Oxidative damage to proteins, based on PCC, correlated well with aging and the severity of some ADs (Stadtman and Berlette, 1998; Renke et al., 2000; Martín-Gallán et al., 2003; Renke et al., 2007). There is appreciable evidence that NT, PCC and other markers of protein oxidation are enhanced in diabetes and many other ADs, which may contribute to the pathogenesis of these diseases (Stadtman and Berlette, 1998; Oates et al., 1999; Martín-Gallán et al., 2003; Morgan et al., 2005; Ohmori and Kanayama, 2006; Khan et al., 2006; Khan and Ali, 2006; Renke et al., 2007).

TCE is known to generate free radicals, causes increased lipid peroxidation and induces autoimmune response both in vivo and in vitro (Channel et al., 1998; Khan et al., 2001; Zhu

et al., 2005; Wang et al., 2007a, 2007b, 2008). However, potential mechanisms by which TCE-induced RONS generation elicit an autoimmune response and their contribution to disease pathogenesis remains largely unexplored. We hypothesize that TCE-induced overproduction of RONS leads to a variety of RONS-mediated structural modifications of the endogenous proteins (increased formation of nitrotyrosine and protein carbonyl), which could lead to generation of neoantigens. After antigen processing, these neoantigens could elicit autoimmune responses by stimulating T and B lymphocytes, especially Th1 cells. To test this hypothesis and establish a link between RONS and induction/exacerbation of autoimmune response as a result of TCE exposure, we examined serum levels of NT and iNOS, analyzed NT and iNOS expression in the livers and kidneys, quantitated protein carbonyls in serum, livers and kidneys, and also determined the release of Th1 and Th2 cytokines (IL-2, IL-4, IL-10 and INF- γ) in the splenocyte cultures of autoimmune-prone MRL $^{+/+}$ mice treated with TCE for 6 or 12 weeks. Our results not only support that TCE exposure enhances/induces oxidative/nitrosative stress, but also suggest that increased nitration and carbonylation of proteins could be a potential mechanism in the etiology of ADs.

Materials and methods

Animals and treatment

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 hr light/dark cycle. The animals were provided 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 University of Texas Medical Branch. The mice were divided into 4 groups of 6 animals each. The TCE exposure groups received ip injections of 10 mmol/kg TCE (purity 99+%; Aldrich, Milwaukee, WI) in corn oil in a final volume of 100 μ l, an exposure regimen previously shown to lead to an early induction of autoimmunity in MRL $^{+/+}$ mice (Khan et al., 1995, 2001; Wang et al., 2007b, 2008). The control mice received an equal volume of corn oil only. The TCE treatments were given every 4th day for 6 or 12 weeks, and animals were weighed on a weekly basis. After 6 or 12 weeks of TCE treatment, the animals were euthanized under nembutal (sodium pentobarbital) anesthesia, and blood was withdrawn from the inferior vena cava. Individual sera, 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 analysis of iNOS, nitrotyrosine and protein carbonyls. Splenocytes were isolated immediately for the determination of cytokines (INF- γ , IL-2, IL-4 and IL-10) as described below.

Quantification of nitrotyrosine and iNOS in serum

Nitrotyrosine (NT) levels in the mouse serum was quantitated by an ELISA kit (Cell Sciences, Norwood, MA). The serum iNOS was measured using an ELISA established in our laboratory earlier (Wang et al., 2007a). Briefly, flat bottomed 96-well microtiter plates were coated with anti-iNOS monoclonal antibodies (Transduction Laboratories, Lexington, KY; diluted 1:1000 in coating buffer) overnight at 4 °C. The plates were washed with TBS-Tween 20 and the non-specific binding sites were blocked with TBS containing 1% BSA (Sigma, St. Louis, MO) at room temperature for 1 h. After washing extensively with TBS-Tween 20, 100 μ l of 1:40 diluted serum samples were added to duplicate wells of the coated plate and incubated at room temperature for 2 h. The plates were washed five times with TBS-Tween 20 and then 100 μ l of rabbit anti-mouse iNOS IgG2 (Alpha Diagnostic Int'l, San Antonio, TX) was added and incubated at room temperature for 1 h. The plates were washed extensively and 100 μ l of goat

anti-rabbit IgG-horseradish peroxidase (Upstate, Lake Placid, NY) was added and incubated at room temperature for 1 h. After washing, 100 μ l of TMB substrate was added to each well. The reaction was stopped after 10 min by adding 100 μ l 2M H₂SO₄ and the OD was read at 450 nm on a Bio-Rad Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

Quantitation of nitrotyrosine in the livers and kidneys

For the quantitation of nitrated proteins in the livers and kidneys of control and TCE-treated mice, liver or 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 nitrotyrosine (NT) concentration in the supernatants was quantitated by using an ELISA kit (Cell Sciences) following the manufacturer's instructions.

Western blot detection of iNOS in the livers and kidneys of mice

iNOS in the livers or kidneys of MLR +/+ mice was also detected by Western blot analysis as described in our previous study (Wang et al., 2007a). Briefly, liver or kidney proteins from control and TCE-treated mice were isolated using a lysis buffer (Pierce, Rockford, IL), and protein concentration in the lysates was determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA). Fifty μ g of protein dissolved in sample buffer was loaded onto a 12% Novex Tris-Glycine Gel (Invitrogen, Carlsbad, CA), resolved by electrophoresis, and subsequently transferred to nitrocellulose membrane. The membrane was incubated with TBS with 0.1% Tween-20 and 5% non-fat dry milk at room temperature for 2 h and subsequently probed with rabbit polyclonal anti-iNOS antibody for 2h. Blots were washed thoroughly and incubated with IgG-horseradish peroxidase-conjugated goat anti-rabbit antibody (Upstate) for 1 h. iNOS bands were detected by using enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ). The density of iNOS bands was analyzed with Eagle Eye II software (Stratagene, La Jolla, CA).

RNA isolation and real-time PCR analysis for iNOS gene expression in liver and kidney

RNA isolation—RNA was isolated as described in our earlier studies (Wang et al., 2005, 2008). Briefly, total RNA was isolated from liver and kidney tissues using RiboPure kit (Ambion, Austin, TX) as per manufacturer's instructions. To eliminate contaminating genomic DNA, RNA preparation was treated with RNase free DNase I (DNA-free kit, Ambion, Austin, TX). The total RNA concentration was determined by measuring the absorbance at 260 nm. RNA integrity was verified electrophoretically by ethidium bromide staining and by measuring A260/A280 ratio.

Real-time PCR—The real-time PCR was performed as described earlier (Wang et al., 2005, 2008). Briefly, cDNA was prepared from isolated RNA by using SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) described earlier (Wang et al., 2005). Quantitative real-time PCR employing a two-step cycling protocol (denaturation and annealing/extension) was carried out using the primers (forward 5'-TGTCTGCAGCACTTGGATCA and reverse 5'-AACTTCGGAAGGGAGCAATG) by the Smart Cycler System. For each cDNA sample, parallel reactions were performed in triplicate for the detection of mouse iNOS and 18S. The reaction samples in a final volume of 25 μ l contained 2 μ l of cDNA templates, 2 μ l primer pair, 12.5 μ l iQ SYBR Green Supermix and 8.5 μ l water. 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: 15s at 95 °C for denaturation, 30s at 65 °C for annealing, 30s at 72 °C for extension, and an additional 6s 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 (Simpson et al., 2000; Rajeevan et al., 2001). 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:

$$\text{Fold change} = 2^{-\Delta\Delta C_T}, \text{ where } \Delta\Delta C_T = (C_{TICE} - C_{T18S}) - (C_{TCONTROL} - C_{T18S}).$$

Determination of protein carbonyl content in serum

Carbonyl content in the mouse serum was quantitated by the protein carbonyl assay (Levine et al., 1990; Lushchak et al., 2005) with slight modification. Briefly, 100 μ l of mouse sera was incubated with 400 μ l of 10 mM 2,4-dinitrophenylhydrazine (DNPH) or 400 μ l of 2.5 M HCl. After one hour incubation at room temperature 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 minutes at 4 °C. The OD of the supernatants was read at 370 nm on a Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories). Protein carbonyls were calculated using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Detection of protein carbonyl content in liver and kidney

Carbonyl levels in the livers and kidneys were quantitated by the protein carbonyl assay (Levine et al., 1990; Lushchak et al., 2005). The liver and kidney homogenates (10%, w/v) were made in phosphate-buffered saline (PBS, pH 7.4) containing protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 10,000 g at 4 °C for 15 min and the protein carbonyls in the supernatants were analyzed by the method described above for serum.

Isolation of splenocytes and determination of Th1 and Th2 cytokines in splenocyte cultures

Splenocytes were prepared by passing a portion of the spleen through a stainless steel mesh and suspended in RPMI 1640 culture medium. The cell suspension was centrifuged at 1000 $\times$ g for 5 min at 4 °C. The cell pellet was resuspended in Hank's solution and laid on to an equal volume of Histopaque-1083 (Sigma, St. Louis, MO). After centrifugation at 400 $\times$ g for 30 min, the interface (containing splenocytes) was transferred into a fresh tube and washed twice with RPMI 1640 without serum. 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 total splenocytes were counted (Khan et al., 2006). 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) were added, respectively, 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 INF- γ , IL-2, IL-4 and IL-10 into the cultures was quantitated using specific ELISA kits (Biosource, Camarillo, CA).

Statistical analyses

All data are expressed as means $\pm$ SD. Comparison between the two groups was made by *p* value determination using Student's *t* test. A *p* value less than 0.05 was considered to be statistically significant.

Results

Nitrotyrosine and iNOS levels in the serum of TCE-treated mice

NT formation is considered to be a biomarker of RNS production and iNOS catalyzes the formation of nitric oxide (Beckman et al., 1996; Radi, 2004). To assess the possible contribution of nitrosative stress in TCE-induced autoimmune response, we determined the serum levels of nitrotyrosine and iNOS in control and TCE-treated mice. As evident from Fig. 1A, TCE exposure led to significant increases in serum NT levels at both 6 and 12 weeks in comparison with their respective controls, but the increases were much greater at 12 weeks, which were also significantly higher than 6-week treatment group. Similarly, the iNOS levels were also significantly higher in the sera of TCE-treated mice in comparison to their respective controls at both time points. Interestingly, when the two TCE-treated groups were compared, the increases in iNOS levels were significantly higher at 12 weeks in comparison to 6 weeks (Fig. 1B).

Effect of TCE exposure on nitrotyrosine levels in the livers and kidneys of mice

NT levels, quantitated by specific ELISA, in control and TCE-treated mice are presented in Fig. 2. NT formation in the TCE-treated animals was significantly increased at both 6 and 12 weeks in comparison with the controls, but the increases were much greater at 12 weeks, which were also significantly higher in livers of animals treated with TCE for 6 weeks (Fig. 2A). Similar pattern and even higher levels of NT formation was found in kidneys (Fig. 2B). Our results thus show greater formation of nitrated proteins in both livers and kidneys of TCE-treated mice. The response in both livers and kidneys was even greater at 12 weeks, thus resembling a pattern similar to NT levels in serum (Fig. 1A).

iNOS in the livers and kidneys of mice treated with TCE

The iNOS protein expression was also determined by Western blot analysis in the livers and kidneys, major organs where TCE is known to generate free radicals and lead to autoimmune damages (Cojocel et al., 1989; Atkinson et al., 1993; Channel et al., 1998; Griffin et al., 2000b; Lash et al., 2005; Cai et al., 2008). Since serum iNOS showed greater response at 12 weeks, Western analysis for iNOS was conducted in 12-week liver and kidney samples. The Western results show that iNOS expression increased significantly both in livers [2.8 folds, Fig. 3 (A and B)] and in kidneys [3.7 folds, Fig. 3(C and D)] of TCE-treated mice compared to the controls.

iNOS mRNA expression in the livers and kidneys of mice treated with TCE

To further evaluate the impact of TCE exposure on iNOS and thus, nitrosative stress, the iNOS mRNA expression was analyzed using real-time PCR in both livers and kidneys of mice treated for 12 weeks. Figs. 4A and 4B show the results of real-time PCR analyses of iNOS mRNA from control and TCE-treated in livers (Fig. 4A) and kidneys (Fig. 4B). The amount of mRNA in each sample was calculated as the ratio between the iNOS and the endogenous control (18S rRNA). As evident from the figures, the mRNA levels of livers and kidneys of TCE-treated mice showed significant increases (2.2- and 2.5-folds, respectively), in comparison to controls. The increased mRNA levels, as detected by real-time RT-PCR, in the livers and kidneys coincide with increased protein expression evaluated in these tissues as determined by Western blot (Fig. 3) and also correlates with iNOS levels in the serum (Fig. 1B).

Protein carbonyl content (PCC) in the sera, livers and kidneys of mice treated with TCE

PCC is not only a biomarker of oxidative stress, but also provides evidence of oxidative protein damage (Khan et al., 1999; Renke et al., 2000; Morgan et al., 2005). To assess the status of protein oxidation (protein carbonyl) in TCE-induced autoimmune response, we first

determined the serum levels of PCC in the control and TCE-treated mice. Our data show carbonyl content in the sera was significantly increased at both 6 and 12 weeks in comparison to their respective controls (Fig. 5A). To evaluate the extent of oxidative stress exerted on cells or organs, protein carbonyls in the livers and kidneys, the major organs targeted by TCE, were analyzed. As evident from Fig. 5B, PCC in the livers of TCE-treated mice was significantly increased at both 6 and 12 weeks (2.1 and 3.5 folds, respectively) in comparison with the controls, but the increases were much greater at 12 weeks and also significantly higher in comparison to mice exposed to TCE for 6 weeks. Similar pattern of increases in PCC (1.4 and 2.1 folds, respectively) was also observed in kidneys of mice treated with TCE for 6 and 12 weeks (Fig. 5C), suggesting increased protein oxidation (carbonylation) as a result of TCE exposure.

Effect of TCE exposure on the release of Th1 vs. Th2 cytokines into splenocyte cultures

Imbalance of Th1 and Th2 cytokines plays an essential role in the immune dysregulation observed in ADs, both in humans and murine models (Takahashi et al., 1996; Segal et al., 1997; Lit et al., 2007). To investigate the effect of TCE treatment on cytokine release and to evaluate their possible involvement in TCE-induced autoimmune response, splenocytes isolated from TCE-treated mice (both 6 and 12 weeks) and their respective controls were cultured with or without ConA or anti-mouse CD3/CD28, and the release of INF- γ , IL-2, IL-4 and IL-10 into the culture supernatants was determined. Splenocytes from TCE-treated mice secreted significantly higher levels of IL-2 (Fig. 6A) than did splenocytes from untreated control mice following 72h stimulation with ConA or CD3/CD28. Similar pattern and greater increases in INF- γ release into the splenocyte cultures with the ConA or CD3/CD28 stimulation were also observed (Fig. 6B). Interestingly, the splenocytes from TCE-treated mice even without stimulation with ConA or CD3/CD28 secreted more INF- γ and IL-2 compared to the unstimulated control cells. However, neither ConA nor CD3/CD28 stimulation led to any significant difference in IL-4 or IL-10 release by the cultured splenocytes from TCE-treated and control mice (data not shown).

Discussion

TCE, a common environmental contaminant and a widely used industrial agent, has been implicated in the development of autoimmune disorders in humans (Flindt-Hansen and Isager, 1987; Kilburn et al., 1992) and it also induces autoimmune response in experimental animals (Khan et al., 1995, 2001; Griffin et al., 2000a; Wang et al., 2007a, 2007b, 2008). Our earlier studies, under experimental conditions similar to this study, consistently showed that TCE treatment in MRL +/+ mice leads to significant elevation in serum anti-nuclear-, anti-ssDNA- and anti-dsDNA-antibodies (Khan et al., 1995; Wang et al., 2007a, 2007b, 2008). However, the mechanisms by which TCE-induces/accelerates the pathogenesis of ADs have not been clearly elucidated. In order to further evaluate the autoimmune response to TCE treatment and possible involvement of Th1 or Th2 cytokines in the process, the release of IL-2, INF- γ , IL-4 and IL-10 into the culture supernatants of splenocytes isolated from TCE-treated and control mice was determined. Splenocytes from TCE-treated mice (both 6 and 12 weeks) secreted significantly higher levels of IL-2 than did splenocytes from untreated control mice following 72h stimulation with ConA or anti-CD3/CD28. Similar pattern and greater increases in INF- γ release into the splenocyte cultures with ConA or anti-CD3/CD28 stimulation were also observed. Even the splenocytes from TCE-treated mice secreted more IL-2 and INF- γ without the stimulation with ConA or anti-CD3/CD28, compared to unstimulated control cells. The data not only show the influence of TCE on greater cytokine release, but further support previous findings that TCE-mediated autoimmune response involves Th1 cell activation (Griffin et al., 2000a; Wang et al., 2008). However, an important focus of this investigation was to delineate the earlier events and pathways by which TCE induces an autoimmune

response. This has lead us to explore the potential involvement of oxidative modification of proteins in TCE-mediated autoimmunity, since a vital part of innate immune response in ADs is the oxidative production of RONS (Ohmori and Kanayama, 2005; Kurien et al., 2006; Habib et al., 2006; Nagy et al., 2007a; Kurien and Scofield, 2008).

Proteins are the key molecules that play the ultimate role in various structural and functional aspects of living organisms. Oxidative modification of proteins by RONS is implicated in the pathogenesis of both normal aging and various diseases (Beal, 2002; Morgan et al., 2005; Khan and Ali, 2006; Renke et al., 2007). NT, a modification product of peroxynitrite (ONOO^-), generally serves as a biochemical marker for peroxynitrite/NO formation and elevated NT level is associated with SLE and other pathological conditions (Oates et al., 1999; Khan et al., 2006; Khan and Ali, 2006; Renke et al., 2007). Furthermore, modification of proteins by ONOO^- could trigger the immunogenicity of these self antigens, leading to a break in immune tolerance (Ohmori and Kanayama, 2005; Khan and Ali, 2006; Kurien et al., 2006; Kurien and Scofield, 2008). MRL $+/+$ mice treated with TCE for 6 or 12 weeks in this study, had significantly higher levels of NT in their sera in comparison to untreated controls, and the increases were much greater at 12 weeks. To further evaluate the modification of tyrosine residues by ONOO^- , NT levels were also examined in livers and kidneys, because they are the major organs where TCE is known to generate free radicals and induce autoimmune disorders (Cojocel et al., 1989; Atkinson et al., 1993; Channel et al., 1998; Griffin et al., 2000b; Lash et al., 2005; Cai et al., 2008). TCE treatment also led to increased NT formation in these organs, with greater increases at 12 weeks. Thus, it is clear from our study that TCE induces increased formation of nitrated proteins in a time-dependent manner (although our study was limited to only two time points). This TCE led oxidative modification of the endogenous proteins may trigger an immunogenic response and thus contribute to the pathogenesis of ADs.

Nitric oxide (NO), which is formed in excessive amounts from the activation of iNOS, is considered to contribute to SLE and other ADs mainly by reacting with superoxide to form ONOO^- (Weinberg et al., 1994; Xia and Zweier, 1997; Cuzzocrea, 2006; Nagy et al., 2007b). There is considerable evidence that overexpression of iNOS is associated with the development and progression of ADs both in human and experimental animals. Furthermore, studies utilizing competitive inhibitors of iNOS suggest a pathogenic role of iNOS in murine ADs (Weinberg et al., 1994; Xia and Zeier, 1997; Gilkeson et al., 1999; Reilly et al., 2002; Karpuzoglu et al., 2006). The increases in iNOS activity have also been shown to be associated with increased formation of NT (Weinberg et al., 1994; Belmont et al., 1997; Karpuzoglu et al., 2006). This led us to test the response of iNOS and its potential contribution in TCE-induced autoimmunity. Our results show not only increased serum iNOS activity in TCE-treated mice, but also its strong association with increased levels of NT. Furthermore, the expression of iNOS protein in livers and kidneys, as analyzed by Western blot, also increased following TCE exposure. To further evaluate its potential role, we also analyzed the expression of iNOS mRNA using real-time PCR in livers and kidneys. To our knowledge, this is the first study to show overexpression of iNOS mRNA in both livers and kidneys of MRL $+/+$ mice following TCE exposure. The increase in iNOS mRNA or protein levels was accompanied by significant increases in liver and kidney NT levels, further supporting that RONS generation could also contribute to TCE-induced autoimmunity.

PCC is one of the most widely used biochemical marker of protein oxidation, and increased PCC is also associated with SLE disease activity and other ADs (Stadtman and Berlette, 1998; Renke et al., 2000; Beal, 2002; Martín-Gallán et al., 2003; Morgan et al., 2005; Renke et al., 2007). To assess our hypothesis that oxidative modification of proteins might be a potential mechanism in TCE-induced autoimmunity, PCC levels were measured in sera, livers and kidneys of MRL $+/+$ mice. It was interesting to note that TCE treatment, both after 6 and 12 weeks, led to significant increases in PCC levels in the sera, livers and kidneys, and the

increases were much greater at 12 weeks. These results not only support our earlier findings that TCE is capable of inducing oxidative stress, triggering oxidative modification of proteins in MRL^{+/+} mice (Khan et al., 2001; Wang et al., 2007a), but also provide evidence for the idea that increased formation of RONS-modified proteins could serve as neoantigen(s) to elicit an autoimmune response and thus present a potential mechanism for tissue damage.

Taken together, the results of this study show that TCE exposure leads to increased nitration and carbonylation of proteins. Increased iNOS activity and production of RONS and/or ONOO⁻ can contribute to oxidative modification of proteins. More importantly, our data along with the evidence that TCE induces autoimmune response (Khan et al., 2005; Wang et al., 2007a, 2007b, 2008), also suggest an association between oxidative modification of proteins and autoimmunity. The modification of proteins, such as nitration or carbonylation, may alter immunogenicity of self antigens (converting them to neoantigens), and may lead to an autoimmune response by stimulating T cells (especially activation of Th1 cells). Further studies to establish a clear link between oxidative/nitrosative stress and autoimmune response through exposure to such chemicals as TCE, could be a step forward in the understanding the mechanisms of ADs.

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A

B

Fig. 1.

Nitrotyrosine and iNOS levels in the sera of MRL^{+/+} mice treated with TCE for 6 or 12 weeks. Nitrotyrosine levels (A) and iNOS levels (B) were determined by specific ELISAs. The results are means $\pm$ SD of 6 animals in each group. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. 6 weeks TCE-treated mice.

Fig. 2. Nitrotyrosine levels in the livers (A) and kidneys (B) of MRL +/+ mice treated with TCE for 6 or 12 weeks. Values are means $\pm$ SD of 6 animals in each group. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. 6 weeks TCE-treated mice.

Fig. 3. Western blot analysis for iNOS protein expression in the livers [Fig. 3 (A and B)] and kidneys [Fig. 3(C and D)] of MRL +/+ mice treated with TCE for 12 weeks. (A and C) iNOS expression in control mice (lanes C1-C3) and TCE-treated mice (lanes T1-T3). (B and D) Densitometric analysis of iNOS bands from control and TCE-treated mice. The results are means $\pm$ SD. * $p < 0.05$ vs. controls.

A

Fig. 4. Real-time PCR analysis of iNOS mRNA expression in the livers and kidneys of MRL +/+ mice treated with TCE for 12 weeks. The fold changes of iNOS mRNA ($2^{-\Delta\Delta C_T}$) expression in livers (A) and in kidneys (B) are shown. Values are means $\pm$ SD. * $p < 0.05$ vs. controls.

A

A

Fig. 5. Protein carbonyl content in the sera (A), livers (B) and kidneys (C) of MRL +/+ mice treated with TCE for 6 or 12 weeks. The values are means $\pm$ SD of 6 animals in each group. * $p < 0.05$ vs. controls; # $p < 0.05$ vs. 6 weeks TCE-treated mice.

A

B

Fig. 6. Release of IL-2 (A) and IFN- γ (B) into splenocyte cultures of MRL+/+ mice treated with TCE for 6 or 12 weeks. Splenocytes were incubated with medium alone, ConA (5 μ g/ml) or anti-mouse CD3/CD28 antibodies (2.5 μ g/ml/1 μ g/ml) for 72 h, and the release of IL-2 and IFN- γ into cultures was measured by specific ELISAs. The values are means $\pm$ SD of 6 animals in each group. US: unstimulated (medium alone) cells. * $p < 0.05$ vs. controls.