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Differential immune responses to albumin adducts of reactive intermediates of trichloroethene in MRL +/+ mice

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Abstract

Trichloroethene (TCE) is an industrial degreasing solvent and widespread environmental contaminant. Exposure to TCE is associated with autoimmunity. The mode of action of TCE is via its oxidative metabolism, and most likely, immunotoxicity is mediated via haptenization of macromolecules and subsequent induction of immune responses. To better understand the role of protein haptenization through TCE metabolism, we immunized MRL +/+ mice with albumin adducts of various TCE reactive intermediates. Serum immunoglobulins and cytokine levels were measured to determine immune responses against haptenized albumin. We found antigen-specific IgG responses of the IgG subtypes IgG_1,IgG_{2a}, and IgG_{2b}, with IgG_1 predominating. Serum levels of G-CSF were increased in immunized mice, suggesting macrophage activation. Liver histology revealed lymphocyte infiltration in the lobules and the portal area following immunization with formyl-albumin. Our findings suggest that proteins haptenized by metabolites of TCE may act as neo-antigens that can induce humoral immune responses and T cell-mediated hepatitis.

Keywords

Trichloroethene (TCE); Albumin adducts; Autoimmunity; Autoimmune hepatitis

Introduction

Trichloroethene (trichloroethylene, TCE) is an occupational and ubiquitous environmental contaminant. TCE is also an indoor contaminant, because it is used in various household products. More than 50% of hazardous waste disposal sites are contaminated with TCE (Fay and Mumtaz, 1996), and the chemical commonly leaches into ground water. It is estimated that 34% of U.S. drinking water supplies are contaminated with TCE (ATSDR, 1997). Therefore, human exposure to TCE occurs through inhalation and contaminated drinking water. Studies have also demonstrated dermal TCE exposure and uptake (Giver *et al.*, 2001).

Increased frequency of systemic lupus erythematosus (SLE) and other immunological disorders have been reported in populations that are chronically exposed to environmental chemicals, including TCE, through the consumption of contaminated water (Byers et al., 1988;Kilburn and Warshaw, 1992). Occupational exposure to TCE is also associated with immunological disorders (Reinl, 1957;Saihan et al., 1978;Phoon et al., 1984;Landrigan et al.,

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1987;Lockey et al., 1987;Flindt-Hansen and Isager, 1987;Brasington and Thorpe-Swenson, 1991;Waller et al., 1994;Goon et al., 2001).

Earlier reports from this laboratory support the hypothesis that TCE induces and exacerbates autoimmunity. We have shown that TCE and its metabolite dichloroacetyl chloride are immunogenic in MRL +/+ mice (Khan et al., 1995,1997;Cai et al., 2006), which may occur through protein adduction. Several reports documented covalent modification of proteins by TCE metabolites in rodents (Bolt and Filser, 1977;Stott et al., 1982;Mazzullo et al., 1992). For example, in MRL +/+ mice given simultaneously TCE and diallyl sulfide, an inhibitor of the P450 2E1 enzyme known to metabolize TCE, the concentration of haptenized proteins and the degree and prevalence of autoimmune responses is markedly decreased in comparison to mice treated with TCE alone (Griffin et al., 2000). This finding indicates the importance of TCE metabolism in inducing autoimmune responses. Oxidation of TCE to trichloroethene oxide (TCEO) mediated by P450 2E1 serves as an intermediate in the formation of metabolites with the potential to haptenize proteins (Uehleke and Poplawski-Tabarelli, 1977;Miller and Guengerich, 1983).

A detailed work from the Guengerich laboratory showed that the hydrolysis of TCEO results in reactive intermediates, which have the potential to acylate proteins (Cai and Guengerich, 1999,2000). The hydrolysis intermediate oxyacetyl chloride is capable of forming formyl protein adducts. TCEO can also rearrange to dichloroacetyl chloride (DCAC), which can acylate proteins. Formyl and dichloroacyl adducts are formed during the reaction of bovine serum albumin with TCEO, with formyl adducts surpassing dichloroacyl adducts by 20 to 50-fold (Cai and Guegerich, 2000).

To test the hypothesis that protein adducts of reactive intermediates of TCE are immunogenic and contribute to the induction of autoimmune responses, we generated TCEO, formyl, and dichloroacyl adducts of albumin. Following immunization of MRL +/+ mice with these haptenized proteins, we measured humoral responses and serum cytokine levels, and analyzed liver pathology. Our results indicate induction of autoimmunity in MRL +/+ mice by haptenized albumin, and suggest that formyl albumin may also contribute to autoimmune hepatitis.

Material and methods

Chemicals and reagents

Mouse albumin (Sigma, St. Louis, MO) was used as a carrier protein for the preparation of haptenized protein conjugates. Freund's adjuvant was from Sigma. S-ethyl trifluorothioacetate (purity ~99%) was purchased from Aldrich (St. Louis, MO). Trichloroethene oxide, S-ethyldichlorothioacetate, and acetic formic anhydride were synthesized and characterized by nuclear magnetic resonance (NMR) spectroscopy using a Varian Mercury Plus 300 MHz spectrometer with tetramethylsilane as an internal standard. Mass spectra were obtained using a DESTR matrix assisted laser desorption ionization (MALDI) –time of flight (TOF) mass spectrometer with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix.

Preparation of haptenized protein conjugates

An excess of each hapten was added to 15 mg of mouse albumin in 3 ml $NaHCO_3$ (pH 8.5) over a period of 30 min while stirring at room temperature. Stirring was continued for one hour. A sample of the solution (50μl) was removed to measure protein concentration (Bradford, 1976) and free amino groups (Habeeb, 1996). The remaining solution was dialyzed against phosphate buffer (0.1 M, pH 7.2) and lyophilized. Molecular weights and the number of modified amino groups are summarized in Table 1.

Animals and immunization

Six-week-old female MRL +/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were acclimatized in UTMB's humidity- and temperature-controlled animal care facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, for one week. At the start of the experiments, the average weight was ~28 g. Lab chow and drinking water were provided *ad libitum.* All experiments were performed in accordance to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Mice were randomly divided into groups (5 mice each) representing groups treated with PBS, albumin, or dichloroacyl, formyl, trifluoracetyl or TCEO-adducted albumin. PBS-treated and albumin-immunized groups served as negative controls, and mice immunized with trifluoroacetyl-albumin represented a positive control (Kenna et al., 1993;Christen et al., 1994). Mice were immunized subcutaneously by injection of a total of 50 μg antigen in 200 μl of PBS and complete Freund's adjuvant (CFA; 1:1, v/v) into three sites in the back. After two and four weeks, booster injections were given in the same manner except that incomplete Freund's adjuvant was substituted for CFA. Mice were sacrificed nine days after the second booster immunization. Blood, liver and kidneys were collected. The serum was isolated and stored in small aliquots at −80°C untill further analysis.

Enzyme-linked immunosorbent assay (ELISA) for chemical-specific immunoglobulins

96-well plates (Costar, Cambridge, MA) were coated with 100 μl of antigen (5 μg/ml) in 0.05 M carbonate-bicarbonate solution (pH 9.6) for one hour at room temperature and washed with 50 mM Tris buffered saline (pH 8.0, 0.05% Tween 20). The residual binding was blocked with 50 mM Tris buffered saline (pH 8.0), containing 1% BSA for 30 min. For IgG1 measurements, 10% goat serum was added. The plates were washed again and incubated for two hours at room temperature with mouse serum (1:100 or 1:1000 for IgG1) diluted with blocking solution containing 0.05% Tween 20. After washing, the plates were incubated for one hour with goat anti-mouse HRP-conjugated antibody (IgG, IgG1, IgG2a, IgG2b, or IgM from Bethyl, Montgomery, TX) at a dilution of 1:5000 in blocking solution containing 0.05% Tween 20. After a final wash, the wells were developed with 100 μl of TMB substrate (Sigma) for 5 min. Then, 100 μl of 1 M H_2SO_4 was added to stop the reaction. The absorbance was read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

Serum cytokines

The cytokines IL-1β, IL-6, GM-CSF, TNF-α G-CSF, IL-10 and the chemokine KC were measured using protein multiplex immunoassay kits as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). The fluorescence was measured using a Luminex 100 instrument (Bio-Rad).

Alanine aminotransferase and aspartate aminotransferase

Alanine aminotransferase and aspartate aminotransterase were measured using colorimetric kits from Biotron Diagnostics (Hemet, CA) and a modified protocol for small amounts of serum (12.5 μl). Absorbance was read at 540 nm.

Histopathology

Liver and kidney were fixed in 10% neutral buffered formalin. Tissue sections were stained with hematoxylin and eosin (H & E) for morphological evaluations.

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM) of five samples. For the determination of statistical significance, the data were subjected to the analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test. *P*-values ≤ 0.05 were considered to be statistically significant.

Results

Characterization of albumin adducts

Increases in molecular weight as determined by MALDI-TOF mass spectral analysis indicated successful adduction (Table 1). A TNBS assay was used to determine the extent of adduction, which varied from 34 to 48 amino groups in the adducts (Table 1).

Serum antibodies

To evaluate whether haptenization of albumin by TCE metabolites induced specific antibody responses, we measured albumin-specific serum IgG in PBS-injected mice or mice immunized with albumin or dichloroacyl, formyl, trifluoracetyl or TCEO-albumin adducts. Albumin-specific total serum IgG was not increased in albumin-immunized mice (Fig. 1). However, in mice immunized with any of the albumin adducts, albumin-specific total serum IgG was significantly increased (Fig. 1). Thus, IgG from mice immunized with haptenized albumin cross-reacted with un-adducted albumin. Antibodies raised against any of the adducted albumin preparations also cross-reacted with all other albumin adducts (Table 2). The cross-reactivity to haptenized albumin was always much stronger than that to un-adducted albumin (Table 2).

To determine whether isotype switching following immunization with haptenized albumin preferentially induced certain IgG subtypes, we measured serum levels of specific IgG_1, IgG_{2a}, and IgG_{2b} (Fig. 2). For measurements of IgG_1 titers, we had to dilute sera 1:1000, whereas IgG_{2a} and IgG_{2b} subtypes could be measured at sera dilutions of 1:100. As we observed in the case of total IgG (Table 2), IgG_1 from sera of mice immunized with haptenized albumin was specific for the immunogen, but also showed cross-reactivity to the other albumin adducts, and to a lesser degree to un-adducted albumin (Fig. 2 and data not shown). Hapten-specific titers of IgG_{2a} and IgG_{2b} were lower than IgG_1 titers, but still detectable (Fig. 2).

Serum cytokines

Using a multiplex cytokine immunoassay for the simultaneous detection of seven cytokines, detectable levels could only be measured for tumor necrosis factor (TNF)-α and granulocyte colony-stimulating factor (G-CSF). Serum levels of TNF-α were suppressed in mice immunized with haptenized albumin in comparison to albumin-injected mice (58% suppression for TCEO-albumin; 53% for dichloroacyl-albumin; 49% for trifluoroacetyl-albumin; and 29% for formyl-albumin). In all cases except for formyl-albumin, the suppression was statistically significant at a *P*-value of 0.05. Serum levels of G-CSF were only statistically significantly different from albumin-injected mice in mice immunized with trifluoroacetyl-albumin (63% increase). In all mice immunized with haptenized albumin, but not in those immunized with un-adducted albumin, G-CSF serum levels were increased compared to PBS-injected mice (77 – 126%).

Liver pathology

To determine whether immunization with haptenized albumin induced liver injury, we measured serum levels of alanine aminotransferase and aspartate aminotransferase. No significant change in the serum level of these liver enzymes was found in immunized mice (data not shown).

We also investigated pathology of the liver using histology. In mice immunized with formyl-albumin, lymphocyte infiltration was apparent in the liver lobules and the portal area (in 4 out of 5 mice) (Fig. 3). In mice immunized with trifluoroacetyl-albumin, we observed lipid deposits in hepatocytes. These lipid deposits compressed and displaced the nucleus to the periphery (data not shown). No pathological changes were observed in the other groups (data not shown). We could also not detect pathological changes in the kidneys of any of the immunized mice (data not shown).

Discussion

It has long been recognized that environmental chemicals alter immune responses. Environmental effects on immune responses can lead to immunosuppression (Koller, 1980;Hatch et al., 1985;Moszczynski, 1997) or immunostimulation, resulting in autoimmune diseases. Chloroethenes have been implicated in inducing or accelerating autoimmunity. We and others have reported on the immunogenicity of TCE (Khan et al., 1995;Gilbert et al., 1999). We hypothesize that TCE promotes autoimmunity through a breakdown of self-tolerance by covalent haptenization of self-proteins with TCE metabolites. These newly formed protein adducts would act as neo-antigens, thus circumventing the naturally established tolerance to self-proteins.. Evidence supporting this hypothesis come from observations that T cells can recognize lipid peroxidation products of self-proteins (Wuttge et al., 1999). Further, covalent modifications of pyruvate dehydrogenase have produced a breakdown of immune tolerance in the mouse (Palmer et al., 2004)

Previously, the covalent binding of TCE metabolites to cytochrome P450 2E1 has been reported (Bolt and Filser, 1977;Stott et al., 1982;Mazzullo et al., 1992;Griffin et al., 2000). Metabolic conversion of TCE by P450 2E1 leads to TCEO, which in turn is biotransformed into DCAC and glyoxyl chloride (Uehleke and Poplawski-Tabarelli, 1977;Miller and Guengerich, 1983;Cai and Guengerich, 1999;2000). Glyoxyl chloride forms formyl adducts with proteins, whereas DCAC forms dichloroacyl adducts. Formyl adducts are 20 to 50-fold more prevalent than dichloroacyl adducts *in vitro* (Cai and Guengerich, 2000). Previously, dichloroacyl adducts have been shown to be associated with pulmonary cytotoxicity (Forkert et al., 2006).

To test our hypothesis that protein adducts with TCE metabolites are immunogenic and can act as neo-antigens, we evaluated the antigenicity of TCEO, dichloroacyl, and formyl adducts of albumin. As a negative control, we used un-adducted albumin. We also prepared trifluoroacetyl adducts of albumin for comparison, because trifluoroacetyl-adducted proteins have previously been implicated in autoimmune halothane hepatitis (Kenna et al., 1993;Christen et al., 1994). Homologous albumin was used as a carrier protein to eliminate the potentially confounding effects of heterologous proteins. For example, homologous albumin adducted with acetaldehyde is not immunogenic, whereas the same adducts of heterologous albumin induce immune responses (Yokoyama et al., 1993;Shimada et al., 2002). Similarly, we observed that albumin adducts with formaldehyde also yield differential immune responses depending on homologous or heterologous origins of albumin (Li et al., 2006).

To ensure that immune responses were not due to effects of the adjuvant used in immunization, groups of mice were injected with a mixture of PBS and adjuvant as an additional control. Our results showed minimal responses in mice injected with either PBS or un-adducted albumin, confirming that autoimmunity cannot be due to responses to adjuvant or unaltered albumin. However, mice immunized with any of the adducted albumin preparations generated albumin-specific IgG (Fig. 1), indicating a robust immune response with isotype class switching. Analysis of the IgG subtypes specific for haptenized albumin revealed a preferential isotype switch to IgG_1 (Fig. 2). This observation indicates that immune responses against haptenized

albumin were regulated by T helper type 2 cells (Finkelman et al., 1990;Cai et al., 2006). This finding is consistent with our previous report on antibody responses in MRL +/+ mice exposed to DCAC via intraperitoneal injection and subsequent *in vivo* haptenization of self-proteins (Cai et al., 2006). In humans, systemic autoimmune diseases such as progressive systemic sclerosis and systemic lupus erythematosus are also associated with T helper type 2 responses (Singh et al., 1999). In contrast to our results with intraperitoneally administered DCAC, we also detected IgG_{2a} specific for haptenized albumin, indicating that inflammatory responses occurred. This conclusion is based on the fact that interferon-γ promotes IgG_{2a} production (Finkelman *et al.*, 1988;Bossie and Vitetta, 1991).

Additional evidence for inflammatory responses comes from the observation that in MRL +/+ mice immunized with haptenized albumin, serum concentrations of G-CSF were increased in comparison to PBS-injected mice. This finding indicates activation of macrophages and acute inflammatory responses (Feghali and Wright, 1997). Cytokines exert their effects mainly in an autocrine and paracrine mode at the site of synthesis. Thus, it is not surprising that we could only detect two of the seven cytokines measured (TNF-α and G-CSF). The increased levels of G-CSF measured in the serum of mice nine days after the last booster immunization with haptenized albumin serum levels indicate therefore a robust immune response.

An interesting observation was that the antibody response against albumin was highest in the sera of MRL +/+ mice treated with formyl-albumin. This was accompanied by lymphocyte infiltration into the liver (autoimmune hepatitis), which occurred only in formyl-albumin treated mice. However, liver injury did not progress to affect ALT or AST serum levels. In unrelated experiments, we also observed lymphocyte infiltration into the liver in MRL +/+ mice chronically treated with TCE (unpublished results) as reported earlier (Griffin *et al.*, 2000a). Therefore, exposure to TCE may lead to *in vivo* formyl haptenization of self-proteins, suggesting a potential role for formyl adducts in TCE-mediated autoimmune hepatitis.

In conclusion, our results demonstrate immunogenicity of albumin haptenized with TCE metabolites in MRL +/+ mice, suggesting that neo-antigens may be formed by self-proteins adducted in this manner. Autoimmune diseases may then occur due to the lack of T cell tolerance to haptenized self-proteins.

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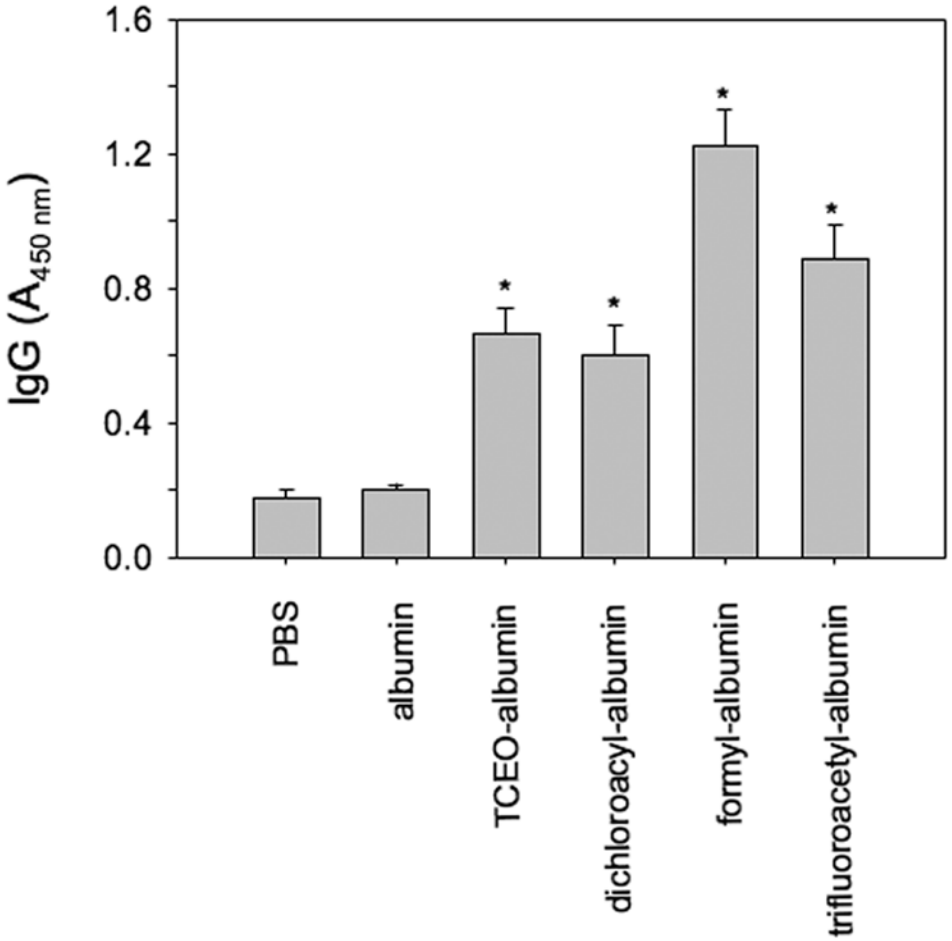

Fig 1.

Serum concentration of albumin-specific IgG in immunized MRL +/+ mice. Control mice were injected with a mixture of PBS and adjuvant or un-adducted albumin. The immunogen (antigen used in the immunization) is indicated on the abscissa. Reactivity of immune sera (1:100 dilution) against un-adducted albumin was measured by ELISA. The data are expressed as absorbance at 450 nm. Bars represent the mean ± SEM of five mice per group tested individually. * $P \leq 0.05$ compared to groups immunized with PBS or albumin.

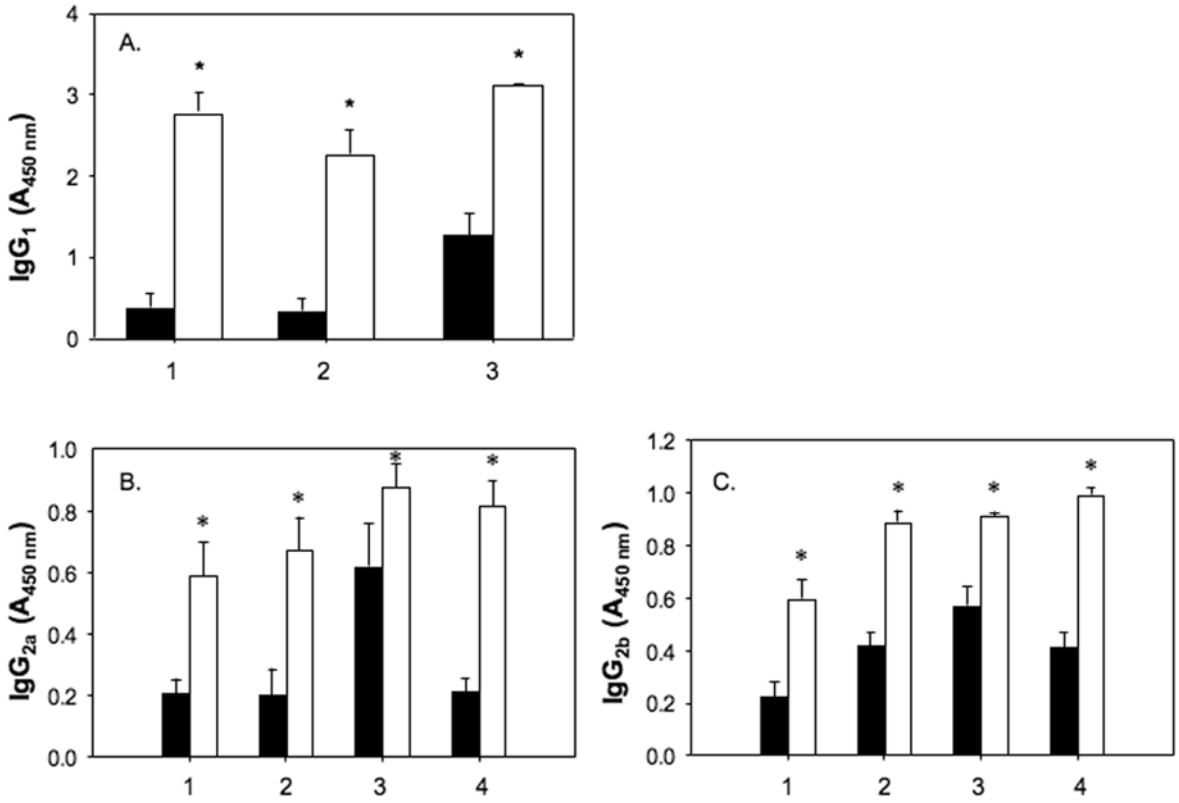

Fig 2.

Serum concentration of immunogen-specific IgG subtypes and cross-reactivity with un-adducted albumin. Sera from mice immunized with (1) TCEO- , (2) dichloroacyl-, (3) formyl-, and (4) trifluoroacyl-albumin were tested by ELISA for IgG subtypes reactive against un-adducted albumin (black bars) and immunogen (open bars). A. Serum concentration of IgG_1 specific for albumin or the haptenized albumin used as immunogen. Sera were diluted (1:1000). B. Serum concentration of IgG_{2a}. Sera were diluted (1:100). C. Serum concentration of IgG_{2b}. Sera were diluted (1:100). Bars represent the mean ± SEM of five mice per group tested individually. * $P \leq 0.05$ compared to albumin as an antigen.

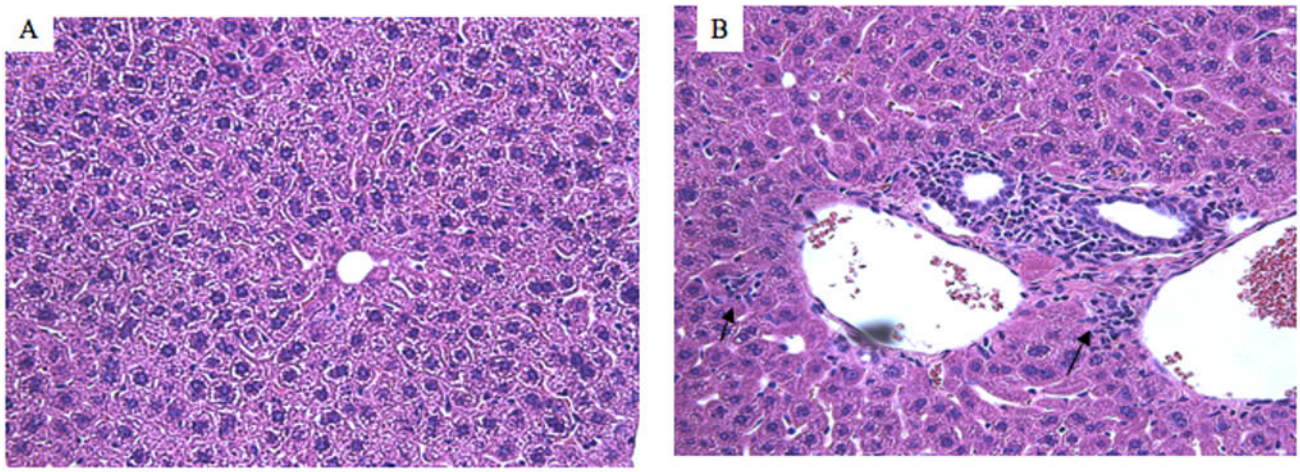

Fig 3.

Liver histopathology of mice immunized with albumin (A) or formyl-albumin (B). Tissue sections were stained with H & E. Magnification 200x. The arrows in B indicate lymphocyte infiltration.

Table 1

Molecular weights and numbers of modified amino acids in albumin adducts

Protein	Molecular weight (MALDI-TOF)	Number of modified amino groups (TNBS assay)
albumin	65,935	0
TCEO-albumin	66,997	34
dichloroacyl-albumin	69,149	41
formyl-albumin	67,250	48
trifluoroacyl-albumin	67,216	40

Table 2

Reactivity of IgG in sera from MRL +/+ mice immunized with albumin adducts

Antiserum against	Antigen used in ELISA				
	albumin	TCEO-albumin	dichloroacyl-albumin	formyl-albumin	trifluoroacyl-albumin
TCEO-albumin	0.27 ± 0.07*	1.83 ± 0.16	1.07 ± 0.12*	1.39 ± 0.21*	0.99 ± 0.14*
dichloroacyl-albumin	0.44 ± 0.11*	2.04 ± 0.05	1.92 ± 0.04	1.61 ± 0.60*	1.94 ± 0.08
formyl-albumin	0.81 ± 0.15*	1.77 ± 0.08*	1.45 ± 0.09*	2.10 ± 0.04	1.55 ± 0.08*
trifluoroacyl-albumin	0.66 ± 0.14*	1.93 ± 0.03	1.81 ± 0.03	1.96 ± 0.05	1.86 ± 0.05

* $P \leq 0.05$ when compared to the immunogen (n = 5).