# Immunochemical Detection of Protein Adducts in Mice Treated with Trichloroethylene

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Trichloroethylene has been shown to produce tumors in rodents and is a suspect human carcinogen. In addition, a number of case reports raise the possibility that trichloroethylene can induce an autoimmune disorder known as systemic sclerosis. To investigate whether covalent binding of reactive trichloroethylene metabolites may be involved in the mechanisms underlying these toxic responses, we have developed a polyclonal antibody that can recognize trichloroethylene−protein adducts in tissues. The antibody was prepared by immunizing a rabbit with dichloroacetic anhydride-modified keyhole limpet hemocyanin. Enzyme-linked immunosorbent assay data indicated that the serum antibody recognized dichloroacetic anhydride-modified rabbit serum albumin, but not unmodified protein. In addition, $N^{\epsilon}$-dichloroacetyl-L-lysine was the most potent inhibitor of antibody binding to dichloroacetic anhydride-modified rabbit serum albumin, indicating that the antibody recognizes primarily dichloroacetylated lysine residues. Immunoblots revealed the presence of two major trichloroethylene adducts at 50 and 100 kDa in liver microsomal fractions from male B6C3/F1 mice treated with trichloroethylene. The formation of trichloroethylene adducts was both dose and time dependent. Furthermore, the 50-kDa adduct was found to comigrate on a polyacrylamide gel with cytochrome P450 2E1. These data show that reactive metabolites of trichloroethylene are formed *in vivo* and bind covalently to discrete proteins in mouse liver. The data also suggest that one of the protein targets is cytochrome P450 2E1. Further studies will be necessary to elucidate the relationship between covalent binding of trichloroethylene and trichloroethylene toxicity.

## Introduction

Trichloroethylene (1,1,2-trichloroethene, TRI)[1] has been used extensively in industry as a solvent for the degreasing of metals. TRI has also been used as a dry cleaning agent, a lubricant, and an extractant in food processing and has had limited use as an inhalation anesthetic. The annual production of TRI in the United States is about 130 000 metric tons, and it has been estimated that 3.5 million people in this country are occupationally exposed to TRI (*1*). Because of its widespread use, TRI has become a major air pollutant and groundwater contaminant and is the most frequently detected organic pollutant at many hazardous waste sites (*2*).

Acute TRI exposure is known to induce a number of toxic effects in experimental animals, including CNS depression, impaired cardiac function, mild hepatotoxicity, and nephrotoxicity (*2*); similar effects have been observed in occupationally exposed humans (*3*). Chronic exposure to TRI is known to increase the incidence of liver and lung tumors in mice and kidney tumors in male rats (*2*, *4*, *5*). On the basis of these animal studies, TRI has been classified as a suspect human carcinogen. In addition, clinical case reports have indicated an association between TRI exposure and autoimmune disease, such as systemic sclerosis, systemic lupus erythematosus, and fasciitis (*6*−*9*).

Several studies have shown that metabolic activation is a requirement for TRI-induced cytotoxicity and carcinogenicity (*1*, *2*, *5*); however, the molecular mechanisms underlying these toxicities are not known. Although the production of liver tumors in B6C3/F1 mice is thought to be related to the ability of the TRI metabolite trichloroacetic acid to induce peroxisome proliferation (*4*), a role for covalent binding of reactive metabolites has not been entirely ruled out. TRI has been shown to initiate or accelerate an autoimmune response in MRL mice (*10*). Covalent binding of reactive TRI metabolites to protein may be responsible for initiating an immune response, directed against either the hapten (TRI), the protein (autoantigen), or a novel epitope on the protein that forms as a consequence of TRI binding to the protein (*11*).

TRI is known to undergo initial biotransformation by both oxidative (i.e., cytochrome P450) and conjugative (i.e., GSH transferase) pathways, either of which has the potential to generate reactive metabolites capable of binding covalently to protein (*1*, *2*, *5*). On the basis of *in vitro* studies, Miller and Guengerich (*12*, *13*) suggested that TRI forms an oxygenated intermediate complex with cytochrome P450 which, on chemical grounds, could be expected to rearrange to form a number of reactive

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[1] Abbreviations: TRI, trichloroethylene; DCA, dichloroacetyl; KLH, keyhole limpet hemocyanin; RSA, rabbit serum albumin; ELISA, enzyme-linked immunosorbent assay; DCA-RSA, dichloroacetylated rabbit serum albumin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DQ-COSY, double quantum filtered correlation spectroscopy.

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metabolites including TRI oxide, dichloroacetyl chloride, and chloral. In addition, conjugation of TRI by GSH followed by $\beta$-lyase-mediated metabolic activation is thought to result in the generation of reactive thioketene or thionoacyl chloride derivatives (*5*), which may be responsible for the nephrotoxic effects of TRI observed in male rats.

Although a variety of studies have reported that TRI undergoes metabolic activation and protein covalent binding *in vitro* and *in vivo* (*13*−*17*), confirmation of covalent binding *in vivo* using [$^{14}$C]TRI has been difficult since most of the label associated with protein arises as a consequence of metabolic incorporation of TRI metabolites into the amino acid ($C_2$) pool of proteins (*17*). Thus, the identity of the adduct(s) and the reactive metabolites from which they are formed remain to be established.

The present studies were undertaken to determine whether TRI adducts could be detected *in vivo* using an immunochemical approach (*11*). This was accomplished by raising polyclonal antibodies against dichloroacetylated proteins, which were assumed to have the highest probability of detecting a variety of TRI-derived adducts containing from one to three chlorine atoms. Furthermore, dichloroacetylated proteins would be expected to arise from dichloroacetyl chloride, a putative reactive metabolite of TRI (*2*, *13*). We report that anti-dichloroacetyl (anti-DCA) antibodies, collected from the serum of a rabbit immunized with dichloroacetylated keyhole limpet hemocyanin (KLH), recognized dichloroacetylated rabbit serum albumin (RSA) but not unmodified protein. Of importance, these antibodies were able to detect TRI−protein adducts in the livers of TRI-treated mice, which confirms that TRI undergoes metabolic activation and covalent binding to protein *in vivo*.

## Experimental Procedures

**Materials.** TRI (99% purity), dichloroacetic anhydride, $N^{\epsilon}$-acetyl-L-lysine, and mono-, di-, and trichloroacetyl chloride were obtained from Aldrich Chemical Company (Milwaukee, WI). L-Lysine monohydrochloride was purchased from NovaBiochem (La Jolla, CA). Anti-cytochrome P450 2E1 was a generous gift of Dr. Magnus Ingelman-Sundberg, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden. KLH, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), and the ImmunoPure IgG Orientation kit were obtained from Pierce Chemical Company (Rockford, IL). The alkaline phosphatase substrate kit was obtained from Bio-Rad Laboratories (Hercules, CA). Enhanced Chemiluminescence Western blotting detection reagents were from Amersham Life Science (Buckinghamshire, England). All other chemicals were reagent, electrophoretic, or HPLC grade and used without further purification.

**Preparation and Detection of Anti-DCA Antibodies.** Dichloroacetic anhydride (80 $\mu$L; 250 mM final concentration) was added dropwise to the immunogenic carrier protein KLH (40 mg) in 2 mL of sodium phosphate (pH 10) with vigorous mixing. The reaction mixture was incubated at room temperature for 1 h and then dialyzed three times with 4 L of 10 mM phosphate-buffered saline (pH 7.2). A nonrelated carrier protein, RSA, was derivatized in a similar manner for use as the solid-phase antigen in an enzyme-linked immunosorbent assay (ELISA).

One female New Zealand White rabbit was immunized sc at 15 sites along the back and im in the right and left hindquarters with 1.5 mg of dichloroacetylated KLH suspended in 5 vol of Freund's complete adjuvant. The rabbit was boosted 4 weeks later with 500 $\mu$g of dichloroacetyl KLH in 2 vol of Freund's incomplete adjuvant.

Anti-DCA serum antibodies were detected by an ELISA as described previously (*18*), with the use of dichloroacetylated rabbit serum albumin (DCA-RSA) as the test antigen. Serial dilutions of DCA-RSA (50 $\mu$L) in 0.06 M carbonate buffer (pH 9.6) were applied to microtiter plate wells and incubated overnight at 4 °C. The wells were washed, and rabbit serum (100 $\mu$L, diluted 1:500 to 1:1 093 500, v/v) was added and allowed to incubate for 2 h at room temperature. Specific antibody binding was detected with the use of goat anti-rabbit alkaline phosphatase (1:2000, v/v) and *p*-nitrophenyl phosphate as substrate. The absorbance was measured at 410 nm using a Dynatech plate reader.

A competitive ELISA was performed essentially as described above, except that 1 $\mu$g of solid-phase antigen was applied to each microtiter plate well. Rabbit serum (1:2500, v/v), incubated overnight in the presence of an equal volume of various concentrations of inhibitors, was then added to the plates, and the assay was carried out as described above.

**Affinity Purification of Rabbit Serum.** Rabbit antiserum was affinity purified using a column consisting of dichloroacetic acid conjugated to diaminodipropylamine immobilized on agarose gel. The affinity column was prepared by incubating dichloroacetic acid (2 mM) for 2 min with 10 mM EDC in 12.5 mM sodium phosphate (pH 5). This mixture was then added to a 50% suspension of diaminodipropylamine immobilized on 4% beaded agarose (Pierce) in 200 mM sodium phosphate (10 mL, pH 8) and incubated at room temperature for 24 h. The beads were washed with 10 mM Tris-HCl buffer (pH 7.5) and incubated with rabbit serum (1 mL) at room temperature for 1 h. The suspension was transferred to a column and washed with 2 column vol each of Tris-HCl buffer and Tris-HCl buffer containing 0.5 M NaCl. The antibody was then eluted with 0.2 M glycine (pH 2.6) and immediately neutralized with 1 M Tris (pH 8).

**Synthesis of Antibody Inhibitors.** $N^{\epsilon}$-Monochloroacetyl-L-lysine, $N^{\epsilon}$-dichloroacetyl-L-lysine, and $N^{\epsilon}$-trichloroacetyl-L-lysine were synthesized as described previously (*19*). Briefly, preparation of each lysine derivative was carried out by reacting L-lysine monohydrochloride (10 mmol) with the corresponding *S*-ethyl monochloro-, dichloro- and trichlorothiolacetate (15 mmol) in 10 mL of aqueous 1 N sodium hydroxide for 2 h at room temperature. The dichloro- and trichloroacetyl lysine derivatives precipitated from the reaction mixture and were collected by vacuum filtration. These lysine derivatives were then purified by recrystallization from absolute ethanol. The monochloroacetyl lysine derivative was purified by HPLC using a Vydac reverse-phase $C_{18}$ semi-preparative column (10 mm × 25 cm; Separations Group, Hesperia, CA). Monochloroacetyl lysine was eluted using an isocratic solvent system consisting of 0.1% aqueous trifluoroacetic acid/0.084% trifluoroacetic acid in acetonitrile (99:1, v/v), at a flow rate of 3 mL/min.

Liquid secondary ion mass spectrometry of the chloroacetyl lysine derivatives was performed in the first stage of a Jeol HX110/HX110 tandem mass spectrometer in the Medical University of South Carolina Mass Spectrometry Research Resource Facility. Approximately 1 $\mu$g of each compound dissolved in water (1 $\mu$L) was loaded onto the probe tip; glycerol was used as the matrix. Sample ionization was achieved by bombardment with 9 keV of cesium ions.

Proton NMR spectra of the chloroacetyl lysine derivatives dissolved in $Me_2SO$-$d_6$ (Sigma, St. Louis, MO) were acquired in the Medical University of South Carolina NMR Research Resource Facility on a Varian VXR 400 NMR spectrometer operating at 400 MHz with conventional quadrature detection. Spin−spin correlations were determined by employing a double quantum filtered correlation spectroscopy (DQ-COSY) pulse sequence collected in the phase-sensitive format. A total of 512 1024-point free induction decays were collected, resolution enhanced and converted from Lorenzian-to-Gaussian transformation functions, and then Fourier transformed to a 2 K × 2 K data matrix.

The purity of the chloroacetyl lysine derivatives was determined on a Waters HPLC system (Waters Associates, Milford,

MA) consisting of a Vydac $C_{18}$ reverse-phase analytical column (4.6 mm × 25 cm) and a UV absorbance detector (214 nm). The products were eluted using a 60-min linear gradient of 99% solvent A (aqueous 0.1% trifluoroacetic acid) to 99% solvent B (0.084% trifluoroacetic acid in acetonitrile) at a flow rate of 1 mL/min. HPLC analysis indicated a UV purity for each lysine derivative of >99%.

**Animal Treatment and Immunochemical Detection of TRI Adducts.** The presence of TRI−protein conjugates was determined in a time course and dose responsive manner with 12-wk-old B6C3/F1 male mice. Various doses of TRI were administered ip in corn oil (5 mL/kg). Mice dosed with corn oil only and sacrificed at 6 h served as controls. At designated intervals following TRI administration, mice were anesthetized with $CO_2$ and sacrificed by cervical dislocation. A blood sample was removed from the orbital sinus of each mouse for determination of serum alanine aminotransferase levels using a colorimetric assay kit from Sigma.

Livers from mice in each treatment group were excised, minced, and homogenized in 3 w/v of 0.25 M sucrose buffer (pH 7.5) containing 10 mM HEPES, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 1 $\mu$M leupeptin, and 1 $\mu$M pepstatin, utilizing a Dounce homogenizer, 6 strokes. An aliquot of each individual homogenate was reserved; then tissues from each group were pooled, frozen in liquid nitrogen, and stored at −80 °C until fractionation procedures were carried out.

Crude subcellular fractions were prepared using the liver homogenates as previously described (*20*). Protein content of tissues was determined according to Lowry et al. (*21*), using bovine serum albumin as a standard. Proteins from the 25000*g* pellet, cytosolic, and microsomal fractions were separated by SDS−PAGE under reducing conditions and then transferred to nitrocellulose as described previously (*22*). Adducts were detected with affinity-purified anti-DCA serum antibodies using an alkaline phosphatase detection system, following reported procedures (*18*, *22*).

The potential that the 50-kDa TRI−protein adduct is cytochrome P450 2E1 was explored by comigration following electrophoresis. Proteins separated by SDS−PAGE were transferred to nitrocellulose; parallel lanes were immunochemically probed with anti-DCA or anti-cytochrome P450 2E1 and visualized using chemiluminescence.

## Results

**Synthesis of Antibody Inhibitors.** To characterize the structural specificity of the serum antibodies (see below), a series of $N^{\epsilon}$-(chloroacetyl)-L-lysine derivatives (i.e., $N^{\epsilon}$-[chloroacetyl]-L-lysine, $N^{\epsilon}$-[dichloroacetyl]-L-lysine, and $N^{\epsilon}$-[trichloroacetyl]-L-lysine) were synthesized as described by Birner et al. (*19*). Structure and purity of the chloroacetyl lysine derivatives were confirmed by HPLC, mass, and NMR spectral analyses. Chromatographic analysis of the purified monochloro-, dichloro-, and trichloroacetyl lysine derivatives (214 nm) showed single peaks with retention times of 9.2, 14.0, and 19.4 min, respectively. Mass spectral analysis of the chloroacetyl lysine derivatives showed molecular ions ($MH^+$) at *m*/*z* 222.7 (monochloro), 256.9 (dichloro), and 290.6 (trichloro), and chlorine isotopic patterns consistent with the number of chlorine atoms for each derivative. In addition, DQ-COSY analysis confirmed that chloroacetylation had occurred on the $\epsilon$-amino group of lysine (Table 1). Of interest, monochloroacetyl lysine was found to react with ethanethiol (liberated from *S*-ethyl monochlorothiolacetate) during its synthesis to form the corresponding thioether as a major byproduct of the reaction. This observation raises the possibility that a monochloroacetylated lysine adduct, if formed *in vivo*, may not be stable.

**Table 1. 400 MHz $^1$H-NMR Spectral Parameters of $N^{\epsilon}$-Chloroacetylated Lysine Derivatives**

| | chloroacetyl Lys[a] | | dichloroacetyl Lys[a] | | trichloroacetyl Lys[a] | |
|---|---|---|---|---|---|---|
| proton | shift ($\delta$)[b] | multiplicity (*J*, Hz) | shift ($\delta$)[b] | multiplicity (*J*, Hz) | shift ($\delta$)[b] | multiplicity (*J*, Hz) |
| NH | 8.21 | t (5.5) | 8.59 | t (5.5) | 8.99 | t (5.5) |
| $H_3N^+$ | n.o.[c] | | 8.21 | bs | 8.20 | bs |
| $H_\alpha$ | 3.85 | t (6.2) | 3.88 | m | 3.87 | m |
| $H_\beta$ | 1.74[d] | m | 1.74[d] | m | 1.75[d] | m |
| $H_{\beta'}$ | 1.75[d] | m | 1.75[d] | m | 1.76[d] | m |
| $H_\delta$ | 1.34[d] | m | 1.35[d] | m | 1.36[d] | m |
| $H_\gamma$ | 1.42[d] | m | 1.43[d] | m | 1.50[d] | m |
| $H_\epsilon$ | 3.08 | q (6.5) | 3.11[b] | q (6.8) | 3.18 | q (6.6) |
| $CH_xCl_y$ | 4.03 | s | 6.44 | s | | |

[a] The test compounds were fully protonated as the trifluroacetate salts. [b] In $Me_2SO$-$d_6$ downfield from TMS ($\delta$ 0.00 ppm). [c] Not observed due to the presence of a strong water peak in the spectrum. [d] Chemical shift determination based on the center of the DQ-COSY cross peak. Abbreviations: s, singlet; t, triplet; q, quartet; m, multiplet; bs, broad singlet.

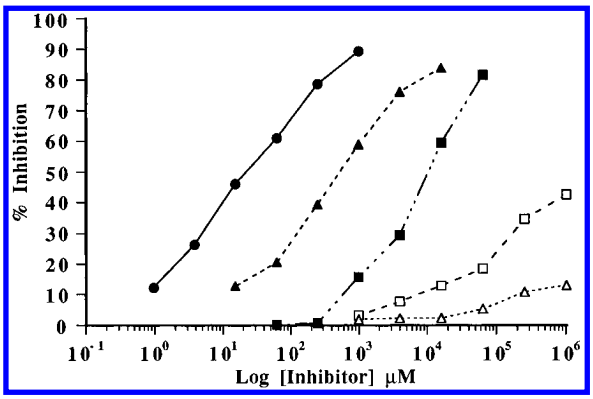


**Figure 1.** Competitive inhibition of binding of anti-DCA serum antibodies to the solid phase antigen, DCA-RSA (1 $\mu$g/well). Serial dilutions of inhibitors were incubated overnight with affinity purified rabbit antiserum (1:2500, v/v). The inhibitors used in the competitive ELISA were $N^{\epsilon}$-(dichloroacetyl)-L-lysine (●), $N^{\epsilon}$-(trichloroacetyl)-L-lysine (▲), $N^{\epsilon}$-(chloroacetyl)-L-lysine (■), $N^{\epsilon}$-(acetyl)-L-lysine (□), and L-lysine monohydrochloride (△).

**Characterization of Antibody Specificity.** In an ELISA, serum from a rabbit immunized with dichloroacetylated KLH was found to react with the solid-phase antigen (DCA-RSA) but not with unmodified RSA (data not shown). In a competitive ELISA, using affinity purified antiserum (Figure 1), the $N^{\epsilon}$-(chloroacetyl)-L-lysine derivatives were at least 3 orders of magnitude more effective as inhibitors of antibody binding to DCA-RSA than was L-lysine ($IC_{50}$ >1 M) or $N^{\epsilon}$-(acetyl)-L-lysine ($IC_{50}$ 0.6 M). Dichloroacetyl lysine was the most potent inhibitor tested, with an $IC_{50}$ of about 15 $\mu$M. Trichloroacetyl and monochloroacetyl lysine were less potent, with $IC_{50}$ values of 0.6 and 10 mM, respectively. TRI, trichloroacetic acid, and dichloroacetic acid, which were also tested for inhibitory activity, had no effect on antibody binding below concentrations that resulted in antibody inactivation due to nonspecific (i.e., denaturing) effects.

**Immunochemical Detection of TRI Adducts *in Vivo*.** In order to confirm the existence of TRI−protein adducts *in vivo* and also to determine whether the anti-DCA antibody could detect such adducts, mice were given a single dose of TRI (1000 mg/kg) and sacrificed 1, 3, 6, and 9 h later. The livers were then removed and homogenized, and subcellular fractions were prepared. Microsomal, 25000*g* pellet, and cytosolic proteins were then separated by SDS−PAGE, transferred to nitrocellulose, and probed with anti-DCA antibodies (Figure 2).

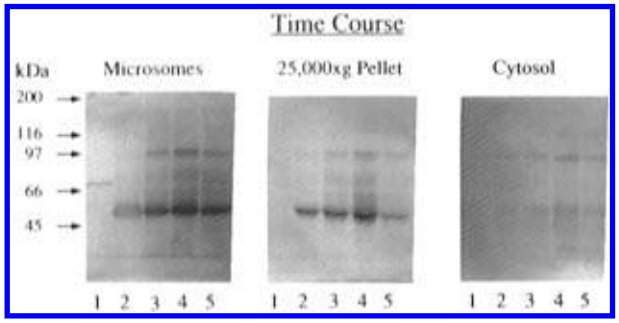


**Figure 2.** Immunoblot detection of liver protein adducts using affinity purified anti-DCA antiserum following SDS−PAGE of (left panel) microsomal, (center panel) 25000*g* pellet, and (right panel) cytosolic proteins (200 $\mu$g of protein/lane) from male B6C3/F1 mice given TRI (1000 mg/kg, ip in corn oil). Mice were sacrificed 1 (lane 2), 3 (lane 3), 6 (lane 4), and 9 h (lane 5) after TRI administration. Mice treated with corn oil and sacrificed at 6 h served as controls (lane 1).

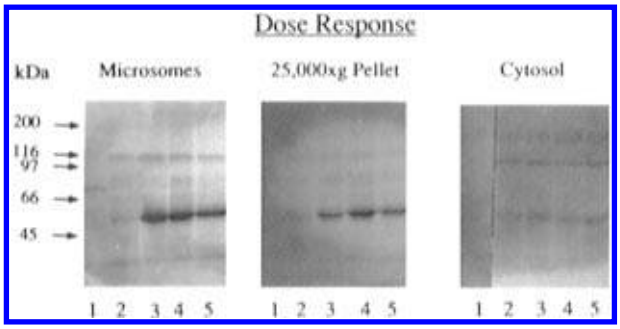


**Figure 3.** Effect of dose of TRI on the amount of liver protein adducts detected by the anti-DCA antibody in (left panel) microsomal, (center panel) 25000*g* pellet, and (right panel) cytosolic proteins (200 $\mu$g of protein/lane) from male B6C3/F1 mice. Lane 1: corn oil control; lane 2: 250 mg/kg; lane 3: 500 mg/kg; lane 4: 1000 mg/kg; lane 5: 2000 mg/kg TRI.

In the microsomal fraction of corn oil-treated mice (Figure 2, lane 1), a single 70-kDa protein reacted immunochemically with the anti-DCA antibody; however, this reaction was not observed consistently, and its significance is not yet known. In contrast, two major adducts, at 50 and 100 kDa, were detected in liver proteins from TRI-treated mice. As shown in Figure 2 (left panel, lanes 2−4), the amount of the 50- and 100-kDa adducts in the microsomal fraction increased up to 6 h following treatment and then declined by 9 h. Similar results were observed in the 25000*g* pellet (Figure 2, center panel, lanes 2−4); however, an additional protein band was observed in this fraction at approximately 95 kDa. In the cytosol (Figure 2, right panel), the 50- and 100-kDa adducts were observed; however, the intensity of staining in this fraction was much less than that observed in microsomes.

Adduct formation in the liver was also dose-dependent from 250 to 1000 mg/kg (Figure 3). In the microsomes and the 25000*g* pellet, the 50-kDa adduct exhibited the highest intensity of staining when using the anti-DCA antibody, with the 100-kDa adduct staining less intensely. Similar bands are observed at all dose levels, with the most concentrated staining in the 50- and 100-kDa protein fractions. In the cytosolic fraction, staining was much less intense; however, both the 50- and 100-kDa adducts are detectable.

In order to confirm the specificity of TRI adduct detection, microsomal and 25000*g* proteins from TRI-treated mice were probed with the anti-DCA antibody in the presence of 500 $\mu$M of the chloroacetyl lysine

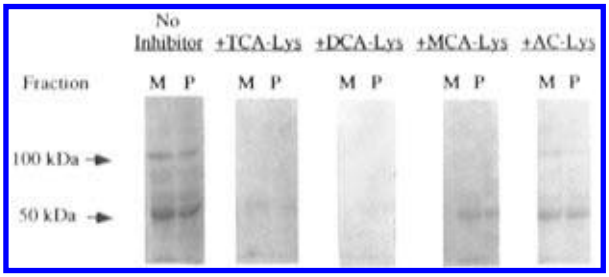


**Figure 4.** Inhibition of immunoblot detection of protein adducts in the microsomal (M) and 25000*g* pellet (P) fractions from liver homogenates of B6C3/F1 mice given TRI (1000 mg/kg). Mice were sacrificed 6 h after TRI administration. anti-DCA antiserum was incubated overnight in the absence (no inhibitor) or presence of 500 $\mu$M $N^{\epsilon}$-(trichloroacetyl) lysine (TCA-Lys), $N^{\epsilon}$-(dichloroacetyl) lysine (DCA-Lys), $N^{\epsilon}$-(chloroacetyl) lysine (MCA-Lys), and $N^{\epsilon}$-(acetyl) lysine (AC-Lys) prior to immunoblot analysis.

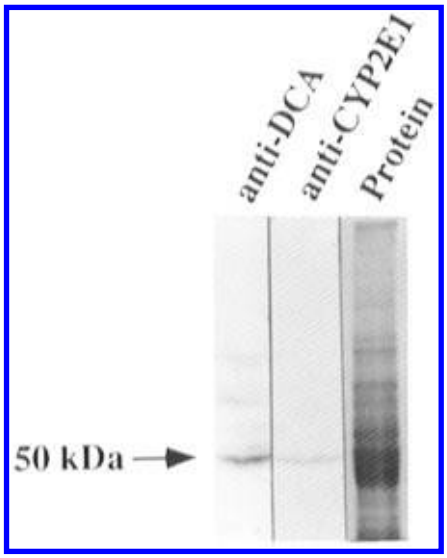


**Figure 5.** Immunoblot detection of protein adducts following SDS−PAGE of liver microsomal protein (200 $\mu$g protein/lane). Mice were sacrificed 6 h after administration of TRI (1000 mg/kg) One lane was stained with anti-DCA antiserum (anti-DCA), and the other was stained with anti-cytochrome P450 2E1 (anti-CYP2E1). One lane of the SDS−PAGE gel was stained with Coomassie blue to visualize all proteins.

inhibitors. As shown in Figure 4, all of the chloroacetyl lysine derivatives were inhibitory. Dichloroacetyl lysine completely inhibited antibody binding under the described experimental conditions, whereas trichloroacetyl and monochloroacetyl lysine were less potent inhibitors, respectively; this pattern was also seen in the competitive ELISA. Acetyl lysine was the least effective inhibitor tested.

**Immunoblot of Liver Microsomal Protein Using Anti-CYP2E1 Antisera.** Since TRI has been shown previously to be a mechanism-based inhibitor of cytochrome P450 (*12*), the possibility that the 50-kDa protein was cytochrome P450 was investigated. Liver microsomal protein from mice treated with 1000 mg/kg TRI and sacrificed at 6 h was divided into two aliquots, each of which was separated on a polyacrylamide gel, and transferred to nitrocellulose. One lane was stained with the anti-DCA antibody, and the other was stained with an antibody raised against cytochrome P450 2E1, an isoform known to be responsible for TRI oxidation in rats (*23*) and humans (*24*). As shown in Figure 5, a single 50-kDa protein was detected in the lane probed with anti-cytochrome P450 2E1. This protein band comigrated

with the 50-kDa protein adduct detected in the lane probed with anti-DCA antibodies.

**TRI-Induced Hepatotoxicity.** Serum alanine aminotransferase levels were determined on all mice from each of the treatment groups. Alanine aminotransferase levels for mice treated with TRI were not found to be statistically different from those of control mice (data not shown).

## Discussion

These studies describe the preparation of a rabbit polyclonal antibody against dichloroacetylated KLH, which is highly specific for the dichloroacetyl moiety on protein (Figure 1). When the anti-DCA antibody was used to probe liver protein from TRI-treated mice (Figures 2 and 3), two major protein adducts, at 50 and 100 kDa, were detected. Adduct formation was found to be both dose and time dependent in all subcellular fractions examined; however, the amount of TRI adduct formation was highest in the microsomal fraction. In addition, anti-DCA specific binding to TRI adducts on immunoblots was inhibited completely by $N^{\epsilon}$-(dichloroacetyl)-L-lysine (Figure 4).

Of interest, the formation of TRI adducts appeared to be highly selective, in that most proteins in the liver did not react with the anti-DCA antibodies. This observation is in contrast to the situation seen with acetaminophen, where the reactive metabolite *N*-acetyl-*p*-benzoquinone imine has been shown by immunochemical methods to covalently bind first to a small, selective group of hepatic proteins, and later in the course of toxicity (after 2−4 h), to a relatively large number of proteins (*22*, *25*). The reason for the high degree of selectivity observed with TRI covalent binding is not yet known. However, since the presumed target for TRI reactive metabolites (i.e., the $\epsilon$-amino groups of lysine in cellular protein) is widely available in the cytosol and within cellular organelles, the lack of general binding indicates that the TRI reactive metabolite is not kinetically free in the cell. One possibility is that the selectivity of binding is related to the ability of TRI to inactivate cytochrome P450 by a mechanism-based process (*12*, *13*, *26*). That is, a TRI-oxygenated cytochrome P450 intermediate complex rearranges to a reactive intermediate that binds covalently to the apoprotein and/or the heme prosthetic group. Since hepatic microsomal cytochrome P450 2E1 is thought to be the major isoform responsible for the oxidation of TRI in rodents (*23*, *27*), we postulate that the 50-kDa TRI-adducted protein is cytochrome P450 2E1. This postulate is supported by the data presented in Figure 5, which shows that the 50-kDa TRI adduct comigrates electrophoretically with a protein recognized by an antibody raised against cytochrome P450 2E1. The identity of the 100-kDa adduct is not yet known; however, it seems unlikely that it is a dimer of the 50-kDa adduct, since it did not react immunochemically with anti-cytochrome P450 2E1.

The intermediary role of acyl chlorides in the metabolism of haloalkenes and haloalkanes has been widely assumed based primarily on the appearance of haloacetic acid metabolites in microsomal incubations and as urinary metabolites (*19*, *28*). However, recent evidence suggests that haloacetic acid metabolites can arise by alternate metabolic pathways, as has been shown for the TRI metabolite, dichloroacetic acid, which can arise in mice via reductive dechlorination of trichloroacetic acid (*29*). Thus, the status of acyl chlorides in general, and dichloracetyl chloride in particular, as metabolites is in doubt. In the present studies, the high specificity of the anti-DCA antibody toward the dichloroacetyl moiety is significant because it suggests that some, if not all, of the TRI adducts are formed by acetylation of hepatic protein by a dichloroacetyl chloride metabolite of TRI. The validity of this approach is supported by the studies of Pohl and colleagues, who showed that a polyclonal antibody raised against trifluoroacetylated protein was able to recognize protein adducts formed in rats exposed to halothane (*30*).

Since metabolic incorporation of TRI metabolites into the amino acid pool does not interfere with immune recognition of chemical−protein adducts, it will be possible to use the anti-DCA antibody as a tool to investigate the role of covalent binding in the mechanisms underlying acute and chronic TRI toxicity. The antibody also has the potential for use as a dosimeter for TRI, which may be useful for assessing the risk associated with environmental exposure. Another application is the potential to determine whether anti-DCA antibodies are present in the sera of systemic sclerosis patients. This type of response has been observed previously with serum from halothane hepatitis patients, which recognized trifluoroacetylated proteins (*31*). Additionally, the proteins shown to bind covalently to reactive metabolites of TRI can be purified, through immunoaffinity or other forms of chromatography (*32*), and can be examined for changes in structure and/or activity. The ability to detect and identify the proteins covalently modified by TRI will enhance our understanding of the mechanism(s) of chemical-induced cytotoxicity and may elucidate the potential role of adduct formation in the production of a sclerosis-like disease.

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