Chronic Exposure to a Trichloroethylene Metabolite in Autoimmune-Prone MRL+/+ Mice Promotes Immune Modulation and Alopecia

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The industrial solvent trichloroethylene (TCE) is a widespread environmental contaminant known to impact the immune system. In the present study, female MRL+/+ mice were treated for 40 weeks with trichloroacetaldehyde hydrate (TCAH), a metabolite of TCE, in the drinking water. The results were compared with the data from an earlier study in which MRL+/+ mice were exposed to TCAH for 4 weeks. Following a 40-week exposure, the mice developed skin inflammation and dose-dependent alopecia. In addition, TCAH appeared to modulate the CD4⁺ T-cell subset by promoting the expression of an activated/effector (i.e., CD62Llo) phenotype with an increased capacity to secrete the proinflammatory cytokine interferon-γ. However, unlike what was observed after only 4 weeks of exposure, TCAH did not significantly attenuate activation-induced cell death (AICD) or the expression of the death receptor FasL in CD4⁺ T cells. Some metalloproteinases (MMPs) are thought to play a role in susceptibility to AICD by inducing FasL shedding. Thus, both the 4- and 40-week sera were tested for MMP-7 levels in an attempt to explain the disparate results of TCAH on AICD and FasL expression. Serum MMP-7 levels were significantly higher in mice exposed to TCAH for 4 weeks. In contrast, the serum MMP-7 levels were increased in all the mice by 40 weeks when compared with a nonautoimmune strain. Taken together, a chronic exposure to TCAH promotes alopecia and skin inflammation. The early effects of TCAH on MMP-7 levels may provide a mechanism by which TCAH promotes skin pathology.

Key Words: TCAH; rodent; CD4⁺ T cell; alopecia.

INTRODUCTION

Trichloroethylene (TCE) is an organic solvent that is primarily used in industry as a metal degreaser. Because of its widespread commercial use and improper disposal, TCE has also become a major environmental pollutant found in air emissions, water supplies, and soil. TCE is a common contaminant found at Superfund sites and has been identified in at least 852 of the 1416 hazardous waste sites proposed for inclusion on the Environmental Protection Agency National Priorities List (ATSDR, 1997). Although most TCE exposures occur in the occupational setting (Bruckner et al., 1989; Wu and Schaum, 2000), the general population can be exposed via environmental contamination through drinking water, air, or food. As a result, it has been estimated that 10% of the nonoccupationally exposed population has detectable levels of TCE in the blood (Ashley et al., 1994).

TCE has been associated with adverse health effects including autoimmune disease (i.e., lupus and scleroderma) in susceptible individuals (Byers et al., 1988; Flindt-Hansen and Isager, 1987; Hansen and Isager, 1988). Direct evidence of this association has been lacking until recently. A mouse model using autoimmune-prone MRL+/+ mice was developed to study the ability of environmental toxicants, such as TCE, to promote autoimmunity (for review see, Gilbert et al., 1999). The MRL+/+ mouse strain was chosen because a genetic predisposition is thought to be an important aspect of the multifactorial nature of autoimmune disease, including xenobiotic-induced autoimmune disease (Kono et al., 2001; Pollard et al., 2001). The MRL+/+ mouse strain is over 99.6% homozygous to MRL/lpr mice but lacks the lpr mutant Fas gene that causes fulminant lupus-like disease early in life (50% mortality at 6 months). MRL+/+ mice appear normal during the first 6 months of life but eventually develop a milder form of the lupus-like disease (50% mortality at 17 months). The mechanism that makes MRL+/+ mice autoimmune prone is not known, but the disease that develops in MRL/lpr and MRL+/+ mice is dependent on activated, interferon-γ (IFN-γ)–producing CD4⁺ T cells and is characterized by increased levels of total antinuclear antibodies (Balomenos et al., 1998; Haas et al., 1997).

Previous studies in our laboratory using the MRL+/+ model showed that low-dose TCE given in the drinking water initiated...
the generation of autoimmune hepatitis in these mice following a chronic exposure period (Griffin et al., 2000a,b). TCE is metabolized by the oxidative enzyme cytochrome P450 2E1 (CYP 2E1) into oxidative by-products, which include trichloroacetaldehyde hydrate (TCAH) (for review see, Davidson and Beliles, 1991). Experiments in which MRL+/+ mice were exposed to TCE and an inhibitor of CYP 2E1 demonstrated that the immunological effects of TCE required metabolic activity (Griffin et al., 2000c). Further studies conducted in our laboratory showed TCAH activated CD4+ T cells in vivo following a chronic exposure (Blossom et al., 2004). CD4+ T cells from MRL+/+ mice treated in the drinking water with low, occupationally relevant doses of TCAH, unlike CD4+ T cells from water-treated control MRL+/+ mice, demonstrated functional and phenotypic signs of activation, as evidenced by increased IFN-γ production in association with an increased percentage of CD62L+CD4+ T cells. Interestingly, it was also found in the same study that the CD4+ T cells from the TCAH-treated mice showed a decreased susceptibility to the activation-induced cell death (AICD) form of apoptosis following restimulation in vitro. It was recently demonstrated that TCAH attenuated CD4+ T-cell AICD in vitro by increasing metalloproteinase (MMP) activity (i.e., MMP-7), which subsequently facilitated FasL shedding (Blossom and Gilbert, 2006). Although a short-term exposure of MRL+/+ mice was sufficient to activate CD4+ T cells and inhibit their apoptosis, no autoimmune disease pathology was found after 4 weeks. Thus, it seemed likely that a longer exposure to TCAH would be necessary in order to generate tissue pathology like that observed in mice treated long-term with TCE (Griffin et al., 2000b). Consequently, the present study was undertaken to expand our evaluation of TCAH in vivo following a chronic exposure.

**MATERIALS AND METHODS**

**Animals and treatment.** All mice used for the experiments described in the current study were purchased from The Jackson Laboratories (Bar Harbor, ME), maintained in the animal facility at Arkansas Children’s Hospital Research Institute, and housed in polycarbonate ventilated cages (Animal Care Systems, Littleton, CO). The animals were provided standard laboratory chow and drinking water ad libitum and were acclimated for 1 week prior to exposure. All studies were approved by Animal Care and Use Committee of the University of Arkansas for Medical Sciences. For the chronic exposure study, 4- to 6-week-old female MRL+/+ mice were given TCAH (purity 99%; obtained from Sigma, St Louis, MO) resuspended in deionized water. Freshly made solutions of TCAH were provided every 2–3 days to ensure maintenance of the dose. Eight mice per treatment group received either TCAH or deionized water at concentrations of 0, 0.1, 0.3, or 0.9 mg/ml for 40 weeks. These doses were used previously in a 4-week study and are known to reflect levels that can be encountered following occupational exposure to the parent compound, TCE (Blossom et al., 2004; Davidson and Beliles, 1991). Based on average water consumption and body weight, the MRL+/+ mice given drinking water containing TCAH at 0.1, 0.3, and 0.9 mg/ml were exposed to the toxicant in amounts of approximately 13, 46, and 143 mg/kg/day. For the analysis of serum MMP-7 levels for the 4-week time point, a different group of TCAH-exposed female MRL+/+ mice was used as described previously (Blossom et al., 2004). In some experiments, age-matched nonautoimmune female C3H/HeJ mice and young female MRL+/+ mice (water only) were used as controls.

**Pathology.** MRL+/+ mice were evaluated for gross physical manifestations of disease throughout the study. Hair loss in individual mice was assessed by two independent investigators and is presented as the percentage of mice with alopecia per group over time. At the time of sacrifice, livers, kidneys, and macroscopic skin lesions identified at necropsy were fixed in 10% neutral buffered formalin (Fisher, St Louis, MO) and embedded in paraffin. Tissues were sectioned (5 μm thick) and stained with hematoxylin and eosin (H&E) and examined using a light microscope. For the skin sections, the severity of inflammation was graded by a veterinary pathologist in a blinded manner using the following parameters: acanthosis (i.e., dermal thickening), inflammation, fibrosis, vessel dilation, mast cell hyperplasia, and ulceration. The histologic changes in the liver and kidney were numerically scored from 0 to 4 ranging from no change to severe, respectively. The location and composition of all inflammatory infiltrates were recorded.

**Serology.** MRL+/+ mice that were exposed to TCAH for 4 weeks (Blossom et al., 2004) or 40 weeks were bled and evaluated individually for total MMP-7. Sera from age-matched C3H/HeJ mice were used for comparison purposes to demonstrate the propensity of the MRL strain to produce MMP-7. Total immunoglobulin (Ig) and autoantibody (autoab) levels were also evaluated individually in sera of MRL+/+ mice exposed to TCAH for 40 weeks. Age-matched female C3H/HeJ mice and young female (4–6 weeks old) MRL+/+ mice were used as negative controls for the enzyme-linked immunosorbent assays (ELISAs). The mice were anesthetized with Sodium Nembutal (1 mg/kg body weight), and blood was collected from the retroorbital plexus. Blood was allowed to clot at room temperature for at least 1 h and centrifuged at 1000 × g for 30 min. The sera were collected and kept at −20°C until assay. For examination of serum MMP-7 by Western blotting analysis, equal amounts of sera (4 μl) from four independent groups consisting of two mice each per each treatment (n = 4) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 12% Tris-HCL polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose was then immunoblotted with an antibody specific to MMP-7 (rabbit anti-MMP-7; Calbiochem, LaJolla, CA). The appropriate horseradish peroxidase–labeled secondary antibody was then applied. The bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). The images were scanned for densitometric analysis with the Versadoc Imaging System (BioRad, Hercules, CA).

The relative levels of anti–single-stranded DNA (ssDNA), anti–double-stranded DNA (dsDNA), and anti-histone were determined by ELISA as described previously (Blossom et al., 2004) with some modifications. Antigen preparation was as follows: Histone (1 mg/ml from Sigma) or denatured ssDNA, or native dsDNA (1 mg/ml from Sigma) were mixed with reacti-bind coating solution (Pierce Biotechnology) and incubated in a glass tube on a rotator for 10 min. Following an overnight incubation at 4°C, the plates were washed with a solution containing 1× phosphate-buffered saline (PBS) and 0.5% tween-20 (Sigma) and blocked by adding 0.2 ml per well 1× PBS and 10% fetal calf serum (FCS). The plates were then washed, and the serum samples (1:100) were added to the wells (0.1 ml per well) overnight at 4°C. Polyclonal biotinylated goat anti-mouse Ig (Sigma) was diluted 1:1000 and added to the plates for 1 h at room temperature. The plates were developed with extravidin alkaline phosphatase, 0.1 ml per well, and alkaline phosphatase substrate (p-nitrophenol) and measured by an ELISA reader (absorbance 405 nm). The sera of individual mice were also tested for the presence of total
Ig isotypes, IgM, IgG1, and IgG2a by ELISA as described above. All capture and detecting monoclonal antibodies (mAbs) were purchased from BD PharMingen (LaJolla, CA). Capture mAbs included purified IgG1 mAb (clone A85-3, rat IgG1), purified IgG2a mAb (clone R11-89, rat IgG1), or purified IgM mAb (clone 11/41, rat IgG2a). Detecting mAbs included biotinylated anti-mouse IgG1 (clone A85-1, rat IgG1), biotinylated anti-mouse IgG2a, (clone R19-15, rat IgG2a), and biotinylated anti-mouse IgM (clone R6-602, rat IgG2a). Levels of total Ig were quantified using standards consisting of purified mouse myeloma IgM, IgG1, or IgG2a (Zymed Laboratories, San Francisco, CA).

Phenotypic analysis of splenic or lymph node cells. At sacrifice, spleens and mesenteric and inguinal lymph nodes were harvested from individual mice. Cell suspensions were obtained by gently teasing the lymphoid organs with RPMI and sterile forceps to release the cells. Spleen lymphocyte cellularity was determined in individual mice by trypan blue exclusion and light microscopy using a hemacytometer. For staining and flow cytometry, the lymphocytes were pooled accordingly: equal numbers of cells from the eight mice per treatment were pooled into three independent groups for an n = 3 consisting of two to three mice per treatment. Unless otherwise specified, all the antibodies in this study were purchased from BD Biosciences, San Jose, CA. The cells were incubated with either fluorescein isothiocyanate (FITC)-labeled anti-CD62L (clone MEL-14, rat IgG1), and either phycoerytherin (PE)-anti-CD4 (clone GK1.5, rat IgG2a), or PE–anti-CD8 (clone 53-6.7, rat IgG2a) for 30 min at 4°C. The phenotypic analysis of 10,000 events per group was carried out using a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA), and the data are presented as histograms of CD4+ or CD8+ T cells. Nonviable cells, based on low forward scatter and side scatter, were excluded in each sample. Data analysis was performed with the use of WinMDI software. For all groups tested, staining with PE or FITC isotype Ig control was also examined.

Cytokine profile analysis. Equal numbers of splenic lymphocytes from individual mice were pooled into three independent groups per exposure for an n = 3 per group as described above and enriched for CD4+ T cells by positive selection using magnetic bead kits (Dynal Biotech, Lake Success, NY). The CD4+ T cells were incubated at 37°C in medium alone or in a 96-well tissue culture plate containing immobilized anti-CD3ε antibody and anti-CD28 antibody as described (Blossom et al., 2004). Culture supernatants were collected after 48 h and tested for Th1/Th2 cytokines, IFN-γ, interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and IL-4, using cytometric bead array (CBA) kits for flow cytometry (BD PharMingen) according to the manufacturer’s instruction on a FACSCalibur flow cytometer using BD CBA software (BD Biosciences).

Induction and measurement of AICD and FasL on CD4+ T cells. Splenic lymphocytes were pooled for an n = 3 as described above. The cells (2 × 10^6 cells/ml) were placed into six-well plastic tissue culture dishes in RPMI medium supplemented with 2mM l-glutamine, 1mM nonessential amino acids, 1mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10^{-5}M 2-mercaptoethanol, and 10% FCS. AICD was induced by activating the pooled spleen cells with Concanavalin A (type V and VI from Canavalia ensiformis, Sigma; 5 μg/ml) and IL-2 (R&D systems, Minneapolis, MN; 5 ng/ml) for 4 days at 37°C in 6% CO2 humidified air. Viable cells were isolated by passage over Ficoll-Hypaque and then stimulated with immobilized anti-CD3ε antibody (5 μg/ml) for 18 h as described (Blossom et al., 2004). After stimulating the cells to undergo AICD, the cells were stained with PE–anti-CD4 for 30 min at 4°C in conjunction with FITC-labeled Annexin V as described previously (Blossom et al., 2004). The T cells induced to undergo AICD were also examined for their expression of Fas or FasL. Cells were incubated with PE–anti-CD4 antibody and either purified anti-FasL (clone MF14, Armenian hamster IgG3) or Fas (clone Jo2, Armenian Hamster IgG2a) followed by FITC-labeled rat anti-hamster IgG heavy and light chain (Caltag laboratories, San Diego, CA). The analysis of 10,000 events per sample was carried out using a FACSCalibur flow cytometer as described above. Data analysis was performed with the use of WinMDI software. For all groups tested, staining with isotype Ig controls was also examined.

RESULTS

Chronic Exposure to TCAH In Vivo Promoted Alopecia and Skin Inflammation

To test whether a chronic exposure to TCAH promoted the development of autoimmune pathology, female MRL+/+ mice were treated in vivo for 40 weeks with 0, 0.1, 0.3, and 0.9 mg/ml TCAH in the drinking water. At approximately 24 weeks of exposure, it was noted that some of the mice developed diffuse patches of hair loss over all areas of the body. By 40 weeks, 75% of mice exposed to 0.9 mg/ml TCAH and 50% of mice exposed to 0.3 and 0.1 mg/ml TCAH developed alopecia compared with only 25% of water-treated MRL+/+ mice (Fig. 1A). The alopecia found in the 0.9-mg/ml exposure group was statistically significant from that of control mice beginning at week 35 of exposure. In addition to hair loss, some of the mice presented with skin lesions characterized by crusts, scabbing, and ulceration. Five of the eight mice in the 0.9-mg/ml TCAH group had skin lesions that were localized to the dorsal neck region, upper back, and ventral surface (data not shown). In addition, five of the eight mice exposed to 0.3 mg/ml TCAH had similar lesions, and three of these animals had severe tail lesions (data not shown). In contrast, only one mouse in the water control group had a skin lesion in the right flank area, and none of the mice exposed to 0.1 mg/ml TCAH had skin lesions (data not shown). The lesions were subsequently sectioned and stained with H&E. Light microscopic observation of the skin lesions in the TCAH-exposed MRL+/+ mice revealed areas of significant inflammation. In a representative H&E slide from a mouse exposed to 0.9 mg/ml TCAH, Figure 1B shows a focal area of chronic dermatitis characterized by mononuclear inflammation, mast cell hyperplasia, dermal fibrosis, and epidermal thickening. Arrows indicate mast cells in the deep dermis, and the arrowhead points to a region of supplicative inflammation. Figure 1C shows a smaller focus of suppurative inflammation adjacent to a hair follicle on a background of dermal mastocytosis from a different mouse in the 0.9-mg/ml group. Another section of skin from a mouse exposed to 0.9 mg/ml TCAH showed significant mononuclear cell infiltration, mast cell hyperplasia, and fibrosis. The arrow points to vesicle formation between the dermal and epidermal layers (Fig. 1D). This type of pathology is consistent with what is observed in the autoimmune disease, Bullous Pemphigoid. In contrast, the skin lesion that was observed in the water-treated control MRL+/+ mouse demonstrated no significant inflammation (data not shown). Despite the alopecia and skin pathology,
TCAH treatment failed to induce kidney or liver disease commensurate with lupus nephritis or autoimmune hepatitis, respectively, in any of the mice (data not shown). Thus, TCAH promoted a dose-dependent alopecia in association with dermal inflammation.

**TCAH Exposure Did Not Promote Serological Signs of Autoimmunity**

Serum levels of anti-ssDNA, anti-dsDNA, and anti-histone were used as markers of autoimmune disease in the MRL+/- mice, all of which are known to increase in MRL+/- mice with age (Laderach et al., 2003). As expected, serum autoab levels increased in the MRL+/- mice in all exposure groups, as compared with serum from nonautoimmune C3H/HeJ mice or young MRL+/- mice (Fig. 2A). However, TCAH exposure did not increase autoab levels over that of MRL+/- water controls. Instead, TCAH exposure appeared to suppress the increase in serum autoabs that normally accompanies idiopathic disease progression in older MRL+/- mice, although this effect was not significant. Levels of total Igs were also evaluated in the sera. As expected, IgM, IgG2a, and IgG1 increased with age in all the groups (Fig. 2B). However, IgM levels were not elevated to the degree that was shown in water-treated control mice, and this effect was statistically significant. Although all the mice had higher levels of IgG2a relative to IgG1, there was no TCAH affect. Thus, the age-related increase in autoabs or total Ig that is typically associated with idiopathic lupus in the MRL strain was not enhanced by TCAH exposure.

**TCAH Exposure Promoted a CD62L<sup>lo</sup> Phenotype with the Splenic CD4<sup>+</sup> T-Cell Population**

Lymphocytes from the spleens and mesenteric and inguinal lymph nodes were counted at sacrifice to determine cellularity. Although there was a slight decrease in cellularity from the spleens of mice exposed to TCAH, there was no corresponding difference among the groups in terms of spleen weight (Table 1). Mice exposed to 0.3 and 0.9 mg/ml TCAH had significantly fewer CD4<sup>+</sup> T cells per spleen in comparison to controls, yet the other subpopulations were unaffected by TCAH exposure. Surface expression of markers indicative of activation were also evaluated on splenic or lymph node CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells. The only activation marker shown to be altered by TCAH exposure was CD62L. CD62L is an adhesion marker whose expression on T lymphocytes (CD62L<sup>hi</sup>) is associated with a naive phenotype and whose loss of expression (CD62L<sup>lo</sup>) is used as a marker of activated/effector T lymphocytes (Ahmadzadeh et al., 2001; Chao et al., 1997). TCAH increased the percentage of CD4<sup>+</sup> T cells that expressed CD62L<sup>lo</sup> in the spleens. These data are illustrated in a histogram representing one sample from each exposure group (Fig. 3). In contrast to splenic CD4<sup>+</sup> T cells, there was no apparent effect of TCAH in lymph node CD4<sup>+</sup> T cells with respect to...
CD62L expression. Consistent with our previous results, TCAH did not alter CD62L expression in CD8<sup>+</sup> T cells (data not shown). Taken together, the phenotypic analysis revealed that similar to short-term exposure, a chronic treatment with TCAH<sub>vivo</sub> increased the percentage of CD62L<sup>lo</sup> CD4<sup>+</sup> T cells.

CD4<sup>+</sup> T Cells from TCAH-Exposed MRL<sup>+</sup>/<sup>+</sup> Mice Secreted Increased Levels of IFN-γ

IFN-γ is a crucial mediator of disease pathology in the MRL model (Balomenos et al., 1998). The production of this cytokine by CD4<sup>+</sup> T cells was increased in both TCAH- and TCE-exposed mice at early (4 weeks for TCAH and TCE) and

### TABLE 1

<table>
<thead>
<tr>
<th>TCAH (mg/ml)</th>
<th>Weight (mg)</th>
<th>Cellularity (× 10&lt;sup&gt;7&lt;/sup&gt;)</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; (× 10&lt;sup&gt;7&lt;/sup&gt;)</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt; (× 10&lt;sup&gt;7&lt;/sup&gt;)</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt; (× 10&lt;sup&gt;7&lt;/sup&gt;)</th>
</tr>
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<tr>
<td>0</td>
<td>158 ± 54</td>
<td>21.0 ± 6.6</td>
<td>2.33 ± 0.3</td>
<td>1.05 ± 0.6</td>
<td>5.02 ± 1.1</td>
</tr>
<tr>
<td>0.1</td>
<td>120 ± 48</td>
<td>10.9 ± 4.7*</td>
<td>1.42 ± 0.4</td>
<td>0.72 ± 0.3</td>
<td>3.64 ± 1.4</td>
</tr>
<tr>
<td>0.3</td>
<td>109 ± 27</td>
<td>16.0 ± 6.3</td>
<td>1.33 ± 0.3*</td>
<td>1.13 ± 0.3</td>
<td>3.90 ± 0.3</td>
</tr>
<tr>
<td>0.9</td>
<td>137 ± 37</td>
<td>20.3 ± 6.0</td>
<td>1.51 ± 0.4*</td>
<td>0.62 ± 0.1</td>
<td>3.61 ± 1.3</td>
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</tbody>
</table>

**Note.** Equal numbers of spleen cells from the eight mice per treatment group were pooled into three independent groups and stained with FITC–anti-CD4 and PE–anti-CD8, or PE–anti-B220. The percentages were obtained by gating around the PE- or FITC-positive populations. Cell numbers were determined by multiplying the percent gated cells by the total number of lymphocytes counted in the spleens. The numbers in the table represent the mean ± SD cell number from the three independent groups per treatment. FITC, fluorescein isothiocyanate; PE, phycoerythrin; TCAH, trichloroacetaldehyde hydrate.

*Significantly different from control values (p < 0.05) using one-way ANOVA and Tukey’s comparison.
late (32 weeks for TCE) time points (Blossom et al., 2004; Griffin et al., 2000a,b). Likewise, as demonstrated in Figure 4, there was a pronounced dose-dependent increase in CD4+ T-cell IFN-γ production (1580, 3236, 5754, and 5758 pg/ml; 0, 0.1, 0.3, and 0.9 mg/ml TCAH, respectively). In contrast, TCAH did not have a significant effect on IL-2, TNF-α, or IL-4 production by CD4+ T cells. Thus, TCAH appeared to skew CD4+ T cells toward a Th1-like response by increasing the production of IFN-γ.

**Differential Effects of TCAH on Susceptibility of CD4+ T Cells to AICD and MMP-7 Levels Observed at 4 or 40 Weeks of Exposure**

The splenic CD4+ T cells from the MRL+/+ mice were evaluated for their susceptibility to AICD, a parameter shown to be inhibited by TCAH following 4 weeks of exposure (Blossom et al., 2004). In contrast, in MRL+/+ mice exposed to TCAH for 40 weeks, TCAH did not significantly alter AICD susceptibility or affect the expression of the death receptor FasL on CD4+ T cells following restimulation in vitro with anti-CD3 antibody (Table 2). Although there was a slight decrease in the amount of Annexin and FasL staining on CD4+ T cells from TCAH-exposed mice, the effect was not significant.

It is believed that the mechanism by which TCAH attenuated AICD in CD4+ T cells at 4 weeks of exposure was related to its ability to induce FasL cleavage in a MPP-dependent manner. The MPP found to be increased in lymphocyte cultures exposed to TCAH in vitro was MMP-7 (Blossom and Gilbert, 2006). MMP-7 is a known FasL sheddase (Vargo-Gogola et al., 2002). In an effort to understand the disparate results regarding CD4+ T-cell AICD following 4- or 40-week exposure in vivo, levels of the proenzyme form of MMP-7 were evaluated in the sera from the mice in the current study and from serum samples remaining from the 4-week study. MRL+/+ mice that were treated for 4 weeks with TCAH demonstrated a significant and dose-dependent increase in serum levels of MMP-7 (Figs. 5A and 5B). In contrast, at the 40-week time period, even more MMP-7 was detected in all the mice, however; the levels were not enhanced by TCAH exposure. The data in Figure 5C demonstrated that the levels of MMP-7 from water-only MRL+/+ mice at 4 and 40 weeks were much higher when compared with haplotype-matched, nonautoimmune water-only C3H/HeJ mice. Thus, TCAH appeared to exacerbate an existing MRL strain tendency to produce MMP-7 levels after only 4 weeks of exposure.

### TABLE 2

<table>
<thead>
<tr>
<th>TCAH (mg/ml)</th>
<th>% Fas</th>
<th>% FasL</th>
<th>% Annexin V</th>
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<tbody>
<tr>
<td>0</td>
<td>91 ± 2.0</td>
<td>57 ± 9.8</td>
<td>78 ± 3.5</td>
</tr>
<tr>
<td>0.1</td>
<td>91 ± 5.0</td>
<td>60 ± 0.5</td>
<td>77 ± 1.5</td>
</tr>
<tr>
<td>0.3</td>
<td>86 ± 1.7</td>
<td>46 ± 8.3</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>0.9</td>
<td>91 ± 4.9</td>
<td>50 ± 23</td>
<td>68 ± 8.5</td>
</tr>
</tbody>
</table>

**Note.** Equal numbers of splenic cells from each group (n = 3) were pooled and activated with Concanavalin A and IL-2 for 4 days. The cells were Ficoll purified and restimulated on 96-well plates that had been precoated with anti-CD3 antibody. Cells cultured in uncoated plates were used as a control (data not shown). After 18 h, AICD was determined by staining the cells with PE–anti-CD4 antibody and FITC–Annexin V, FITC–anti-FasL, or FITC–anti-Fas antibody and examined by flow cytometry. The percentages indicate FITC staining determined by isotype control fluorescence after gating on the CD4+ T-cell population. The data represent the mean ± SD percentage of CD4+ T cells that were also FITC positive. AICD, activation-induced cell death; FITC, fluorescein isothiocyanate; IL, interleukin; PE, phycoerytherin; TCAH, trichloroacetalddehyde hydrate.

A 40-Week Exposure to TCAH Did Not Induce Overt Toxic Effects

Throughout the study, the mice were monitored once weekly for signs of weight loss, a parameter used in previous studies as an indicator of disease (Griffin et al., 2000b). There were no significant differences in body weight among the groups of animals at any time during the course of the study (data not shown). In addition to monitoring body weight, the TCAH-exposed MRL+/+ mice were evaluated at the end of the 40-week exposure period for any gross physiological changes induced by a chronic exposure to TCAH. Although none of the mice died prior to sacrifice, one mouse in the 0.1-mg/ml TCAH exposure group was eliminated from the study due to suspected hepatocellular carcinoma noted at the time of sacrifice. Subsequent histopathological analysis of the liver confirmed this suspicion. However, further evaluation of liver and kidney histology in the remaining mice at sacrifice revealed no sign of toxicant-induced tissue damage (data not shown). Therefore, with the exception of one animal, TCAH was not shown to be carcinogenic or overtly toxic.

In terms of daily dose, the levels of TCAH given to the mice were within the range of exposure amounts determined in our previous study (Blossom et al., 2004). Table 3 shows that the amount of water consumed by the TCAH-exposed mice calculated to an average daily dose of TCAH of 13, 46, and 143 mg/kg/day (for 0.1, 0.3, and 0.9 mg/ml, respectively). Although all the groups of TCAH-treated MRL+/+ mice consumed less water than control MRL+/+ mice, the doses of TCAH for all groups encompassed physiologically relevant levels of TCAH exposure.

**DISCUSSION**

In the present study, TCAH, the major oxidative metabolite of the environmental toxicant TCE, was administered to
MRL+/+ mice in the drinking water for 40 weeks. Remarkably, TCAH exposure promoted alopecia and skin inflammation but not autoimmune hepatitis. It is not clear how TCAH mediated these skin disturbances. However, more obvious factors that can induce similar pathology in normal laboratory mice can be ruled out. For example, none of the mice showed signs of bacterial infection at necropsy (data not shown). In addition, the alopecia observed in the mice in the present study did not appear to be caused by a barbering (grooming) effect. Barbering in rodents typically occurs around the face (Garner et al., 2004), and the alopecia observed in TCAH-exposed mice covered a significant area of the entire body. In addition, the alopecia was evenly distributed among the cages of mice, indicating that the hair loss was not due to barbering that can occur via a dominant (nonalopecic) cage mate. Thus, the alopecia and skin inflammation in TCAH-exposed mice did not appear to be caused by these non-immunological factors known to promote alopecia and skin inflammation in mice.

Skin disorders in the MRL strain have been known to occur spontaneously. This characteristic led other investigators to use MRL/lpr and MRL+/+ mice as models of cutaneous lupus (Furukawa and Yoshimasu, 2005). Macroscopically, the cutaneous lesions in older MRL+/+ mice are characterized by scab formation and alopecia, particularly on the upper dorsal sites (Furukawa et al., 1984). Histologically, spontaneous skin lesions in MRL+/+ mice have been described as being generally mild and characterized by mononuclear cell infiltration, acanthosis, hyperkeratosis, and fibrosis (Furukawa et al., 1996). In contrast, the

![FIG. 3. MRL+/+ mice given trichloroacetaldehyde hydrate (TCAH) for 40 weeks had a higher percentage of splenic CD62Llo-expressing CD4+ T cells. Equal numbers of spleen or mesenteric and inguinal lymph node cells from eight mice per treatment group were pooled into three independent subgroups consisting of two to three mice for an n = 3. The pooled cells were then two-color stained for phycoerythrin–anti-CD4 monoclonal antibody (mAb) and fluorescein isothiocyanate–anti-CD62L mAb and subjected to flow cytometry. The flow cytometric histograms are representative of a single staining group. The darker histograms represent CD62L staining of CD4+ cells from mice treated with TCAH. The lighter histograms represent CD62L staining of CD4+ T cells from mice treated with water alone. The numbers indicate the percent CD62Llo CD4+ T cells from MRL+/+ mice treated with water versus TCAH.](image-url)
distribution of the skin lesions and the pathology in the TCAH-exposed animals appeared to be different than typical lesions described in the literature for MRL mice. For example, the TCAH-exposed mice in the present study experienced more aggressive skin lesions that occurred over a larger skin region, including the tail area, than those described in the literature for cutaneous lupus. In addition, the alopecia, skin alterations, and inflammatory infiltrate in TCAH-exposed mice were not associated with increased levels of serum antibodies or lupus nephritis commonly associated with cutaneous lupus in older MRL mice. Given TCAH’s effects on CD4\(^+\) T cells, it is possible that the skin lesions in the TCAH-exposed mice were caused by activated/effector CD4\(^+\) T cells that had infiltrated dermal tissues. It is also possible that the ability of TCAH to increase IFN-\(\gamma\) production by CD4\(^+\) T cells represents a mechanism of promoting alopecia and skin inflammation, since spontaneous alopecia in rodents appears to involve the production of Th1 cytokines (Freysschmidt-Paul et al., 2005).

The CD62L molecule on CD4\(^+\) T cells was also shown to be subject to TCAH regulation in the present study. TCAH exposure promoted CD62L downregulation in cloned Th1 CD4\(^+\) T cells, as well as in naive CD4\(^+\) T cells from MRL+/- mice in vitro (Gilbert et al., 2004) and in vivo following a 4-week exposure with the same concentrations of TCAH used in the study. 

### Table 3

<table>
<thead>
<tr>
<th>TCAH (mg/ml)</th>
<th>Number of mice</th>
<th>Duration of exposure (days)</th>
<th>Average water consumed (ml/day per mouse)</th>
<th>TCAH (mg/kg/day)</th>
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<tr>
<td>0</td>
<td>8</td>
<td>283</td>
<td>6.6 + 0.07</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
<td>5.3 + 0.14*</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>8</td>
<td>5.6 + 0.54</td>
<td>45.5</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>8</td>
<td>6.0 + 0.29*</td>
<td>143</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** Water consumption was measured every 2–3 days. The data are presented as total amount of water consumed/day per mouse. Based on water consumption figures, the amount of TCAH consumed (mg/kg/day) could be calculated. TCAH, trichloroacetaldehyde hydrate.

*These data are significant (\(p < 0.05\) by one-way ANOVA and Tukey’s comparison).
The present study (Blossom et al., 2004). It is not known how TCAH regulates CD62L expression. Mature mouse CD62L is constitutively expressed on a variety of cell types, including naive CD4+ and CD8+ T lymphocytes as a 90-kDa glycoprotein (Chao et al., 1997). Under conditions that promote T-cell activation, CD62L expression is downregulated and progressively lost over time as a result of shedding due to the presence of a disintegrin and metalloprotease (ADAM-17 or TACE) (Smalley and Ley, 2005). The functional importance of the CD62Llo population in TCAH-exposed mice is not known. However, the loss of CD62L has been shown to be important in allowing T-cell extravasation into inflammatory sites in non-lymphoid tissues (Galkina et al., 2003), a feature that may be important in promoting the skin pathology observed in TCAH-exposed animals. Alternatively, the mechanism by which TCAH regulates CD62L expression may be similar to how it modulates FasL expression, since both require MMP activity for cleavage from the cell surface.

FIG. 5. Trichloroacetaldehyde hydrate (TCAH) promotes metalloproteinase-7 (MMP-7) levels in the serum of MRL+/- mice as early as 4 weeks of exposure. Two separate groups of eight female MRL+/- mice per treatment were exposed to TCAH for 4 or 40 weeks (0, 0.1, 0.3, or 0.9 mg/ml in the water). (A) Equal amounts of sera were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with an antibody specific for mouse MMP-7. Each lane represents pooled sera from four independent groups consisting of two mice for an n = 4 per treatment. (B) The Western blots were evaluated by densitometric analysis of scanned gels ± SD from pooled sera shown in (A) and expressed as percent adjusted volume. Statistical differences were determined by one-way ANOVA with Tukey’s comparison; *p < 0.05 or **p < 0.01. (C) Equal amounts of protein obtained from sera of two water-only female MRL+/- mice and two age-matched, water-only female C3H/HeJ mice were tested for pro-MMP-7 levels as in (A).
In the present study, TCAH exposure in vivo only had a slight effect on CD4$^+$ T-cell AICD and FasL expression. AICD that is dependent on Fas-FasL interactions is thought to comprise a mechanism to limit the expansion of activated CD4$^+$ T cells, some of which are self-reactive (Green et al., 2003). The importance of AICD is underlined by the fact that defects in the Fas-FasL pathway are found in several idiopathic autoimmune diseases in mice and in humans (Szodorai et al., 2003; Zhang et al., 2001). TCAH inhibited apoptosis by decreasing FasL expression in an MMP-dependent manner (Blossom and Gilbert, 2006). FasL expression on the T-cell surface is regulated at the level of transcription, as well as by cleavage of membrane-bound FasL into the less bioactive form of soluble FasL by MMP-7 and MMP-3 (Oyaizu et al., 1997; Tanaka et al., 1998; Vargo-Gogola et al., 2002). MMPs are usually produced as proenzymes that are controlled by a balance between activation by other enzymes and inactivation by interaction with tissue inhibitors of metalloproteinases.

There was an interesting correlation between serum MMP-7 levels and TCAH’s effects on CD4$^+$ T-cell AICD. TCAH exposure promoted high levels of the proenzyme form of MMP-7 in association with a significant attenuation of AICD and FasL expression on CD4$^+$ T cells after only 4 weeks of exposure in a group of mice from a separate study (Blossom et al., 2004). However, in the current study, after 40 weeks of exposure, MMP-7 levels were elevated in all groups of mice, as compared with a nonautoimmune strain, when TCAH’s effects on AICD were less pronounced. This discrepancy may be related to the inherent ability of the MRL$^+$/+ strain to produce certain MMPs. Tissue levels of MMP-2 and MMP-9 were shown to be much higher in MRL$^+$/+ mice as compared with C57BL/6 mice (Gourevitch et al., 2003). It is this characteristic of higher baseline MMP activity observed in both the MRL/lpr and MRL$^+$/+ strains that gives them an unusual capacity for wound healing (McBrearty et al., 1998). It is also possible that the MRL strains’ propensity to produce MMPs may also play a role in its susceptibility to develop autoimmune disease. Studies to evaluate the effects of TCAH in the nonautoimmune mice C3H/HeJ mice are currently underway in our laboratory.

It is interesting that TCE and TCAH induced pathology in completely different target organs following chronic exposure. It is possible that the TCE-induced liver pathology is due to the capacity of TCE, but not TCAH, to form adducts with cellular proteins, that is, CYP 2E1, in the liver (Halmes et al., 1996). Indeed, TCE adducts can stimulate the production of adduct-specific antibodies in the serum (Griffin et al., 2000a). Thus, TCAH-exposed mice demonstrated many of the same alterations in CD4$^+$ T cells seen in the TCE-treated mice. However, the TCAH-treated mice did not develop CYP 2E1 adducts (data not shown) or autoimmune hepatitis. Exposure to TCAH is apparently sufficient to alter CD4$^+$ T-cell function, MMP-7 activity, and promote skin pathology, but exposure to the parent compound TCE is needed to generate autoimmune hepatitis specifically. Why TCAH exposure targets the skin and hair follicles is not known, but studies to address the possibilities will be planned in the future.

The findings presented in the current study provide a link between immune-mediated disease pathology and chronic toxicant exposure. Humans can be exposed to TCAH as a result of occupational or environmental exposure to TCE. The Occupational Safety and Health Administration states that the 8-h permissible exposure limit for TCE should not exceed a time-weighted average of 100 ppm (ATSDR, 1997). Thus, the concentrations of TCAH used here to treat the mice may be considered functionally relevant. The doses of TCAH used in this study were low, nontoxic, and administered to the mice in the drinking water over a long period of time, a situation that may more realistically portray exposures for humans in the occupational setting. Thus, the information provided in this study has the potential to aid in human risk assessment for not only TCE but also other environmental toxicants.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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two common environmental toxicants, trichloroacetaldehyde hydrate and trichloroacetic acid. J. Autoimmun. 23, 211–220.


