Coexposure to Mercury Increases Immunotoxicity of Trichloroethylene

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We have shown previously that chronic (32 weeks) exposure to occupationally relevant concentrations of the environmental pollutant trichloroethylene (TCE) induced autoimmune hepatitis (AIH) in autoimmune-prone MRL+/+ mice. In real-life, individuals are never exposed to only one chemical such as TCE. However, very little is known about the effects of chemical mixtures on the immune system. The current study examined whether coexposure to another known immunotoxicant, mercuric chloride ($HgCl_2$), altered TCE-induced AIH. Female MRL+/+ mice were treated for only 8 weeks with TCE (9.9 or 186.9 mg/kg/day in drinking water) and/or $HgCl_2$ (260 μg/kg/day, sc). Unlike mice exposed to either TCE or $HgCl_2$ alone, mice exposed to both toxicants for 8 weeks developed significant liver pathology commensurate with early stages of AIH. Disease development in the coexposed mice was accompanied by a unique pattern of anti-liver and anti-brain antibodies that recognized, among others, a protein of approximately 90 kDa. Subsequent immunoblotting showed that sera from the coexposed mice contained antibodies specific for heat shock proteins, a chaperone protein targeted by antibodies in patients with AIH. Thus, although TCE can promote autoimmune disease following chronic exposure, a shorter exposure to a binary mixture of TCE and $HgCl_2$ accelerated disease development. Coexposure to TCE and $HgCl_2$ also generated a unique liver-specific antibody response not found in mice exposed to a single toxicant. This finding stresses the importance of including mixtures in assessments of chemical immunotoxicity.

Key Words: mercury; trichloroethylene; MRL+/+ mice; Co-exposure; liver; autoimmune hepatitis; CD4+ T cells cytokines; autoimmunity.

There is increasing evidence that chronic exposure to certain toxicants at levels too low to be overtly toxic can harm human health by altering immune function. Although many of the environmental chemicals that affect the immune system in humans are suppressive, others such as trichloroethylene (TCE) can be proinflammatory. TCE is a solvent and degreasing agent whose improper disposal has led to it becoming a widespread environmental pollutant. Although exposure to TCE is relatively common, it rarely occurs at levels thought to be directly toxic.

However, a number of epidemiological reports and case studies have linked chronic low-level TCE exposure to a variety of autoimmune diseases in humans including lupus, scleroderma, bullous pemphigoid, diabetes, and immune-mediated hepatitis (Byers *et al.*, 1988; Dubrow and Gute, 1987; Flindt-Hansen and Isager, 1987; Gist and Burg, 1995; Hansen and Isager, 1988; Lockey *et al.*, 1997; Saihan *et al.*, 1978; Yanez Diaz *et al.*, 1992). Even if overt disease is not diagnosed, signs of immune activation including increased numbers of T cells and elevated serum levels of autoantibodies (antinuclear antibodies [ANA]) have been associated with chronic exposure to a domestic water supply contaminated with TCE (Byers *et al.*, 1988; Clark *et al.*, 1994; Kilburn and Washaw, 1992; Nietert *et al.*, 1998). Similarly, occupational exposure to TCE resulted in significantly increased serum levels of the T cell–derived proinflammatory cytokine interferon-gamma (IFN-γ) (Iavicoli *et al.*, 2005). Aside from humans, mice chronically exposed (26–48 weeks) to low-level TCE have demonstrated an increased incidence of autoimmune hepatitis (AIH) (Griffin *et al.*, 2000b) and increased production of lupus-related autoantibodies (Cai *et al.*, 2008; Keil *et al.*, 2009).

Delineating the role of TCE in the development of autoimmune disease would be easier if it were understood why only some people seemingly exposed to similar amounts of the toxicant develop autoimmune disease. This discrepancy may be due in part to genetic differences in susceptibility factors. However, equally important is the fact that no individual is exposed to a single toxicant but rather a unique but ill-defined set of environmental toxicants with potentially interactive effects. Multiple chemicals can have discrete mechanisms of action with the cumulative effect on the immune system difficult to predict. Little is known about the possible modulating effects of other chemicals on TCE-induced immunotoxicity. Most coexposure studies with TCE have used the liver or kidney as endpoints. Only a few studies have examined the effects of TCE-containing mixtures on the immune system, and because these examined a mixture of at least four chemicals, it was impossible to determine the contribution of TCE (Vodela *et al.*, 1997; Yang, 1993). The weight-of-evidence approach to examining mixtures dictates that a mixture is broken down into

binary mixtures in order to examine the interaction potential of each pair. Consequently, this study examined TCE immunotoxicity in the context of a TCE-containing binary mixture.

The second chemical that was selected for examination here was mercury. Human exposure to mercury is widespread because of its existence as a natural element in the environment and its anthropogenic release from industrial use. A recent analysis of women in the National Health and Nutrition Examination Survey 1999–2006 data sets showed that inorganic mercury detection increased dramatically from 2% in 1999–2000 to 30% in 2005–2006 with compelling evidence of age-dependent mercury deposition (Laks, 2009). Mercury has been implicated as a cofactor in systemic human autoimmunity, where studies showed that mercury exposure increased levels of antinucleolar antibodies (ANoA) (Cooper et al., 2004; Gardner et al., 2010). The ability of mercury to promote autoimmunity has been especially well documented in mice where it promotes autoantibodies specific for fibrillarin and other nuclear antigens, such as chromatin, and induces immune complex-mediated lupus nephritis (Hultman et al., 1996; Pollard et al., 1997).

The 2007 Comprehensive Environmental Response, Compensation and Liability Act Priority List of Hazardous Substances, compiled by the Agency for Toxic Substances and Disease Registry after considering toxicity, frequency, and potential for human exposure, listed mercury and TCE as 3rd and 16th, respectively, out of 275 environmental toxicants. They are both considered Superfund chemicals and are often found together. According to the National Priorities List, approximately 44% of the active Superfund sites contaminated with TCE also list mercury as a contaminant. Because exposure to both TCE and mercury is not unlikely, the question of how exposure to a binary mixture of these toxicants affects the immune system is a relevant one. The two toxicants appear to have effects that are overlapping. Both TCE and mercury increase $CD4^+$ T-cell production of IFN-γ and inhibit activation-induced cell death in $CD4^+$ T cells (Gilbert et al., 2006; Laiosa et al., 2007). In view of their similar effects, it is possible that coexposure to mercury and TCE could induce autoimmunity under conditions when single toxicant exposures would not. This possibility was tested here.

MATERIALS AND METHODS

Mice. Eight-week-old female MRL+/+ mice (Jackson Laboratories, Bar Harbor, ME) were housed in polycarbonate ventilated cages and provided with laboratory drinking water *ad libitum*. TCE (purity 99+%; Aldrich Chemical Co. Inc., Milwaukee, WI) was suspended in drinking water with 1% emulsifier Alkamuls EL-620 from Rhone-Poulenc (Cranbury, NJ). The mice (six mice per group) received 0, 0.1, or 2.0 mg/ml TCE in their drinking water for 8 weeks. Freshly made TCE-containing drinking water was provided every 2–3 days. Subsets of mice were injected sc twice per week for 8 weeks with 100 μl PBS alone or containing 40 μg mercuric chloride ($HgCl_2$). The mice were weighed weekly. In addition to prebleeds, retro-orbital blood samples were obtained at 4, 6, and 8 weeks of treatment. All studies were approved by the Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Histopathology. At the time of sacrifice, dorsal skin, the right kidneys, and the caudal lobes of the livers were fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Liver, skin, and kidney sections were examined microscopically and scored in a blinded manner by a veterinary pathologist for the severity of mononuclear cell infiltration and other tissue-specific changes in morphology based on a four-point scale. The location and cellular composition of all inflammatory infiltrates were recorded. A chi-square test was conducted to compare cumulative tissue pathology scores in toxicant-treated versus control mice.

Western blotting to detect tissue-specific antibody production. Equal amounts (30 μg) of microsomal or cytosolic liver protein (prepared as described; Halmes et al., 1996) or brain (cerebellum) protein obtained from the control or treated MRL+/+ mice were separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto nitrocellulose, and subsequently immunoblotted with pooled sera (1:1000) from control or treated MRL+/+ mice collected at 4- or 8-week time points followed by HRP-conjugated goat anti-mouse immunoglobulin G (IgG) (1:10,000). Immunodetection was performed by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). In one set of experiments, recombinant heat shock proteins (HSP)-90α protein (StressMarq Biosciences, Canada) (4 micrograms per lane) was run as the target protein on the SDS-PAGE and then immunoblotted with the pooled 8-week sera as described above. In some cases, 200 μg of pooled microsomal liver or brain protein were incubated with Dynabeads M-280 Streptavidin (Invitrogen Life Sciences, Carlsbad, CA), which had been preconjugated with biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch). The beads were removed using a Dynal MPC magnet, and the beads and remaining protein lysates were separated by SDS-PAGE and then immunoblotted with the goat-anti-mouse IgG.

Quantitative PCR. Fluorescence-based quantitative PCR (qPCR) was conducted using RNA isolated (using RNEasy, Qiagen, Germantown, MD) from frozen ($-80°C$) liver and cerebella of individual mice. For each sample, complementary DNA (cDNA) was synthesized with random hexamers and 0.5 μg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). PCR primer sequences were designed using the National Center for Biotechnology Information gene databases and synthesized by IDT, Inc. (Coralville, IA) using PrimerQuest. All qPCR reactions were carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories) and the IQ5 iCycler (Bio-Rad Laboratories, Inc.) according to manufacturer's protocols. No-template controls were included to detect primer-dimer artifacts. DNA melting curve analysis was used as another control against primer-dimer formation and to ensure the lack of contaminating DNA in the RNA preparations. C_q values > 36 were not reported because of implied low efficiency. The relative gene expression was calculated using the delta-delta C_q method based on normalization to the reference gene eukaryotic translation elongation factor 2. The C_q value of this reference gene did not vary more than 5% among the treatment groups and was found to be superior to *β-actin*, *18S ribosomal RNA*, and glyceraldehyde-3-phosphate dehydrogenase in terms of copy number and treatment-associated invariance in the tissues tested. Primer optimization was conducted, and the PCR amplification efficiency for the reference gene and the genes of interest was > 90% but < 110%, with the R^2 value > 0.9. The results were reported as fold change in liver or brain tissue from treated compared with expression in tissue from control mice.

Phenotypic analysis of spleen cells. The flow cytometric analysis of spleen cells from six individual mice per group was conducted as previously described (Blossom et al., 2007). The phenotypic analysis of 30,000 events per sample was carried out using a CyFlow ML (Partec GmbH, Munster, Germany), and the data were presented as mean ± SE of the different cell subsets. Nonviable cells, based on low forward scatter and side scatter, were excluded in each sample. Fluorescence Minus One controls and isotype Ig controls were included.

Cytokine analysis. $CD4^+$ T cells isolated from the spleens of individual mice using Dynabeads Mouse CD4 (Invitrogen Corp.) were stimulated with

immobilized anti-CD3 antibody (10 µg/ml) and anti-CD28 antibody (1 µg/ml) for 24 h. The 99+% culture supernatants were examined using ELISA kits for IFN-γ (OptEIA Set Mouse IFN-γ, BD Biosciences, San Diego, CA), interleukin (IL)-17 (RayBiotech, Inc., Norcross, GA), or osteopontin (Assay Designs, Detroit, MI).

Detection of ANA, ANoA, and total IgG. ANA and ANoA were examined in the sera obtained from each animal after 4, 6, or 8 weeks of toxicant exposure. This was done using indirect immunofluorescence and commercially available fixed HEp-2 cell substrates (Antibodies, Inc., Davis, CA). The sera from individual mice were tested at 1:50, 1:100, and 1:200 dilutions. Detecting antibodies specific for mouse IgG_1 and IgG_{2a} were used to examine isotype-specific ANA and ANoA. Each ANA/ANoA result was interpreted under blinded conditions, and the results were represented as the mean reciprocal titer that provided a positive score. Total levels of IgG_1 and IgG_{2a} in the blood from individual mice were determined in triplicate wells using an ELISA as described (Blossom *et al.*, 2007). A standard curve was used to determine concentrations of total IgG_{2a}, although consistent problems with the myeloma used to generate the IgG_1 standard curve resulted in total IgG_1 levels being reported in terms of relative optical density.

Statistics. The data are presented as means and SDs. Most assays, unless stated in the figure legends, were conducted using samples from six individual mice per treatment group. Among-group comparisons of mean values were made using a one-way ANOVA and *post hoc* analysis. Chi-square distribution was used to determine the association between the categorical variables for tissue pathology and treatment groups using SAS v 9.2 (SAS Institute, Inc., Cary, NC). The threshold for statistical significance was set at $\alpha = 0.05$.

RESULTS

Coexposure Promoted AIH

Based on water intake, body weight, and measured TCE degradation in the water bottles, the mice given water with TCE present at 0.1 or 2.0 mg/ml were exposed to an average of 9.9 or 186.9 mg/kg/day TCE, respectively. The current 8-h permissible exposure limit (PEL) established by the Occupational Safety and Health Administration for TCE is 100 ppm or approximately 76 mg/kg/day. Thus, the concentrations of TCE used in the current study were approximately 13 or 246% of the PEL. The mice that were exposed to $HgCl_2$ received approximately 260 µg/kg/day of this second toxicant. To put this in perspective, the Environmental Protection Agency has designated the Lowest-Observed-Adverse-Effect-Level for autoimmune effects following chronic oral exposure to $HgCl_2$ as 317 µg/kg/day.

Histological analysis of liver, kidney, and skin from each mouse was conducted to examine possible TCE- and/or $HgCl_2$-induced tissue pathology. Based on cumulative scores concerning mononuclear cell infiltration, fibrosis, necrosis, and hepatocellular enlargement, liver pathology from mice exposed to $HgCl_2$ and either 0.1 or 2.0 mg/ml TCE, 0.1 was significantly different from that of control mice ($\chi^2_{3df} = 8.311$; $p = 0.040$ and $\chi^2_{3df} = 8.000$; $p = 0.046$, respectively). Representative liver sections from an $HgCl_2$- and TCE-treated mouse are presented in Figure 1. The livers of mice exposed to $HgCl_2$ or TCE alone for 8 weeks exhibited no significant pathology. None of the mice exhibited significant kidney or skin pathology regardless of treatment. Taken together, the results indicate that although an 8-week exposure to TCE alone at the concentrations used here
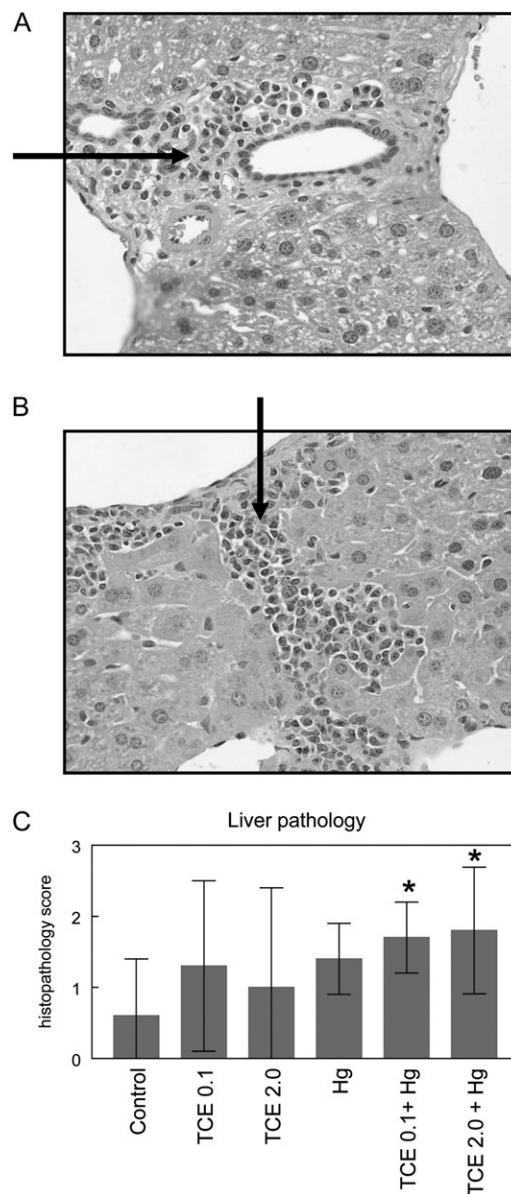
FIG. 1. Coexposure to TCE and $HgCl_2$ promoted AIH. Shown are representative liver sections (×400) demonstrating portal inflammation with fibrosis (A) and centrilobular inflammation (B) in liver sections from mice exposed to both TCE (2 mg/ml) and $HgCl_2$. The arrows indicate areas of mononuclear infiltration. (C) Cumulative histopathology scores for liver sections. *Statistically different from the results obtained using liver sections from control mice.

was not sufficient to promote pathology, coexposure to both TCE and $HgCl_2$ did induce significant early liver pathology. Thus, coexposure to $HgCl_2$ appeared to enhance the autoimmune-promoting effects of TCE.

Physical Characteristics

Body weight was not affected by exposure to TCE and/or $HgCl_2$ (Fig. 2). A decrease in water consumption was seen in several of the groups exposed to TCE and/or $HgCl_2$. However,
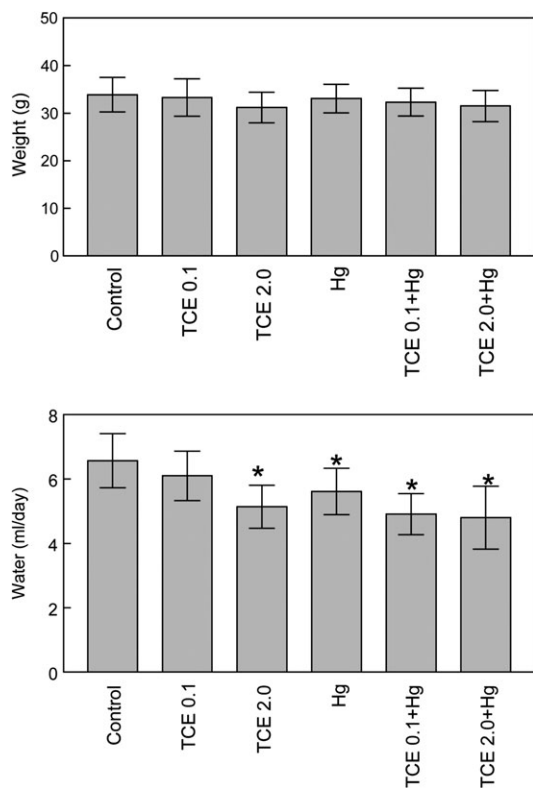
FIG. 2. Effects of TCE and HgCl$_2$ on weight gain and water consumption. Female MRL+/+ mice were given water alone (controls), water with TCE, and/or HgCl$_2$ for 8 weeks. Body weight was monitored over time and is presented as mean ± SD. Water consumption was monitored over time and is presented as mean ± SD per mouse per day for the entire 8-week time period. *Statistically different from the results obtained from control mice.

FIG. 3. Effects of TCE and HgCl$_2$ on splenic cellularity. Spleen cells from six individual mice per group treated with water alone (controls), water with TCE, and/or HgCl$_2$ for 8 weeks were evaluated for total cell number. The spleen cells were also evaluated for total numbers of CD4$^+$ T cells, CD8$^+$ T cells, B cells, and CD4$^+$ CD25$^+$ cells. The data are presented as the mean ± SD.

because the toxicant-treated mice exhibited normal weight gain and showed no signs of dehydration (e.g., piloerection, lethargy, or recessed eyes), it was not clear that their decreased water consumption was functionally significant.

Coexposure Did Not Alter Lymphoid Organ Cellularity

Cellularity of the spleen and lymph nodes from individual mice was examined as a potential measurement of toxicant-induced immunotoxicity. As shown in Figure 3, no differences in total spleen cell numbers were observed in any of the groups. Subsets of splenocytes were also examined. None of the toxicant combinations altered numbers of total splenic CD4$^+$ T cells, CD8$^+$ T cells, or B cells. The number of splenic CD4$^+$ CD25$^+$ T cells (a phenotype often used to designate T$_{Reg}$ cells but also found on some activated non-T$_{Reg}$ CD4$^+$ T cells) was also unaltered by toxicant exposure. Similarly, none of the treatment regimens altered the percentages of CD4$^+$ T cells or CD8$^+$ T cells in lymph nodes (data not shown). Thus, the tissue pathology observed in the coexposed mice could not be attributed to an alteration in the percentage of the measured lymphoid subsets.
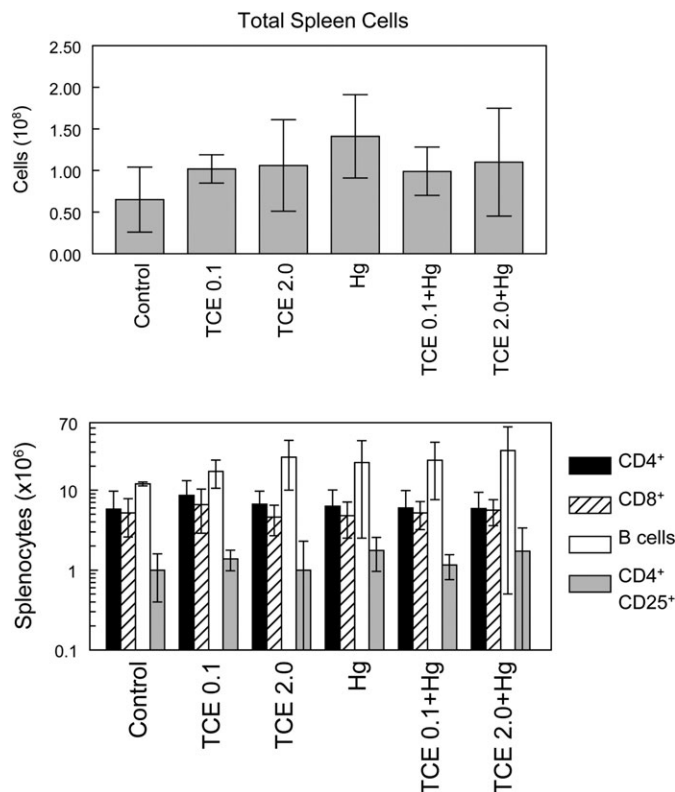
Effects of Coexposure on Cytokine Production

CD4$^+$ T-cell production of cytokines associated with autoimmune inflammation, including IFN-γ, IL-17, and osteopontin, was examined using culture supernatants of isolated CD4$^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies. As shown in Figure 4A, intragroup variation was large resulting in only a few groups demonstrating statistical difference from controls. Exposure to 0.1 mg/ml TCE alone increased production of both IL-17 and osteopontin. Osteopontin was also produced in higher amounts by CD4$^+$ T cells from the mice exposed to TCE (2.0 mg/ml) and HgCl$_2$. Because blood levels of osteopontin, unlike many other cytokines, can change during autoimmune disease development, we also examined this cytokine in the mouse sera. Interestingly, mice exposed to either concentration of TCE alone demonstrated increased levels of osteopontin in the blood (Fig. 4B). Thus, levels of IFN-γ, IL-17, and osteopontin did get upregulated in response to TCE and/or HgCl$_2$, even if these effects were not directly correlated to pathology.
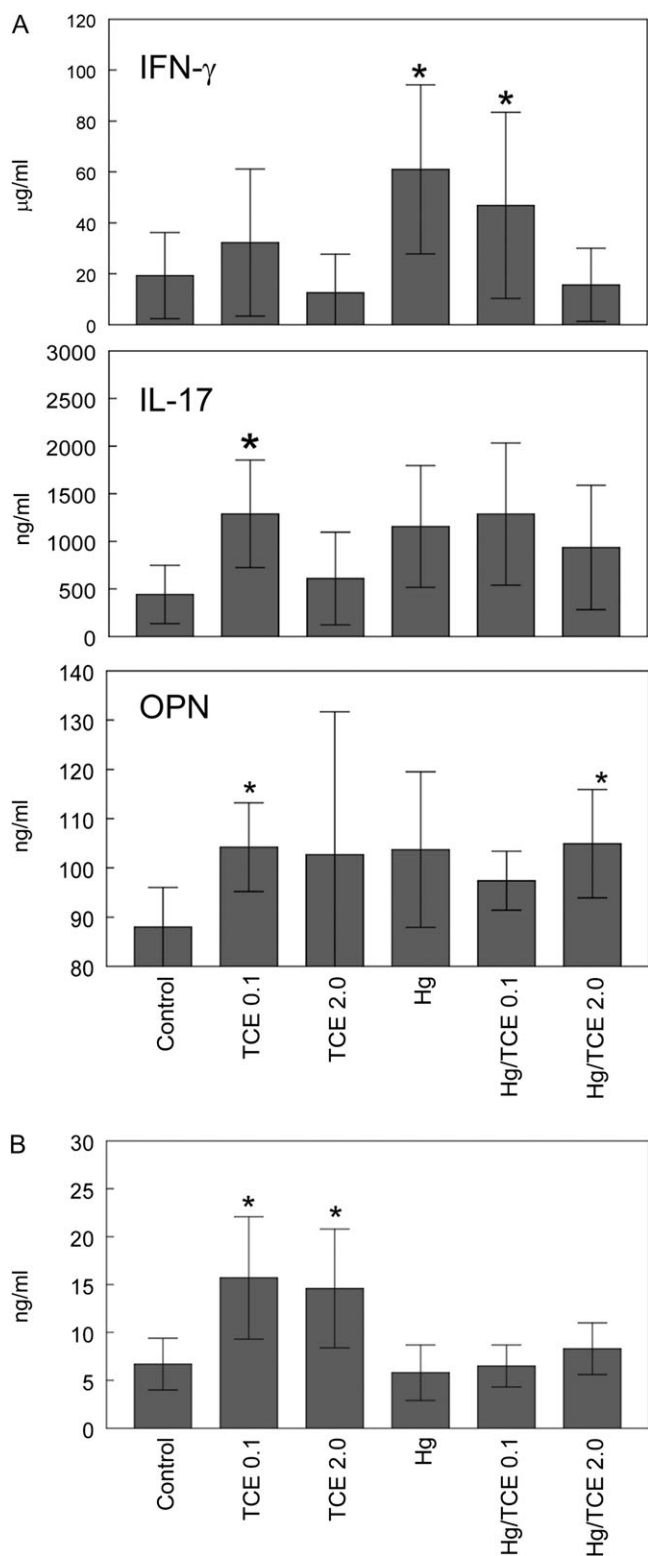
FIG. 4. Effects of TCE and HgCl$_2$ on cytokine production. (A) CD4$^+$ T cells isolated from the spleens of individual mice were stimulated with anti-CD3 and anti-CD28 antibodies. The resulting culture supernatants were tested for levels of IFN-γ, IL-17, and osteopontin. (B) Sera were tested for levels of osteopontin. The data are presented as the mean ± SD, with asterisks designating results statistically different from controls.

Alterations in Liver and Brain Gene Expression

Hepatic expression of genes that might promote liver inflammation was examined for a potential role in the generation of the liver-reactive antibodies. No increase was observed in the expression of proinflammatory cytokines IL-1β or tumor necrosis factor-α (TNF-α) (Fig. 5). Expression of IFN-γ was undetectable in the liver. Depletion of the immunosuppressive cytokine transforming growth factor-β (TGF-β) in the liver has been shown to promote T cell–mediated inflammatory liver disease (Gorham *et al.*, 2001). Although TGF-β expression was decreased in some of the treatment groups in this study, the decrease did not correlate with liver pathology. Neither *CYP2E1* nor *CYP1A2*, genes that encode for TCE-metabolizing enzymes, was increased in the liver tissue from any treatment groups (data not shown). Only betaine homocystein methytransferase (*BHMT*) was increased in the livers from the coexposed mice. Because *BHMT* upregulation is usually thought of as a compensatory protective mechanism designed to maintain levels of *S*-adenosylmethionine, the likelihood that this alteration promoted autoimmunity seems low. However, this effect did underscore the additive effect of coexposure on liver gene expression.

The HgCl$_2$ did not induce measurable nephritis. However, the central nervous system is even more sensitive to the effects of mercury than the kidney. Consequently, toxicant-induced alterations in the expression of genes associated with inflammation and repair were examined in the cerebellum, a brain region likely to demonstrate inflammatory and oxidative stress–related events. As shown in Figure 5, TCE at the concentrations used here did not alter expression of the genes selected. However, when used in combination with HgCl$_2$, TCE increased expression of brain-derived neurotrophic factor (*BDNF*), an active member of the neurotrophin family that is often altered in brain-associated disease states. Unlike in the liver, coexposure also increased expression of the proinflammatory cytokine *IL-1β* in the cerebellum. Neither TCE nor HgCl$_2$ alone altered expression of these two genes. Exposure to HgCl$_2$ and/or TCE did not increase glutathione *S*-transferase, *Lymphotoxin-α* (Fig. 5), or other inflammation-related genes such as inducible nitric oxide synthase, *TNF-α*, or *IL-6* (data not shown). Even though the functional consequences of the coexposure-induced increased expression of *BDNF* and *IL-1β* in the cerebellum are not yet known, this finding once again demonstrated that mixtures can be more powerful than single chemicals.

Coexposure Increased Serum Parameters of Autoimmunity

At certain concentrations, TCE has been shown to transiently increase the production of ANA in MRL+/+ mice (Cai *et al.*, 2008). HgCl$_2$, on the other hand, is most noted for its ability to induce ANoA that target fibrillarin (Pollard *et al.*, 1997). Although the role of ANA and ANoA in tissue pathology is not clear, they can be used for diagnostic
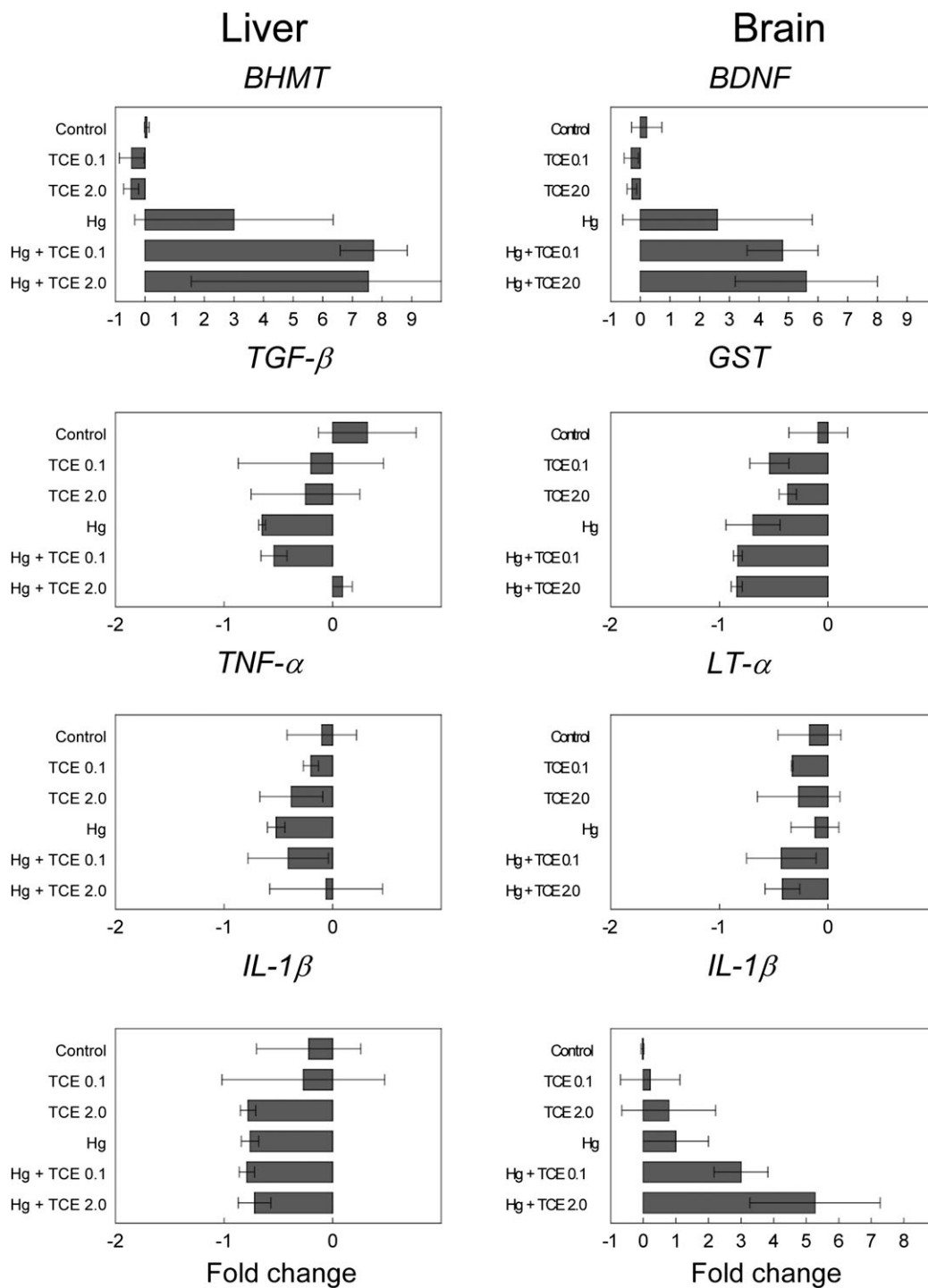
FIG. 5. Effects of coexposure to TCE and $HgCl_2$ on gene expression in liver and cerebellum. qPCR was performed using liver or cerebellum from three individual mice per treatment group collected after 8 week exposure to TCE and/or $HgCl_2$.

purposes. Consequently, both ANA and ANoA were examined in the serum of individual mice exposed to TCE and/or $HgCl_2$. As shown in Figure 6, IgG_1 ANA levels were increased at the final time point in the mice exposed to both Hg and 2.0 mg/ml TCE, whereas IgG_{2a} ANA was increased at that time point in all the groups exposed to $HgCl_2$. Levels of ANoA were low and not altered in any of the groups (data not shown). Autoimmunity can also be associated with an increase in total
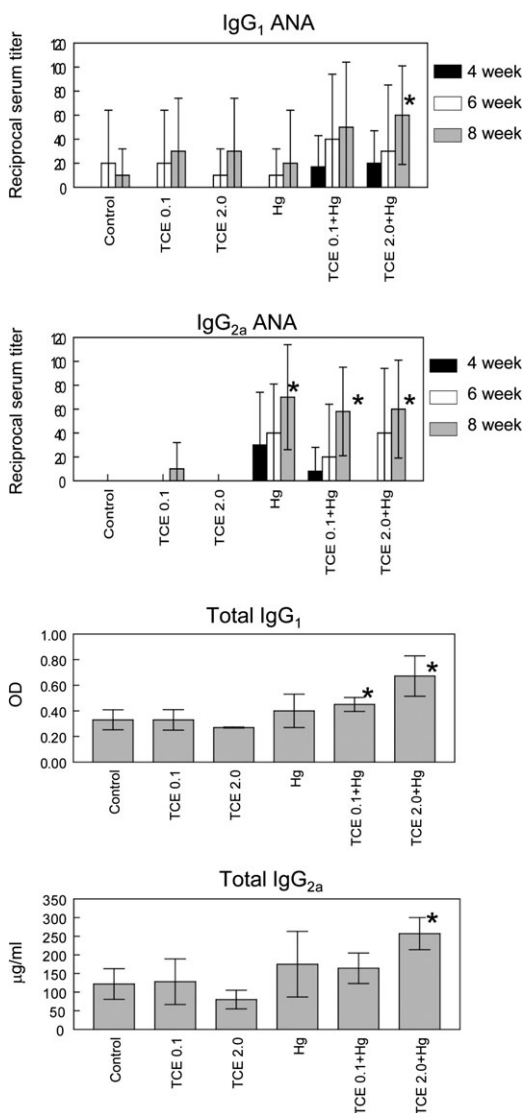
FIG. 6. Coexposure to TCE and $HgCl_2$ increased autoantibody production. Sera obtained at 4, 6, or 8 weeks from individual mice treated with water alone (controls), water with TCE, and/or $HgCl_2$ were evaluated for IgG_1 and IgG_{2a} ANA. The ANA results are presented as the inverse of the highest dilution that provided a positive score. These results duplicate those found in an earlier analysis conducted with fewer sera but a wider dilution range. Levels of total IgG_1 and IgG_{2a} in the sera collected at the 8-week time point are also shown. *Statistically different from results in control mice.

Ig levels. As shown in Figure 5, coexposure to TCE and $HgCl_2$ increased levels of total IgG_1 and IgG_{2a}. This effect was also evident at the 6-week time point (data not shown). Taken together, although none of the mice developed detectable nephritis, the immune pathology most often associated with autoantibody production, coexposure to $HgCl_2$ and TCE did increase levels of ANA and total IgG_1 and IgG_{2a}. Once again, coexposure promoted serum parameters of autoimmunity, whereas single exposures were ineffective at the time points and concentrations used here.

Toxicant-Induced Tissue-Reactive Antibodies Minimal at 4 Weeks

TCE has been shown to stimulate the time- and dose-dependent generation of antibodies that react with liver proteins (Gilbert *et al.*, 2009). The autoantibodies induced by $HgCl_2$ are best known as targeting nucleolar proteins; their specificity for tissue proteins such as those found in the liver has not been extensively examined. Because autoantibodies can be used to help identify the autoantigens targeted by an autoimmune response, we tested the sera from the mice exposed here to TCE and/or $HgCl_2$ for their immunoreactivity with different target tissues including microsomal liver and cerebellum.

The tissue-specific reactivity was tested using sera collected at 4 and 8 weeks. The anti-liver staining pattern produced by sera collected at the 4-week time point showed little differences among the groups exposed to TCE alone (Fig. 7A). However, liver proteins between 30 and 37 kDa were detected by antibodies in the sera mice exposed to $HgCl_2 \pm$ TCE. Another exception was the appearance of an antibody in the mice treated with TCE (2.0 mg/ml) and $HgCl_2$ that recognized a protein between 75 and 100 kDa in liver tissue. Interestingly, a brain protein of similar molecular weight was stained by sera from the same treatment group.

All the Western blots obtained using 4-week sera, regardless of treatment group, revealed a protein of approximately 50 kDa in both brain and liver. A subsequent analysis in which brain and liver tissue from all the groups were immunoblotted with only the secondary goat anti-mouse IgG detected bands at a similar molecular weight (Fig. 7B). Densitometric analysis showed no differences among the groups in terms of the amount of the 50 kDa detected in liver or brain. Immunoprecipitation with anti-mouse IgG removed the 50-kDa protein from the protein (Fig. 7C), further suggesting that both brain and liver from the MRL+/+ mice, regardless of treatment, contained mouse IgG, the heavy chain of which is approximately 50 kDa. The same 50-kDa protein was found in microsomal liver protein from non-autoimmune C57Bl/6 and C3H/HeJ mice (Fig. 7D). The significance of IgG in the microsomal fraction of liver protein is not known; however, the detection of the 50-kDa protein in the liver and brain lysates provided an internal reference point for the individually stained Western blots.

Coexposure for 8 Weeks Promoted Unique Patterns of Tissue-Reactive Antibodies

Unlike the 4-week sera, sera from mice exposed to TCE and/or $HgCl_2$ for 8 weeks revealed several interesting differences in immunoreactivity among the groups. In case reactivity depended upon toxicant-induced adduct formation, protein from both control and treated mice was used as a target for immunoblotting. For example, a preparation of pooled sera from mice treated with TCE at 0.1 mg/ml in the water was tested for its ability to stain control brain protein as well as brain protein from the mice treated with TCE at 0.1 mg/ml.
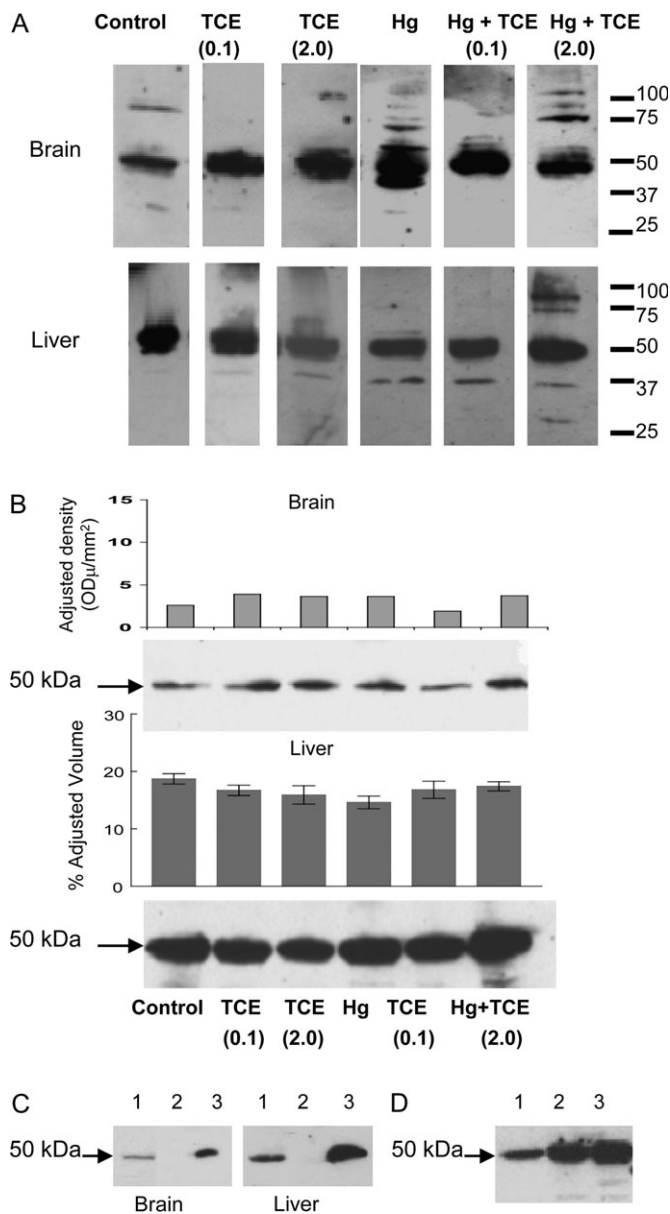
FIG. 7. Detecting TCE- and HgCl$_2$-induced antibodies after 4 week exposure. (A) Pooled microsomal liver or brain protein collected from control MRL+/+ mice (treated with water alone for 8 weeks) was separated by SDS-PAGE and immunoblotted using pooled sera from pooled control or treated MRL+/+ mice collected at the 4-week time point, followed by a goat anti-mouse IgG antibody. (B) Brain or microsomal liver proteins from control MRL+/+ mice or MRL+/+ mice exposed to either TCE and/or HgCl$_2$ for 8 weeks were separated by SDS-PAGE and immunoblotted with goat anti-mouse IgG antibody alone. Densitometric analysis of the 50-kDa protein was conducted. This experiment was repeated with brain tissue, and mean densitometric values ($\pm$ SD) from three experiments are presented. (C) Pooled brain or microsomal liver protein from control MRL+/+ mice was separated by SDS-PAGE and immunoblotted with goat anti-mouse IgG antibody (lane 1). Alternatively, IgG was immunoprecipitated from the protein lysates using beads conjugated with donkey anti-mouse IgG; the remaining lysates (lanes 2) or resulting beads (lanes 3) were separated by SDS-PAGE and immunoblotted with goat anti-mouse IgG. (D) Microsomal liver protein from an untreated MRL+/+ mouse (lane 1), C57BL/6 mouse (lane 2), or C3H/HeJ mouse (lane 3) was separated by SDS-PAGE and immunoblotted with goat anti-mouse IgG antibody.

Using this approach, we found that sera from mice exposed to either 0.1 or 2.0 mg/ml TCE in drinking water contained antibodies that recognized liver microsomal proteins around 30–37 kDa (Fig. 8). Interestingly, this reactivity was observed regardless of whether the source of the liver tissue was control mice or the same mice as the source of sera. Unlike exposure to TCE alone, exposure to TCE plus HgCl$_2$ or HgCl$_2$ alone generated little or no antibodies specific for 30- to 33-kDa liver proteins. Indeed, the liver-specific immunoblotting detected using sera from mice exposed to HgCl$_2$ alone was similar to that of control mice. However, sera from mice exposed to both HgCl$_2$ and TCE produced a unique staining pattern for liver microsomal proteins of approximately 90 kDa as well as proteins of approximately 20–30 kDa. Neither of these sets of proteins was detected by sera from mice exposed to TCE or HgCl$_2$ alone. Thus, coexposure to TCE and HgCl$_2$ generated liver-specific antibodies not found in the blood of mice exposed to either toxicant alone. Antibodies generated by exposure to TCE and HgCl$_2$ appeared to recognize liver microsomal proteins from either control or toxicant-treated mice. In other words, the toxicant-induced liver-specific antibodies were not restricted to haptenated proteins. Very similar results were obtained if cytosolic rather than microsomal liver protein was used as the target of the immunoblotting.

When antibodies reactive with brain antigens were examined, it appeared that sera from mice exposed to TCE alone differed very little when compared with sera from control mice (Fig. 8). In contrast to control sera, sera from mice exposed to HgCl$_2$ alone stained an array of brain proteins. Most striking was the fact that HgCl$_2$ $\pm$ TCE stimulated production of an antibody specific for a brain protein of approximately 30–33 kDa. Interestingly, mice exposed to both HgCl$_2$ and TCE, but not those exposed to HgCl$_2$ alone, generated antibodies that recognized a brain protein of approximately 90 kDa. Thus, several of the brain proteins recognized by the sera from the toxicant-treated mice were of similar molecular weight to liver microsomal proteins recognized by the same sera. Also similar was the fact that coexposure to both TCE and HgCl$_2$ generated some brain-specific and liver-specific antibodies not found in sera from mice exposed to either toxicant alone.

A recent proteomic study using sera from humans with idiopathic AIH demonstrated the presence of an autoantibody specific for HSP-90, a 90-kDa protein in the liver plasma membrane (Tahiri *et al.*, 2008). Using recombinant HSP-90 as the target antigen in Western blotting, we found that sera from mice coexposed to TCE and HgCl$_2$ were much more likely than sera from mice exposed to TCE or HgCl$_2$ alone to contain anti-HSP antibodies (Fig. 9).

DISCUSSION

Previous studies have shown that MRL+/+ mice given drinking water with 0.5 or 2.5 mg/ml TCE for 26–32 weeks
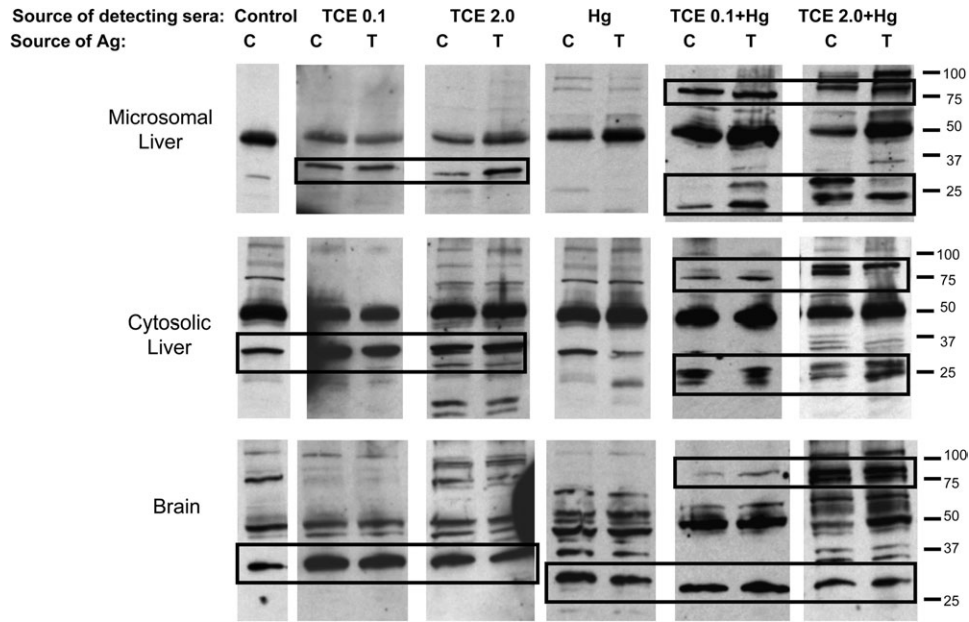
FIG. 8. Coexposure to TCE and HgCl$_2$ for 8 weeks generated unique pattern of anti-liver and anti-brain antibodies. Pooled microsomal liver, cytosolic liver, or brain protein collected at the 8-week time point from control (C) mice (treated with water alone) or from mice treated (T) with TCE and/or HgCl$_2$ was separated by SDS-PAGE. This protein was then immunoblotted using pooled sera from control or treated mice collected at the 8-week time point, followed by a goat anti-mouse IgG antibody. In all cases, the mice that provided the protein in the "T" lane were the same mice that provided the detecting sera.

developed liver inflammation commensurate with AIH (Gilbert *et al.*, 2009; Griffin *et al.*, 2000a). Liver inflammation was not seen in mice exposed to those concentrations of TCE for a shorter (4 weeks) time period. Similarly, in the study here, liver inflammation was not found in MRL+/+ mice treated

FIG. 9. Detecting anti-HSP-90 antibodies after 8 week exposure to TCE and HgCl$_2$. SDS-PAGE was performed using recombinant HSP-90α as the source of protein. The protein was then immunoblotted using pooled detecting sera collected from the different treatment groups at 8 weeks, followed by a goat anti-mouse IgG antibody. This approach was repeated using individual or pairs of pooled sera ($n = 3$ or 4 per group), and the bar graph describes the percentage of these Western blots in which anti-HSP-90 was detected.

here with TCE alone (0.1 or 2.0 mg/ml) in drinking water for 8 weeks. However, modest but significant liver inflammation was observed if the TCE-treated mice were coexposed to HgCl$_2$. Exposure to HgCl$_2$ is not known to promote liver inflammation. It is more likely that HgCl$_2$ augmented the liver inflammation induced by TCE. HgCl$_2$ has similarly been shown in a mouse model of arthritis to enhance rather than initiate disease development (Hansson *et al.*, 2005).
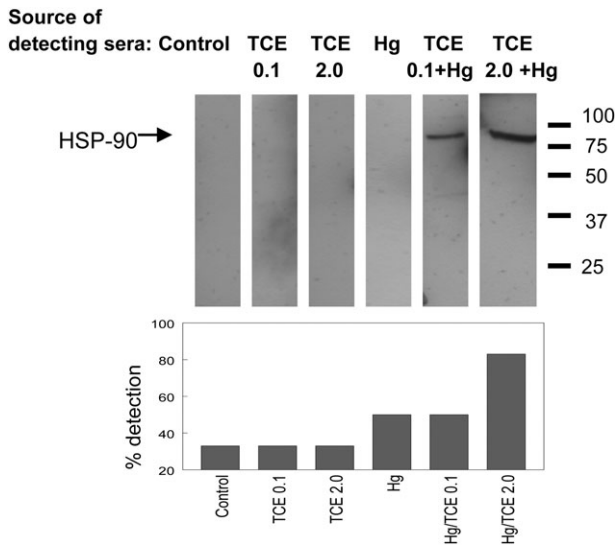
The assessment of autoantibodies and total serum Ig indicated that coexposure to TCE and HgCl$_2$ generated the most robust increase. Exposure to approximately 1.5 mg/kg/week HgCl$_2$ for 4–8 weeks has been shown to alter several serological parameters in mice including the generation of ANoA and an increase in total IgG$_1$ and IgG$_{2a}$. Mouse strains vary in their susceptibility to these effects of HgCl$_2$ with H-2^s mice such as B10.S and A.SW being particularly susceptible. However, MRL+/+ mice are also considered susceptible to the effects of HgCl$_2$ (Pollard *et al.*, 1999). In the current study, the mice exposed to HgCl$_2$ alone did demonstrate increased levels of IgG$_{2a}$ ANA. Conversely, other serum parameters of autoimmunity were only found in mice exposed to both TCE and HgCl$_2$. Because TCE exposure alone was not sufficient to promote the increase in ANA or total IgG$_1$ or IgG$_{2a}$, it must be inferred that HgCl$_2$ contributes to these increases.

TCE and HgCl$_2$ also appeared to work together to generate a unique pattern of liver-associated antibodies. Hepatic seropositivity is an important component of a definitive diagnosis of AIH in humans. In the current study, sera from mice coexposed to TCE and HgCl$_2$ contained antibodies that stained liver

proteins of approximately 90 kDa as well as proteins in the 25- to 35-kDa range. Unlike coexposure, exposure to just TCE or $HgCl_2$ alone did not appear to stimulate antibodies for these liver proteins.

Sera from the mice were also tested for brain-reactive antibodies. Neither TCE nor $HgCl_2$ as individual chemicals is known for their ability to generate anti-brain antibodies. In this study, sera from mice treated with TCE alone immunoblotted brain protein in a manner indistinguishable from control sera. Immmunoblotting using sera from mice exposed to $HgCl_2$, alone or together with TCE, demonstrated reactivity with a brain protein between 25 and 37 kDa. However, coexposure to $HgCl_2$ and TCE generated unique brain-reactive antibodies not found in mice exposed to either toxicant alone. Most notable was the immunoblotting of a brain protein of approximately 90 kDa similar to that seen using liver as the target antigen. Thus, similar to the findings obtained used liver as the target tissue, coexposure appeared to generate a unique pattern of brain-specific autoantibodies.

HSP-90 was considered as a candidate for the high molecular weight liver and brain protein targeted by autoantibodies in the coexposed mice. HSP-90 is an abundant and widely distributed protein found in both liver and brain. Using recombinant HSP-90 as an antigen, Western blotting showed that this protein was recognized by antibodies in sera from mice coexposed to TCE and $HgCl_2$. HSP-90 is a constitutively expressed protein that can be upregulated by various environmental stresses including high temperature, infections, and chemical exposure. HSPs associate with a broad arrays of proteins in the cell, and there is extensive evidence that HSP-protein complexes generated in the cell and released after cell death are very immunogenic, generating immune responses to both protein and HSP (Nemoto et al., 1997). How exactly HSPs act as adjuvants is not known but HSPs complexed to peptides have been shown to enhance peptide processing and Major Histocompatability complex class II presentation to T cells (Tobian et al., 2004).

Blood and/or tissue levels of HSP-90, as well as anti-HSP-90 autoantibodies, have been shown to be increased in several autoimmune diseases including rheumatoid arthritis, Systemic Lupus Erythematosus, and AIH (Hayem et al., 1999; Ripley et al., 2001; Tahiri et al., 2008). Anti-HSP-90 antibodies have been shown to develop in MRL+/+ mice as they age, becoming detectable when they develop lupus around 40 weeks of age (Faulds et al., 1995). Because the MRL+/+ mice in the current study were approximately 20 weeks of age at the end of the experiment, it appears that coexposure to TCE and mercury accelerated the generation of anti-HSP-90 antibodies. It is not clear whether these antibodies are pathogenic, protective, or merely serve as markers of inflammation. AIH is after all thought to be primarily T-cell mediated. The generation of anti-HSP-90 antibodies may involve activation of HSP-90–specific $CD4^+$ T cells. Alternatively, these antibodies may be generated via linked recognition by $CD4^+$ T cells

specific for some other self-antigen complexed to HSP-90. In either case, the pathogenicity of the $CD4^+$ T cells involved in anti-HSP-90 antibody production remains to be determined.

Toxicant-induced liver inflammation in the current study may depend on toxicant-induced protein alterations. TCE exposure has been shown to modify liver proteins in MRL+/+ mice in a manner that increases their immunogenicity (Halmes et al., 1996). It has been proposed that metabolites of TCE form adducts with self-proteins in the liver and stimulate an immune response against the modified (haptenated) proteins. Haptenated proteins have been shown to have unusually immunostimulatory potential demonstrated at the level of antibody production and at the level of T-cell proliferation (Willis et al., 2003). However, there is some disagreement over whether haptenated proteins promote an immune response to the haptenated protein, the carrier protein, or both. If TCE-induced haptenation does promote liver-specific autoimmunity in the present study, it must be concluded that coexposure to mercury somehow enhances this effect because autoimmunity was not observed in mice exposed for 8 weeks to TCE alone. Second, if haptenation is involved in initiating liver-specific autoimmunity, the response it generates must encompass both modified and unmodified liver proteins; the sera from the coexposed mice reacted similarly to tissue from untreated and treated mice.

Delineating the mechanisms of immunotoxicity in humans is complicated by the need to consider the cumulative effect of multiple toxicants. The majority of current toxicological research with animal models focuses on a single substance. However, it is possible that exposure to mixtures of immunotoxicants with additive or synergistic effects can promote autoimmunity at concentrations that would not generate measurable responses for single toxicants. This might explain why autoimmunity can be linked to a chemical such as TCE even though it is rarely found at concentrations thought to be directly toxic. On the other hand, simultaneous exposure to an immunostimulatory chemical and one with immunosuppressive capacity could result in a null response. The cumulative effect of multiple toxicants with competing or additive mechanisms presumably determines the final immunological response.

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