Inhibition of CYP2E1 Reverses CD4⁺ T-Cell Alterations in Trichloroethylene-Treated MRL+/+ Mice

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Trichloroethylene is an organic solvent that is primarily used as a degreasing agent for metals. There is increasing evidence in both humans and animal models that trichloroethylene promotes the development of autoimmunity, but little is known about the mechanisms that mediate the effect of trichloroethylene on the immune system. Metabolic activation of trichloroethylene is considered an obligatory pathway for other known toxicities such as hepatotoxicity, nephrotoxicity, and carcinogenicity. Trichloroethylene is metabolized by the cytochromes P450, primarily cytochrome P450 2E1 (CYP2E1). To investigate whether metabolism by CYP2E1 is required for immunomodulation, we treated autoimmune prone MRL+/+ mice with trichloroethylene in the drinking water for 4 weeks, in the presence or absence of diallyl sulfide, a specific inhibitor of CYP2E1. Using an antibody that recognizes proteins covalently modified by a reactive metabolite of trichloroethylene; two immunoreactive proteins were detected in liver microsomes from trichloroethylene-treated mice. Formation of these trichloroethylene-protein adducts, an indicator of metabolic activation, was completely inhibited in animals that were concomitantly treated with trichloroethylene and diallyl sulfide. The level of CYP2E1 apoprotein in liver microsomes was significantly reduced in the presence of diallyl sulfide. The enhanced mitogen-induced proliferative capacity of T cells from trichloroethylene-treated MRL+/+ mice was reversed if the mice were also treated with diallyl sulfide. In addition, the reduction in interleukin-4 levels secreted by activated CD4⁺ T cells from trichloroethylene-treated mice was reversed if the mice were also treated with diallyl sulfide. Taken collectively, metabolism of trichloroethylene by CYP2E1 is responsible, at least in part, for the CD4⁺ T cell alterations associated with exposure to this environmental toxicant.

Key Words: trichloroethylene; autoimmunity; MRL+/+; diallyl sulfide; CYP2E1; metabolism.

Trichloroethylene (1,1,2-trichloroethene) is a volatile organic compound that is used in industry as a degreasing agent for metals. Trichloroethylene is also used as a polymer precursor, a dry cleaning agent, and a cleaning agent for computer chips. It has been estimated that more than 400,000 workers employed at 23,225 plants in the United States may be exposed to trichloroethylene annually (Gist and Burg, 1995). Trichloroethylene is released to aquatic systems from industrial discharges to streams and can leach to ground water from landfills (ATSDR, 1993). Approximately 34% of the drinking water supplies that have been tested in the United States are believed to have some trichloroethylene contamination (Westrick et al., 1984). Because of its widespread commercial use and improper disposal, trichloroethylene has become a major environmental pollutant, and it is one of the most abundant organic contaminants found in Superfund sites (Gist and Burg, 1995).

Trichloroethylene is extensively metabolized in both humans and laboratory animals, with the major metabolites being trichloroethanol and its glucuronide conjugate, trichloroacetic acid and trichloroacetaldyhyde (chloral). The metabolism of trichloroethylene is primarily through the cytochrome P450 monooxygenase enzymes, principally the 2E1 isozyme (CYP2E1). We have previously shown that trichloroethylene is activated to a reactive intermediate(s) that primarily binds covalently to a 50 kDa microsomal protein, probably CYP2E1 (Halmes et al., 1996, 1997a,b). In addition, metabolic activation of trichloroethylene is a prerequisite for hepatotoxicity associated with exposure (Buben and O'Flaherty, 1985).

The primary concern for the health effects of trichloroethylene has been its carcinogenic potential (ATSDR, 1993). However, trichloroethylene has recently been implicated in the development of autoimmune disorders and immune system dysfunction in humans. Trichloroethylene exposure has been associated with systemic lupus erythematosus (SLE), systemic sclerosis, fasciitis, and altered T-lymphocyte ratios (Byers et al., 1988; Clark et al., 1994; Flindt-Hansen and Isager, 1987; Kilburn and Warshaw, 1992; Lockey et al., 1987; Waller et al., 1994; Yanez Diaz et al., 1992).

Little is known about potential mechanisms by which trichloroethylene promotes an autoimmune response. An animal model of trichloroethylene-induced acceleration of an autoimmune response has been developed in MRL+/+ mice (Khan et al., 1995). Unlike the genetically similar MRL/prpr mice, which develop a spontaneous autoimmune disease early in life, mediated in part by a Th1-type immune response (Takahashi et al., 1994), the MRL+/+ mice develop a delayed autoimmune response that is accelerated by exposure to trichloroethylene (Oh et al., 1996a,b, 1997a,b). In this model, exposure of MRL+/+ mice to trichloroethylene results in a marked increase in autoantibody levels, lymphadenopathy, splenomegaly, and an increased incidence of lupus nephritis (Oh et al., 1996a,b, 1997a,b). These effects are mediated by the Th1-type immune response, as indicated by the induction of IFN-γ and the presence of systemic lupus erythematosus (SLE) autoantibodies (Oh et al., 1996a,b, 1997a,b). In addition, exposure to trichloroethylene results in an increase in the number of leukocytes and lymphocytes, as well as an increase in the number of CD4⁺ T cells, which is associated with the development of autoimmune disease (Oh et al., 1996a,b, 1997a,b). These effects are mediated by the Th1-type immune response, as indicated by the induction of IFN-γ and the presence of systemic lupus erythematosus (SLE) autoantibodies (Oh et al., 1996a,b, 1997a,b).
al., 1996), the MRL+/+ mice, although autoimmune prone, do not normally develop the clinical symptoms of autoimmunity until late in life. However, MRL+/+ mice treated with trichloroethylene have been shown to exhibit similar immune system alterations seen in the MRL/prlpr mice. Using the MRL+/+ mice, we reported that trichloroethylene treatment promoted expansion of the percentage of CD4+ T cells with a Th1 type cytokine profile. These mice also expressed high levels of CD44, a cell-adhesion receptor that is upregulated following T cell activation (Griffin et al., 2000). This alteration in CD4+ T-cell activity may be involved in the accelerated autoimmune response in MRL+/+ mice exposed to trichloroethylene. Because metabolic activation of trichloroethylene is an obligatory pathway for its hepatotoxic and carcinogenic effect, the present study was undertaken to examine if metabolism by CYP2E1 is also required for the immunomodulatory effects following trichloroethylene exposure.

**MATERIALS AND METHODS**

**Materials.** Trichloroethylene (99+ % purity) and diallyl sulfide (DAS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The emulsifier Alkamuls EL-620 was from Rhone-Poulenc (Cranbury, NJ). Constant rate osmotic pumps with a 200 μl capacity, 0.25 μl/h rate and a 4-week duration were from Alzet (Palo Alto, CA). Concanavalin A (Con A) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Reagents for the Coomassie protein assay were from Pierce (Rockford, IL). Enhanced chemiluminesence Western blotting detection reagents were purchased from Amer sham Life Science (Piscataway, NJ). Rabbit-anti-CYP2E1 antibody was a generous gift of Dr. Magnus Ingelman-Sundberg, Department of Medical Biochemistry and Biophysics, Karolinska Institut, Stockholm. Radiolabeled thymidine (3[H]) was purchased from ICN pharmaceuticals (Costa Mesa, CA).

The antibodies used to detect dichloroacetylated protein-adducts formed by a reactive metabolite of trichloroethylene have been previously described (Halmes et al., 1996). All media, supplements and secondary antibodies used in Western blotting were obtained from Gibco BRL (Grand Island, NY). Recombinant IL-4 was purchased from R & D Systems (Minneapolis, MN). All chemicals and reagents were of the highest purity and quality available.

**Animals and treatments.** Eight-week-old female MRL+/+ mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed in polycarbonate cages, provided with hardwood-chip bedding and maintained on a 12-h light/dark cycle. The animals were provided lab chow and drinking water ad libitum and were acclimated to the upper level of 38°C. The animals were anesthetized with CO2, and blood was collected from the retro-orbital plexus. The spleens were teased apart in RPMI 1640 and CD4+ T cells were isolated using anti-mouse CD4 magnetic beads following manufacturer’s instructions (Dynal, Lake Success, NY). CD4+ T-cell purity was determined by flow cytometry to be ≥95%. Following purification, 2 × 10⁵ cells per well were plated in microtiter plates in RPMI 1640 media supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10⁻⁵ M 2-mercaptoethanol and 10% fetal calf serum. Concanavalin A was added to wells in triplicate at concentrations of 0, 0.4, 1.0, and 4.0 μg/ml. After 72 h at 37°C, the cells were pulsed with 0.5 μCi/well of [³H]-thymidine for an additional 24 h, and the incorporated radioactivity was measured using a Matrix-96 Direct Beta counter (Packard, Meriden, CT).

**Interleukin-4 secretion.** Splenic CD4+ T cells (2 × 10⁵ per well) were incubated for 72 h at 37°C in a 96-well ELISA plate containing immobilized anti-CD3 mAb [(Hamster IgG, clone 145–2C11, Pharmingen, La Jolla, CA) 50 μl of 10 ng/ml for 24 h at 20°C] and soluble anti-CD28 mAb [(Hamster IgG, clone 37.51, Pharmingen, La Jolla, CA) 10 μg/ml]. After 72 h, the supernatants were removed and tested using capture and detecting mAb’s for IL-4 [rat IgG, clone 11B11 and rat IgG, clone BVD6-24G2 (Pharmingen, La Jolla, CA), respectively]. Cytokine concentrations were determined by comparison to a standard curve generated using mouse recombinant IL-4.

**Clinical chemistry.** Serum levels of alanine aminotransferase were measured according to the manufacturer’s instructions with a kit from Sigma Diagnostic Inc (St. Louis, MO). Renal function was determined by measuring blood urea nitrogen levels following manufacturer’s instructions (Sigma Diagnostics Inc., St. Louis, MO).

**Statistical analysis.** When appropriate, statistical significance was determined using one-way analysis of variance with a predetermined significance level of p ≤ 0.05. To determine statistical differences between groups, Student-Neuman-Keuls post-hoc test was used. Data is presented as mean ± standard error of the mean (SEM) for n = 5 mice per group.

**RESULTS**

**Metabolic Activation of Trichloroethylene Is Inhibited by DAS**

To determine if metabolism of trichloroethylene is required for the immunomodulation associated with exposure to the compound, inhibition studies were conducted using DAS, a selective CYP2E1 inhibitor (Reicks and Crankshaw, 1996). We have previously shown that reactive metabolite(s) of trichloroethylene covalently bind to specific proteins in mouse liver and kidney were fixed in neutral buffered formalin and processed for hematoxylin and eosin staining. The other section of liver was used for fraction preparation and Western-blot analysis.

**Western-blotting analysis.** Liver microsomes from individual animals were prepared as previously described (Halmes et al., 1996). Microsomal protein concentration was determined by Coomassie protein assay using bovine serum albumin as the standard. Proteins from the microsomal fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and then transferred to nitrocellulose as previously described (Halmes et al., 1996). Immunoblots were stained using affinity-purified anti-dichloroacetyl antiserum as previously reported (Halmes et al., 1996). The membranes were then stripped of antibodies according to instructions in Amersham’s Enhanced Chemiluminescence (ECL) kit, and reprobed with anti-CYP2E1 antiserum (Eliasson et al., 1988; Johansson et al., 1988). Trichloroethylene-protein adducts and CYP2E1 were visualized using peroxidase-labeled secondary goat anti-rabbit antibodies with an ECL substrate. Immunoblots were scanned using a laser densitometer and the intensity of CYP2E1 staining for individual animals was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
and rat liver tissue (Halmes et al., 1996, 1997a), and human (Griffin et al., 1998) hepatocytes. To determine if metabolic activation of trichloroethylene occurs in MRL+/+ mice treated with the compound, Western blots of liver microsomal protein isolated from MRL+/+ mice were treated for 4 weeks with 2.5 mg/ml trichloroethylene, DAS, trichloroethylene and DAS, or diluent were developed using anti-dichloroacetyl antibodies. As shown in Figure 1, liver proteins from trichloroethylene-treated mice contained two major proteins at 50 and 100 kDa that were detected using anti-DCA antibody. Diallyl sulfide completely inhibited formation of the 50 and 100 kDa trichloroethylene-protein adducts. No immunochemical staining with the anti-DCA antibody was detected in microsomes from control or DAS only treated MRL+/+ mice.

To determine if CYP2E1 protein expression was affected in any of the treatment groups, the nitrocellulose was stripped of antibodies and re-probed for CYP2E1 protein expression. CYP2E1 staining was significantly reduced by 83% in liver microsomal protein from MRL+/+ mice treated with DAS, when compared to liver microsomal protein staining from control mice (Fig. 2). There was also a reduction in CYP2E1 staining in liver microsomal proteins from mice treated with trichloroethylene alone. However, the immunochemical staining of CYP2E1 in mice treated with trichloroethylene alone was significantly more intense than that found in liver proteins from mice treated with DAS alone, or with DAS and trichloroethylene (Fig. 2). This data indicates that DAS inhibited CYP2E1 immunoochemical staining. Trichloroethylene also inhibited CYP2E1 staining, although the degree of inhibition was much less than with DAS.

Inhibition of Trichloroethylene-induced T-cell Mitogenic Response

To determine the importance of trichloroethylene metabolism in the T-cell activation observed in trichloroethylene-treated mice, CD4+ T cells isolated from mice concomitantly treated with DAS and trichloroethylene were tested for their responsiveness to the mitogen Con A. The 4.0 μg/ml dose gave the optimal proliferative response (Fig. 3) and baseline proliferative response was not altered in any treatment group. There was a significant, approximately 2-fold increase in Con A-induced proliferation observed in CD4+ T cells isolated from animals treated for 4 weeks with trichloroethylene as compared to CD4+ T cells isolated from control mice (Fig. 3). However, the increased responsiveness to Con A observed in CD4+ T cells isolated from mice treated with trichloroethylene was reversed if the mice were treated with DAS as well as trichloroethylene. Treatment of mice with DAS alone did not significantly alter the ability of the CD4+ T cells to respond to Con A (Fig. 3). These data suggest that the increased mitogenic response of CD4+ T cells isolated from trichloroethylene-treated mice require metabolism of trichloroethylene.

DAS Reverses Trichloroethylene-induced Inhibition of T-cell Interleukin-4 Production

Trichloroethylene treatment of MRL+/+ mice has been shown to reduce the percentage of CD4+ T cells that secrete IL-4, and correspondingly increase the percentage of CD4+ T cells that secrete INF-γ (Griffin et al., 2000). To determine if...
metabolism of trichloroethylene by CYP2E1 is required for this alteration in cytokine production, CD4\(^+\) T cells isolated from control mice and from mice treated with trichloroethylene in the presence or absence of DAS were examined for their ability to secrete IL-4 when stimulated in vitro. CD4\(^+\) T cells from mice treated with trichloroethylene for 4 weeks produced significantly less IL-4 than CD4\(^+\) T cells isolated from control mice (Fig. 4). This reduction in IL-4 production by CD4\(^+\) T cells from mice treated with trichloroethylene was completely reversed if mice were treated with both trichloroethylene and DAS. CD4\(^+\) T cells isolated from mice treated with DAS alone produced IL-4 levels similar to that of controls. This finding indicates that the ability of trichloroethylene to alter IL-4 production by CD4\(^+\) T cells requires metabolism of this compound.

**Physical Characteristics Unaltered by Treatment with Trichloroethylene**

We have previously reported that MRL\(^{+/+}\) mice treated with trichloroethylene for 4 weeks via the oral route, at the dose used in this experiment, caused no measurable hepatic or renal damage (Griffin et al., 2000). Similarly, in the present study, serum levels of alanine aminotransferase and blood urea nitrogen were unaffected by trichloroethylene treatment when compared to control levels (data not shown). Body, liver, and spleen weight measurements in the present study revealed that no significant physical changes were associated with trichloroethylene, DAS or DAS-plus-trichloroethylene treatments (data not shown). Histological examination of hematoxylin and eosin-stained slides showed no pathological changes in the liver or kidney with any treatment (data not shown). This indicates that the alterations in CD4\(^+\) T cells measured were not due to direct hepatic or renal toxicity.

**DISCUSSION**

Metabolic activation of trichloroethylene appears to be a prerequisite for many of its effects in vivo. For example, Buben and O’Flaherty (1985) found a linear correlation between hepatotoxicity and urinary metabolites indicating a direct relationship between metabolism of trichloroethylene and toxicity. Metabolism of trichloroethylene is also required for tumor formation in mice and rats (Davidson and Beliles, 1991; Goeptar et al., 1995; Green, 1991). Although studies relating metabolic activation of trichloroethylene and induction of cancer are numerous, studies relating metabolic activation of trichloroethylene and immunomodulation are few. The present study investigates whether the immune system changes previously reported in trichloroethylene-treated mice (Griffin et al., 2000; Khan et al., 1995) require metabolism of trichloroethylene by CYP2E1. Specifically we examined whether DAS, an inhibitor of the primary metabolizing enzyme of trichloroethylene, CYP2E1, affected the trichloroethylene-induced alterations in T-cell function.

To confirm the effectiveness of DAS in the present study, we examined the effect of this inhibitor on the CYP2E1-mediated production of trichloroethylene-protein adducts, as a marker of metabolic activation. The trichloroethylene-protein adducts were visualized with an antibody specific for dichloroacetylated proteins. We have previously shown that several liver and lung microsomal proteins are covalently modified by a reactive
metabolite of trichloroethylene (Griffin et al., 1998, 2000; Halmes et al., 1996, 1997a,b). The most predominant trichloroethylene-protein adducts are of 50 and 100 kDa. Similarly, in the present study, two major immunoreactive bands at 50 and 100 kDa were detected in a Western blot analysis of liver microsomal proteins from MRL+/- mice treated with trichloroethylene. However, if the mice were concomitantly treated with trichloroethylene and DAS, metabolic activation of trichloroethylene to a reactive intermediate capable of covalent binding was completely blocked. Although the functional role of these protein adducts has not yet been determined, blocking the production of these adducts in the presence of DAS constituted evidence that this inhibitor was blocking metabolic activity of CYP2E1 in our system.

Inhibition of trichloroethylene-protein adducts suggests that DAS is suppressing the metabolic activity of CYP2E1. Such DAS-induced inhibition has been shown to play a role in the ability of DAS to act as a chemoprotective agent by selectively inhibiting metabolic activation of several toxic compounds (Jin and Baillie, 1997). It has been shown that inhibition of the initial metabolic event facilitated by CYP2E1 constitutes the primary protective action of DAS (Yang et al., 1994). However, the finding that DAS inhibits protein levels of CYP2E1 suggests that DAS may also work in our system by suppressing CYP2E1 protein levels. Along these lines, Reicks and Crankshaw (1996) reported that DAS inhibits both the activity of CYP2E1, and CYP2E1 protein expression in rats following DAS treatment. In addition to DAS, trichloroethylene also somewhat suppressed protein levels of CYP2E1. This reduction in protein expression may be related to the inhibitory effect of trichloroethylene on CYP2E1 enzyme activity as previously noted (Halmes et al., 1997b). Taken together, this data indicates that DAS is acting to inhibit CYP2E1 mediated metabolic activation of trichloroethylene.

With evidence that DAS was effectively inhibiting CYP2E1-mediated metabolic activation of trichloroethylene, we examined the effects of CYP2E1 inhibition on trichloroethylene-induced immune system alterations. This examination centered on CD4+ T cells because we have previously shown that trichloroethylene significantly alters the activation state and cytokine profile of this lymphocyte subset (Griffin et al., 2000). The importance of CD4+ T cells in mediating trichloroethylene-induced autoimmunity is mirrored in other models of idiopathic and experimental autoimmune diseases: CD4+ T cells can often transfer autoimmune disease, while depletion of this T cell subset often suppresses disease induction and/or progression (Gilbert et al., 1999). In addition, similar to trichloroethylene-induced autoimmunity (Griffin et al., 2000), idiopathic systemic immune diseases such as systemic lupus erythematosus, scleroderma, rheumatoid arthritis, and Sjogren’s syndrome are accompanied by an increase in the percentage of activated/memory CD4+ T cells (Gilbert et al., 1999).

The CD4+ T cells in MRL+/- mice, unlike the CD4+ T cells in MRL lpr/lpr mice, are normally not hyperproliferative. However, in accordance with our previous findings demonstrating increased activation of CD4+ T cells in mice treated with trichloroethylene (Griffin et al., 2000), the CD4+ T cells isolated from MRL+/- mice treated for 4 weeks with trichloroethylene exhibited an increased mitogenic response. However, the increased responsiveness of the T cells to Con A was reversed if the mice were treated with both trichloroethylene and DAS. In addition to their increased mitogenic response, the CD4+ T cells isolated from MRL+/- mice treated with trichloroethylene also exhibited a reduction in IL-4 production. A decrease in the percentage of CD4+ T cells that secreted IL-4, and an increase in the percentage of CD4+ T cells that secreted IFN-γ were similarly observed in an earlier study, after 4 weeks of trichloroethylene treatment (Griffin et al., 2000). This finding supports the hypothesis that, at least early in the treatment regimen, trichloroethylene promotes the expansion of Th1 cells. The fact that trichloroethylene skews the CD4+ T cell response toward a Th1-like cytokine profile may help explain the ability of the compound to promote autoimmunity since IFN-γ secretion has been shown to be critical to the development of disease pathology in MRL mice (Haas et al., 1997). It seems likely that IL-4 inhibits the Th1 cell bias needed for disease pathology, since studies on lupus-prone mice have shown that constitutive production of IL-4 through expression of an IL-4 transgene suppresses the development of lupus nephritis (Reiminger et al., 1996). In the present study, it was shown that inhibiting CYP2E1 with DAS in vivo completely reversed the decreased IL-4 production observed in CD4+ T cells isolated from trichloroethylene-treated mice. The reversal of the enhanced mitogenic response, in addition to the reversal of the altered cytokine profile following inhibition of CYP2E1 with DAS in vivo suggests that metabolism of trichloroethylene is an obligatory pathway in the alterations in CD4+ T cells observed following trichloroethylene treatment.

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CYP2E1 with DAS completely reversed the decreased IL-4 production observed in CD4+ T cells isolated from trichloroethylene-treated mice. The reversal of the enhanced mitogenic response, in addition to the reversal of the altered cytokine profile following inhibition of CYP2E1 with DAS in vivo suggests that metabolism of trichloroethylene is an obligatory pathway in the alterations in CD4+ T cells observed following trichloroethylene treatment.


