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Oxidative and Nitrosative Stress in Trichloroethene-Mediated Autoimmune Response

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Abstract

Reactive oxygen and nitrogen species (RONS) are implicated in the pathogenesis of several autoimmune diseases. Also, increased lipid peroxidation and protein nitration are reported in systemic autoimmune diseases. Lipid peroxidation-derived aldehydes (LPDAs) such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are highly reactive and bind proteins covalently, but their potential to elicit an autoimmune response and contribution to disease pathogenesis remain unclear. Similarly, nitration of protein could also contribute to disease pathogenesis. To assess the status of lipid peroxidation and/or RONS, autoimmune-prone female MRL +/+ mice (5-week old) were treated with trichloroethene (TCE), an environmental contaminant known to induce autoimmune response, for 48 weeks (0.5 mg/ml via drinking water), and formation of antibodies to LPDA-protein adducts was followed in the sera of control and TCE-treated mice. TCE treatment led to greater formation of both anti-MDA- and anti-HNE-protein adduct antibodies and higher serum iNOS and nitrotyrosine levels. The increase in TCE-induced oxidative stress was associated with increases in anti-nuclear-, anti-ssDNA- and anti-dsDNA- antibodies. These findings suggest that TCE exposure not only leads to oxidative/nitrosative stress, but is also associated with induction/exacerbation of autoimmune response in MRL +/+ mice. Further interventional studies are needed to establish a causal role of RONS in TCE-mediated autoimmunity.

Keywords

Trichloroethene; Oxidative Stress; MDA; HNE; iNOS; Nitrotyrosine; Autoimmune Diseases

1. Introduction

Autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and scleroderma are chronic and life-threatening disorders that affect ~3% of United States population, and are among the leading causes of death for women under the age of 65 (Jacobson et al., 1997; Walsh et al., 2000). Despite high prevalence of these diseases, molecular mechanisms underlying systemic autoimmune response remain largely unknown. Reactive oxygen and nitrogen species (RONS)-mediated oxidative damage has been implicated in the pathogenesis of SLE and other autoimmune diseases (Michel et al., 1997; Hadjigogos, 2003). Highly reactive transient chemical species, i.e., superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and nitric oxide ($\cdot NO$) formed during aerobic metabolism in cells as well as due to phagocyte and neutrophil activation during inflammation, have potential

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to initiate cellular damage to lipids, proteins and DNA (Biemond et al., 1984; Halliwell et al., 1984). Lipid peroxidation, an oxidative degeneration of polyunsaturated fatty acids set into motion by free radicals, leads to formation of highly reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which can bind covalently to proteins resulting in their structural modifications (Grune et al., 1997; Kamanli et al., 2004; Januszewski, 2005). Much attention has been focused on the formation and role of MDA- and HNE-protein adducts in toxic and disease states (Hartley et al., 1999; Khan et al., 1999, 2002; Sampey et al., 2003; Kolodziejczyk et al., 2006). However, role of lipid peroxidation, especially the formation and linkage of MDA- and HNE-protein adducts in the induction of autoimmune diseases remain largely unexplored.

Increasing evidence suggests that autoimmune diseases are multifactorial, and could involve genetic, hormonal and environmental influences. In addition to bacteria and viruses, other environmental factors including such chemicals as trichloroethene (TCE) (Kilburn et al., 1992; Khan et al., 1995), silicon dioxide (SiO₂; Parks et al., 1999), and mercuric chloride (Zheng et al., 2005) are documented to contribute to the induction and/or acceleration of autoimmune diseases. TCE, a volatile organic compound widely used as an industrial solvent and a degreasing agent, is involved in the development of autoimmune disorders and immune system dysfunction both in human and animal studies (Kilburn et al., 1992; Yanez et al., 1992; Khan et al., 1995; Griffin et al., 2000a). Autoimmune disorders were observed in humans following exposure to TCE through contaminated drinking water (Kilburn et al., 1992) or from occupational exposures (Flindt-Hansen et al., 1987; Nietert et al., 1998). Environmental exposure to TCE is associated with several types of immune disorders including SLE (Kilburn et al., 1992), systemic sclerosis (Haustein et al., 1985), and fasciitis (Hayashi et al., 2000), whereas occupational exposure is linked to the development of scleroderma (Flindt-Hansen et al., 1987; Nietert et al., 1998). Our laboratory was the first to use autoimmune-prone MRL^{+/+} mice as an animal model to demonstrate that TCE exposure leads to induction of autoimmune response, as evident from increases in serum anti-nuclear antibodies (ANA) and TCE-specific antibodies (Khan et al., 1995), while other studies with the same model have shown that TCE exposure leads to activation of CD4⁺ cells (Griffin et al., 2000a, 2000b). Despite these human and experimental studies, mechanisms by which TCE induces/accelerates autoimmune disorders remain unknown.

TCE has been shown to generate free radicals and induces lipid peroxidation both in vivo and in vitro (Ogino et al., 1991; Channel et al., 1998; Khan et al., 2001). However, role of TCE-induced lipid peroxidation in the induction/acceleration of an autoimmune response remains unknown. To support our hypothesis that lipid peroxidation and/or RONS might play a role in TCE-induced autoimmune response, and to establish a link between lipid peroxidation/RONS and autoimmune response, we determined the serum profiles of anti-MDA and anti-HNE antibodies, quantitated the serum concentrations of nitrotyrosine and iNOS, examined the serum levels of established autoimmunity markers, including ANA, anti-ssDNA and anti-dsDNA antibodies, and also assessed the relationship between lipid peroxidation and autoimmunity markers in the autoimmune prone MRL^{+/+} mice exposed to TCE. Our studies show that at a relatively low dose, TCE accelerates/induces autoimmune response, and also suggest that lipid peroxidation/RONS may contribute to the autoimmune processes.

2. Materials and methods

2.1. Animals and treatments

Four-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 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 two groups of six each and received TCE (0.5 mg/ml) or drinking water only (controls). The average TCE consumption during the treatment period was 0.817 mmol kg⁻¹ day⁻¹. The choice of low dose TCE exposure was based on previous studies (Griffin et al., 2000a) showing an induction of autoimmune response. The mice were weighed on a weekly basis to monitor weight changes. After 48 weeks of TCE treatment, the animals were euthanized under nembutal (sodium pentobarbital) anesthesia, and blood was withdrawn from the inferior vena cava. Major organs were removed immediately and weighed. Portions of liver from control and TCE-treated mice were frozen immediately for iNOS analysis. Individual sera, obtained following blood clotting and centrifugation, were stored in small aliquots at -70°C till further analysis.

2.2. ELISAs for MDA- and HNE-protein adduct-specific antibodies

ELISAs to analyze anti-MDA- and -HNE-protein adduct-specific antibodies in the mouse serum were performed as described earlier (Khan et al., 2001). Briefly, MDA and HNE adducts of ovalbumin (antigen) were prepared as described by Khan et al. (1997). Free amino groups of ovalbumin that did not react with MDA or HNE were determined by 2,4,6-trinitrobenzene-1-sulfonic acid assay (Habeeb, 1966). Our results showed that treatment of 5 mg/ml of ovalbumin with 50 mM MDA or 8.7 mM HNE modified ~88% and ~65% amino groups of ovalbumin, respectively. Flat-bottomed 96-well microtiter plates were coated with MDA-/HNE-ovalbumin adducts or ovalbumin (0.5 g/well) overnight at 4°C. The plates were washed with Tris buffered saline (TBS)-Tween 20 and the non-specific binding sites were blocked with TBS containing 1% BSA (Sigma) at room temperature for 1 h. After washing extensively with TBS-Tween 20, 50 l of 1:100 diluted serum samples were added to duplicate wells of the coated plates and incubated at room temperature for 2 h. The plates were washed five times with TBS-Tween 20 and then 50 l rabbit anti-mouse IgG-horseradish peroxidase (Chemicon, Temecula, CA; diluted 1:2000 in TBS) was added and incubated at room temperature for 1 h. After washing, 100 l of TMB peroxidase substrate was added to each well. The reaction was stopped after 10 min by adding 100 l 2M H₂SO₄ and the OD was read at 450 nm on a Bio Rad Benchmark plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

2.3. Nitrotyrosine and iNOS determination in serum

Nitrotyrosine (NT) concentration in the mouse serum was quantitated by using an ELISA kit (Cell Sciences, Norwood, MA). The serum iNOS was measured by an ELISA established in our laboratory. 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) at room temperature for 1 h. After washing extensively with TBS-Tween 20, 100 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 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 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 l of TMB peroxidase substrate was added to each well. The reaction was stopped after 10 min by adding 100 l 2M H₂SO₄ and the OD was read at 450 nm on a Bio Rad Benchmark plus microplate spectrophotometer.

2.4. Western blot detection of iNOS in the liver of mice

iNOS in the livers of MLR +/+ mice was also detected by Western blot analysis. Briefly, liver 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 using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Fifty g of protein was dissolved in sample buffer and 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 secondary antibody (Upstate, Lake Placid, NY) for 1 h. iNOS bands were detected by using enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ). The density of protein adduct bands was analyzed with Eagle Eye II software (Stratagene, La Jolla, CA).

2.5. Autoantibodies in the serum

Serum levels of anti-nuclear antibodies (ANA), anti-single stranded DNA (anti-ssDNA) antibodies and anti-double stranded DNA (anti-dsDNA) antibodies were determined by using mouse-specific ELISA kits (Alpha Diagnostic Int'l) as described earlier (Khan et al., 1995).

2.6. 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. Spearman's rank correlation was used to calculate correlation coefficients between anti-MDA-protein adduct antibodies and ANA in the serum. A *p* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Induction of anti-MDA- and -HNE-protein adduct antibodies in the serum of TCE-treated mice

In an attempt to understand the contribution of lipid peroxidation in the pathogenesis of autoimmune diseases, we first determined whether at a relatively low dose TCE is capable of promoting lipid peroxidation and/or induction of specific antibodies against lipid peroxidation-derived aldehyde (LPDA)-protein adducts. As shown in Fig. 1, the levels of serum anti-MDA-protein adduct antibodies in mice treated with TCE for 48 weeks increased significantly in comparison to the controls (Fig. 1A). Similarly, the level of serum anti-HNE-protein adduct antibodies also increased significantly following TCE treatment (Fig. 1B). Since both MDA and HNE are highly reactive aldehydes derived from lipid peroxidation (Esterbauer et al., 1991; Khan et al., 2002; Uchida, 2003), the greater serum levels of anti-MDA and anti-HNE antibodies suggest that TCE not only increased lipid peroxidation, but also the formation of LPDA-protein adducts in the MRL +/+ mice.

3.2. Nitrotyrosine and iNOS levels in the serum

Since oxidative and nitrosative stress could occur simultaneously, possible involvement of nitric oxide in the autoimmune response was evaluated by measuring NT level and iNOS induction because 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). As evident from Fig. 2, NT formation was significantly increased following TCE exposure. Likewise, iNOS level was also increased in TCE-treated mice compared to the controls (Fig. 3).

3.3. iNOS in the livers of mice treated with TCE

To further evaluate if RNS is involved in the pathogenesis of TCE-mediated autoimmunity, the expression of iNOS was also determined in the livers by Western blot analysis. The results show that iNOS expression increased significantly (~ 3 folds) in the livers of TCE-treated mice compared to the controls (Fig. 4).

3.4. Acceleration of autoantibody production in mice treated with TCE

Autoantibodies, such as ANA, anti-ssDNA and anti-dsDNA, have been extensively used as biomarkers of autoimmune diseases (Egner, 2000;Reveille, 2004). To test whether a low dose TCE exposure was capable of inducing/exacerbating autoimmune response, serum samples from control and TCE-treated MRL+/+ mice were analyzed for various autoantibodies including ANA, anti-ssDNA and anti-dsDNA antibodies. TCE exposure resulted in a significant increase in serum ANA levels compared to the control mice (Fig. 5A). Also compared with controls, both anti-ssDNA and anti-dsDNA antibody levels in the serum of TCE-treated mice increased, but did not achieve statistical significance (Fig. 5B, Fig. 5C). Interestingly, the number of mice showing strongly positive response for ANA, anti-ssDNA and anti-dsDNA antibodies was higher in the TCE-treated group (data not shown).

3.5. Correlation between serum anti-MDA-protein adduct antibodies and ANA

To validate our hypothesis that LPDA may be involved in the autoimmune response, we evaluated the relationship of the increases in serum anti-MDA antibodies with serum ANA. A significant correlation was obtained between anti-MDA antibodies and ANA ($r = 0.776$, $p < 0.01$) (Fig. 6). These results also suggest an association among LPDAs formation, LPDA-modified proteins and autoimmune response.

4. Discussion

The etiology of autoimmune diseases is largely unknown but, there is evidence that such diseases are multifactorial and involve genetic, hormonal and environmental influences including chemical exposure (Ahmed et al., 1985;Khan et al., 1995;Cooper et al., 1999). TCE, a common environmental contaminant and widely used industrial agent, has been implicated in the development of autoimmune disorders in humans (Kilburn et al., 1992;Nietert et al., 1998) and induces autoimmune response in experimental animals (Khan et al., 1995;Griffin et al., 2000a). However, the mechanisms by which TCE-induces/accelerates the pathogenesis of autoimmune diseases remain unknown. Even though RONS are implicated in the pathogenesis of SLE, RA and other diseases, an important question being addressed in this investigation is whether oxidative stress contributes to TCE-mediated autoimmunity. This led us to focus our studies in establishing a link between the increased lipid peroxidation and the development of autoimmunity, mediated by TCE at a relative low dose exposure.

Experimental studies have established that TCE exposure leads to lipid peroxidation (Cojocel et al., 1989;Ogino et al., 1991;Channel et al., 1998;Khan et al., 2001). Lipid peroxidation induced by TCE has been associated with liver and kidney damage (Cojocel et al., 1989;Channel et al., 1998). Also, earlier studies in our laboratory proposed a putative role of lipid peroxidation in the induction/exacerbation of autoimmunity (Khan et al., 2001). Lipid peroxidation, which is a major contributor to membrane damage, has also been implicated in the pathogenesis of SLE and other autoimmune diseases (Grune et al., 1997;Kamanli et al., 2004;Frostegard et al., 2005;Lankin et al., 2005). Lipid peroxidation products, especially major aldehydes such as MDA and HNE, can form adducts with proteins (Esterbauer et al., 1991). Covalent binding of LPDAs with endogenous macromolecules may not only alter their physiological functions, but also cause structural modifications generating neoantigens, which consequently could elicit an autoimmune response leading to autoimmune diseases (Khan et

al., 2001;Ahsan et al., 2003;Frostegard et al., 2005). TCE exposure in MRL +/+ mice in this study led to increased serum antibody levels specific for both MDA and HNE, the two most abundant aldehydic products of lipid peroxidation. The increased levels of anti-MDA- and anti-HNE-protein adduct antibodies not only indicate that at relative low dose TCE could promote lipid peroxidation, but also provide support to the idea that increased formation of LPDA-conjugated proteins could serve as neoantigen(s) to produce tissue damage via eliciting an autoimmune response.

There is considerable evidence for induction of oxidative stress as a result of TCE exposure (Ogino et al., 1991;Channel et al., 1998;Khan et al., 2001). Since oxidative and nitrosative stress could occur simultaneously, it was logical to study the possible involvement of RNS whose formation and contribution has not been studied in TCE-induced autoimmune response. Another important finding of this study is the increased nitrosative stress as evident from increases in NT and iNOS expression following TCE exposure. Increased RNS production has been implicated in the pathogenesis of SLE, rheumatoid arthritis (RA), and other autoimmune diseases (Rolla et al., 1997;Liu et al., 2001;Hadjigogos, 2003;Levesque et al., 2004). NT serves as a useful biomarker in identifying the generation of RNS (Beckman et al., 1996;Radi, 2004). Increased iNOS expression and NT formation have been reported in SLE and other autoimmune diseases (Rolla et al., 1997;Liu et al., 2001;Levesque et al., 2004). To our knowledge, this is the first study to show increased nitration of serum protein tyrosines (NT formation) and iNOS expression in both serum and liver of autoimmune-prone MRL+/+ mice following TCE exposure, and are accompanied by significant increases in anti-MDA- and anti-HNE-protein adduct antibodies, further supporting that RONS may also contribute to disease pathogenesis. Alternatively, nitric oxide may react with $O_2^{\cdot-}$ to form peroxynitrite, which on decomposition will lead to $\cdot OH$ and $\cdot NO_2$, and consequently generate LPDAs through lipid peroxidation (Ho et al., 2002).

Another interesting feature of our studies is that a relatively low dose TCE exposure led to induction/exacerbation of an autoimmune response, as evident from increased levels of autoantibodies, such as ANA, anti-dsDNA and anti-ssDNA in the serum, which are established biomarkers of autoimmune diseases (Egner, 2000;Reveille, 2004). The significant increase in ANA following TCE treatment in this study further supports our previous findings that TCE mediates an autoimmune response in MRL +/+ mice (Khan et al., 1995). These findings are interesting and pertinent and suggest that an environmentally-relevant TCE exposure is capable of inducing/exacerbating an autoimmune response in MRL +/+ mice.

One of the major objectives of the present work was to establish a link between lipid peroxidation and autoimmune response in the MRL +/+ mice. The relationship of anti-MDA-protein adduct antibodies with serum ANA levels was established by analyzing the correlation of these two indices. The data show a good correlation ($r=0.776$, $p<0.01$) between serum anti-MDA-protein adduct antibodies and ANA, thus suggesting an association among lipid peroxidation, LPDA-modified proteins and autoimmune response. These studies thus suggest that both oxidative and nitrosative stress may contribute to TCE-mediated autoimmune response in MRL +/+ mice.

In conclusion, our studies demonstrate that long-term, low dose TCE exposure leads to both oxidative and nitrosative stress and induction/exacerbation of autoimmune response. Increased formation of LPDAs such as MDA and HNE, leading to the formation of neoantigens, may elicit autoimmune responses by stimulating T and B lymphocytes. Further, persistent increases in antibodies to LPDA-protein adducts may lead to formation of immune complexes whose deposition in tissues could be a pathogenic mechanism of autoimmune diseases. The proposed sequence of events leading to autoimmune response as results of TCE exposure is presented in Fig. 7. However, extensive interventional studies are needed to establish the causal role of

RONs in TCE-mediated autoimmunity. Establishing the role of oxidative/nitrosative stress in autoimmune diseases through exposure to such chemicals as TCE, could be a step forward toward attributing the role of environmental chemicals in autoimmune diseases.

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Fig 1. Anti-MDA- and anti-HNE-protein adduct antibodies in the serum of MRL^{+/+} mice treated with TCE. Anti-MDA-protein adduct antibodies (Fig. 1A) and anti-HNE-protein adduct antibodies (Fig. 1B) were determined by specific ELISAs. The results represent the means $\pm$ SD of six animals in each group. * $P < 0.05$ versus the vehicle-treated controls.

Fig 2. Nitrotyrosine levels in the serum of MRL +/+ mice treated with TCE. Values are means $\pm$ SD of six animals in each group. * $p < 0.05$ versus controls.

Fig 3. iNOS levels in the serum of MRL +/+ mice treated with TCE. Values are means $\pm$ SD of six animals in each group. * $p < 0.05$ versus controls.

Fig 4.

Western blot analysis for iNOS expression in the livers of MRL +/+ mice. (A) iNOS expression in control mice (lanes 1–3) and TCE-treated mice (lanes 4–6). (B) Densitometric analysis of iNOS bands from control and TCE-treated mice. The results represent the means $\pm$ SD. * $p < 0.05$ versus controls.

Fig 5. Induction of autoantibodies in MRL+/+ mice treated with TCE. Serum levels of ANA (5A), anti-ssDNA antibodies (5B) and anti-dsDNA antibodies (5C). The results represent the means $\pm$ SD. * $p < 0.05$ versus controls.

Fig 6.
The correlation of serum anti-MDA-protein adduct antibodies with ANA. The correlation was established by calculating correlation coefficients between anti-MDA-protein adduct antibodies and ANA.

Fig 7.
The anticipated sequence of events leading to autoimmune diseases following trichloroethene-induced oxidative stress.