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Halothane Metabolism: Immunochemical Evidence for Molecular Mimicry of Trifluoroacetylated Liver Protein Adducts by Constitutive Polypeptides

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SUMMARY

A monoform antibody [anti-TFA antibody] against TFA-protein adducts (TFA-adducts) was obtained by affinity purification of a polyclonal antiserum, raised in rabbits against TFA-rabbit serum albumin, on a *N*-ε-TFA-L-lysine matrix coupled to Affi-Gel 102. The anti-TFA antibody did recognize TFA-adducts of distinct molecular mass on Western blots of hepatocyte homogenates or microsomal membranes obtained from rats pretreated with halothane. The anti-TFA antibody also recognized cross-reactive polypeptides with apparent molecular masses of 52 kDa and 64 kDa on Western blots of hepatocyte homogenates obtained from rats not treated with halothane or metabolites thereof. The 52-kDa and 64-kDa cross-reactive polypeptides were localized in the 3,000 × *g* particulate fraction of liver homogenates. Recognition, on Western blots, of TFA-adducts and both the 52-kDa and 64-kDa cross-reactive polypeptides by anti-TFA antibody was sensitive to competition by *N*-ε-TFA-L-lysine ($IC_{50} < 100$ μM) and *N*-ε-acetyl-L-lysine ($IC_{50} \sim 10$ mM). Treatment with piperidine (1 M) did abolish the recognition of TFA-adducts but not that of the 52-kDa and the 64-kDa cross-reactive polypeptides by anti-TFA antibody on Western blots. In antibody-exchange experiments, anti-TFA antibody was affinity-adsorbed on Western blots to the 52-kDa or the 64-kDa cross-reactive polypeptides of the rat heart, followed by spontaneous transfer to target TFA-adducts present on Western blots of rat liver microsomal membranes. The majority of these target TFA-adducts were recognized by anti-TFA antibody transferring from the source 52-kDa or 64-kDa cross-reactive polypeptides. When examined up to 10 days after exposure of rats to a single dose of halothane, no influence on the constitutive level of expression, in the liver, of either cross-reactive polypeptide was observed. In contrast, TFA-adducts were persistent for >90 hr but <10 days. In addition to the liver, the 52-kDa and the 64-kDa cross-reactive polypeptides were prominently expressed in the heart and the kidney and, to a much lesser degree, in the spleen, the thymus, the lung, and skeletal muscle of the rat. Considerable variation in the level of expression of the 52-kDa and the 64-kDa cross-reactive polypeptides was recognized in livers of the six human individuals tested so far. These data strongly suggest that epitopes on the 52-kDa and the 64-kDa cross-reactive polypeptides confer molecular mimicry of epitopes on TFA-adducts, of which the trifluoroacetyl group is a dominant part.

Fulminate massive liver necrosis ("halothane hepatitis") is the more severe form of hepatotoxicity associated with the anesthetic agent halothane. Upon single or repeated exposure to the anesthetic, ~1 in 30,000 or 1 in 3,000 patients, respectively, is afflicted with halothane hepatitis (1). There is ample evidence suggesting that halothane hepatitis has an immunological basis (2). Most convincingly, sera of halothane hepatitis patients, but not of corresponding control individuals, contain antibodies against prominent liver microsomal heterologous polypeptides (so-called TFA-adducts) (3, 4), of ~100, 76, 59, 57, and 54 kDa, trifluoroacetylated on lysine moieties by the reactive halide CF_3COCl, which arises as a major metabolite upon oxidative cytochrome P450-dependent metabolism of halothane. After halothane pretreatment, such TFA-adducts have previously been detected on the surface of isolated rat hepatocytes (5), in rat and rabbit liver microsomal fractions (6, 7), and in guinea pig centrilobular liver sections (8). Moreover, limited studies seemed to indicate that liver biopsies obtained from halothane hepatitis patients also contained such TFA-adducts (7, 9).

Conceptually, there are several possible mechanisms by which TFA-adducts could be involved in eliciting an immune response. First, TFA-adducts are generated in susceptible individuals only; one or more of the distinct trifluoroacetylated

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ABBREVIATIONS: TFA-, trifluoroacetylated-; RSA, rabbit serum albumin; BSA, bovine serum albumin; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MAP 2, microtubule-associated protein 2; PMSF, phenylmethylsulfonyl fluoride; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay.

polypeptides are immunogenic. Second, in each individual exposed to halothane, TFA-adducts are generated; except in susceptible individuals, none are immunogenic. Third, all TFA-adducts are weakly immunogenic. However, a threshold density of TFA-adducts is necessary to induce an immune response; this threshold is reached in susceptible individuals only. Fourth, generation of TFA-adducts *per se* is not causative for occurrence of anti-TFA antibodies in sera of patients; it is the response of immunocompetent cells to the occurrence of TFA-adducts that is aberrant in patients. Finally, in normal individuals, there may exist a repertoire of self-peptides that are structurally identical to structures of TFA-adducts. As a consequence, tolerance against TFA-adducts might develop before exposure to halothane through thymic selective deletion (10–14) of maturing cells competent for recognition of TFA-adducts. Alternatively, thymic and/or peripheral induction of anergy (15) renders such cells silent towards TFA-motifs. In susceptible individuals only, this tolerance breaks down upon exposure to halothane and the associated generation of TFA-adducts. This last possibility is compatible with the finding that, in studies of halothane-induced hepatotoxicity, we observed generation of TFA-adducts in livers of all rats exposed to halothane, as has been observed previously by other investigators (6, 7, 16). However, these animals did not develop signs of halothane-induced hepatitis; in agreement with this observation, attempts by several groups to develop a truly valid animal model for this drug-induced hepatitis so far have been difficult or even have failed (16–19).

Therefore, we have concentrated our efforts on the detection of presumed structural analogs of TFA-adducts in experimental animals not previously exposed to halothane or its metabolites. In this report, we present the strategy to identify such analogs and report on their occurrence.

Experimental Procedures

Materials. *N*-ε-TFA-L-lysine and *N*-ε-acetyl-L-lysine were purchased from Senn Chemicals (Dielsdorf, Switzerland). *S*-Ethyltrifluorothioacetate, RSA, PMSF, soybean trypsin/chymotrypsin inhibitor, and molecular mass markers (SDS-6H and SDS-7) were all obtained from Sigma (St. Louis, MO). Collagenase/dispase was supplied by Boehringer (Mannheim, FRG). Goat anti-rabbit IgG (H + L) HRP-conjugate, Affi-Gel 102 amino-terminal agarose, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide chloride were from Bio-Rad (Richmond, CA). 4-Chloro-1-naphthol was from Fluka (Buchs, Switzerland). Halothane was obtained from Halothane Laboratories (Hackensack, NJ) and distilled before use. The enhanced chemiluminescence detection system was obtained from Amersham International (Amersham, UK) and used according to the manufacturers' guidelines.

Treatment of animals. Male Sprague-Dawley rats (250–300 g) were pretreated by three daily injections (intraperitoneally) of 80 mg/kg of body weight sodium phenobarbital in PBS. Halothane (10 mmol/kg of body weight, as a 50%, v/v, solution in sesame oil) was administered 18 hr (or the specified period of time; see below) before cells were prepared. In some experiments (as indicated below), animals were not pretreated with sodium phenobarbital. Control animals did not receive halothane.

Preparation of hepatocytes. Animals were fasted for 12 hr before the experiment. After animals were anesthetized [50 mg/kg of body weight pentobarbital (Nembutal), intraperitoneally], single-cell suspensions of hepatocytes were prepared by *in situ* perfusion of collagenase/dispase, as described (20). Separation of hepatocytes from nonparenchymal liver cells was performed according to the method of Smedsrød and Pertoft (20), with slight modifications. Briefly, cell suspensions were filtered through nylon mesh and then centrifuged for 4 min at 30 × *g*, yielding a pellet enriched in hepatocytes and a supernatant fraction enriched in nonparenchymal cells. The pellet was gently resuspended in PBS, pH 7.4, and aliquots of 20 ml were immediately layered on top of a Percoll cushion (15 ml) with a density of 1.070 g/ml and were centrifuged at 130 × *g* for 10 min. Viable hepatocytes (by trypan blue exclusion), which alone penetrated the Percoll layer, were collected and washed once more with PBS. Hepatocytes prepared by passage through a Percoll layer with a density of 1.070 g/ml typically contained <1% Kupffer cells (as tested by peroxidase staining) (20). Finally, the pellet of hepatocytes thus obtained was resuspended in TBS containing 0.5 mM PMSF and 60 μg/ml soybean trypsin/chymotrypsin inhibitor, and a homogenate was prepared by disruption of hepatocytes in TBS by four strokes in a Potter-Elvehjem homogenizer at 4°, followed by sonication for 10 min in a bath-type sonifier (Branson) in an ice-water bath. The homogenates were stored in aliquots at −80° and typically thawed only once, just before the experiment.

Preparation of rat liver microsomes. Rats were sacrificed by decapitation. The livers were removed immediately, rinsed with ice-cold TBS, and minced, and a homogenate (1:5, w/v) was prepared in TBS containing 0.5 mM PMSF and 60 μg/ml soybean trypsin/chymotrypsin inhibitor, by disruption of the tissue by four strokes in a Potter-Elvehjem homogenizer. The homogenate was differentially centrifuged to obtain subcellular fractions at 3,000 × *g*, (nuclei and cell debris), at 20,000 × *g* (mitochondria), and at 105,000 × *g* (microsomes). The microsomal pellet obtained at 105,000 × *g* was washed twice and resuspended, at ~10 mg of protein/ml, in TBS containing 0.5 mM PMSF and 60 μg/ml soybean trypsin/chymotrypsin inhibitor. The other pellets were washed once and resuspended in the same buffer. The supernatant of the 105,000 × *g* centrifugation was also kept and designated as cytosolic fraction. Aliquots were stored at −80° and thawed only once, just before the experiment.

Preparation of tissue homogenates. At the end of the *in situ* perfusion of the liver with collagenase/dispase (see above), rat tissues (i.e., heart, kidney, lung, spleen, thymus, and skeletal muscle) were excised and transferred to ice-cold TBS containing 0.5 mM PMSF and 60 μg/ml soybean trypsin/chymotrypsin inhibitor, rinsed, and minced, and a homogenate was prepared as described above. Aliquots were stored at −80° and thawed only once, just before the experiment. Human liver tissue, obtained from kidney donor individuals, was kindly provided by Prof. U. A. Meyer (Department of Pharmacology, Biocenter of the University, Basel, Switzerland) and taken from the human liver bank established in his laboratory (21). Frozen (−80°) liver sections were thawed in TBS containing 0.5 M PMSF and 60 μg/ml soybean trypsin/chymotrypsin inhibitor, and a homogenate (1:5, w/v) was prepared as described above. Aliquots were stored at −80° and thawed only once, just before the experiment.

Preparation of affinity-purified anti-TFA antibody. RSA was trifluoroacetylated using *S*-ethyltrifluorothioacetate, exactly according to published procedures (22). Polyclonal anti-TFA-RSA sera[1] were obtained, by adhering to published procedures (5), from female New Zealand white rabbits, after initial immunization with TFA-RSA (2 mg emulsified in complete Freund's adjuvant) followed by three weekly booster injections (2 mg of TFA-RSA emulsified in incomplete Freund's adjuvant). A total IgG fraction was obtained through precipitation (twice) of sera (10 ml) with 40% (w/v) ammonium sulfate. Total IgG was resuspended in PBS containing 0.75 M NaCl and 1% BSA (w/v) and was preadsorbed on an Affi-Gel 102 amino-terminal agarose matrix, in order to remove IgG nonspecifically bound to the gel matrix. After preadsorption, the IgG fraction was applied to an Affi-Gel 102 amino-terminal agarose column (1.0 × 10 cm), to which *N*-ε-TFA-L-

[1]The term "anti-TFA-RSA serum" designates the polyclonal antiserum obtained after immunization of rabbits with TFA-RSA. The term "anti-TFA antibody" designates the monoform IgG fraction obtained from anti-TFA-RSA serum through affinity purification on a *N*-ε-TFA-L-lysine matrix. The term "TFA-adduct" designates any polypeptide carrying TFA-amino acid residues, with no reference made to function and identity of that particular polypeptide.

lysine had been coupled by carbodiimide-facilitated chemistry, according to the manufacturers guidelines (BioRad), and which had been equilibrated with PBS containing 0.75 M NaCl and 1% BSA (w/v) (Fig. 1). The IgG-containing solution (~10 ml) was recirculated over the column at 4 ml/hr for 16 hr, followed by washing of the column, with PBS containing 0.75 M NaCl, until $\Delta A_{280\,nm}$ returned to the baseline level; anti-TFA antibody was then eluted with PBS containing 0.75 M NaCl and 50 mM *N*-ε-TFA-L-lysine. Fractions containing material absorbing at 280 nm were collected and tested for reactivity towards TFA-RSA on Western blots. Reactive fractions were pooled, supplemented with 1% BSA (w/v) and 0.05% sodium azide, and dialyzed at 4° against PBS containing 0.05% sodium azide; multiple changes of the dialysis buffer were used to reduce the calculated concentration of remaining *N*-ε-TFA-L-lysine 10^{12}-fold. Aliquots were stored at −80° and typically thawed only once.

Gel electrophoresis and Western blotting. Hepatocyte homogenates (5–10 mg of protein/ml) were mixed 1:1 (v/v) with dissociation buffer (0.5 M Tris·HCl, pH 6.8, 8% SDS, w/v, 20% glycerol, 4 mM EDTA, 40 mM dithiothreitol) and heated to 95° for 10 min. SDS-PAGE was performed according to the method of Laemmli (23), using a 4% stacking and a 10% separating gel. Protein loading was 250 μg/cm slot width. Electrophoresis was typically for 4 hr at 30 mA/gel. Resolved polypeptides were transferred electrophoretically to nitrocellulose, according to the method of Towbin *et al.* (24), at 360 V × hr, using a transfer buffer comprising 15.6 mM Tris, 120 mM glycine, and 20% methanol (v/v), pH 8.3. After transfer, the nitrocellulose was stained with amido black for visualization of proteins, destained, and blocked for 2 hr at room temperature with PBS containing 2% (w/v) dry milk powder and 0.02% (w/v) thimerosal. The nitrocellulose was then cut into strips (3-mm width) and used for antibody overlay. Incubation with the first antibody (appropriately diluted into blocking solution) was for 18 hr at room temperature under constant shaking. After five washes of 5 min each, with blocking solution, incubation with HRP-conjugated second antibody (diluted 1/100 into blocking solution) was for 2 hr at room temperature. After one wash with blocking solution and four washes with PBS for 5 min each, peroxidase activity was developed with 4-chloro-1-naphtol as a substrate. In all experiments, apparent molecular masses of antigenic polypeptides were estimated by comparing their relative mobilities with those of marker proteins of known molecular mass.

Antibody-exchange immunochemistry. Antibody-exchange experiments were performed according to the method of Hammarback and Vallee (25). The principle and validity of antibody-exchange immunochemistry as a tool to identify identical epitopes was convincingly demonstrated by these authors in experiments in which anti-MAP 2 monoclonal antibodies were spontaneously transferred from source MAP 2, immobilized on nitrocellulose chips, to target MAP 2, immobilized on nitrocellulose chips either as a purified protein fraction or as a component of rat brain cytosolic extracts (25). Accordingly, in our experiments, anti-TFA antibody (diluted 1/16 in 500 μl of PBS, corresponding to 18.5 μg of IgG/500 μl) was adsorbed (18 hr) to the 52-kDa and 64-kDa cross-reactive polypeptides[2] present on strips obtained from Western blots of rat heart homogenates. After five washes, of 5 min each, with PBS, the areas of the strips containing either the 52-kDa or the 64-kDa polypeptide/anti-TFA antibody complex were excised (disks of ~3 × 10 mm each). Five disks, constituting the source for anti-TFA antibody, were then coincubated with one target strip, originating from Western blots of liver microsomes obtained from rats 18 hr after exposure to a single dose of halothane. These incubations were done in 700 μl of PBS for 18 hr. After five washes, of 5 min each, with PBS, the target strips only were incubated with HRP-conjugated second antibody (diluted 1/100) for 2 hr. Note that, up to this point, PBS contained 2% (w/v) dry milk powder and 0.1% (w/v) thimerosal throughout and all incubations were done at room temperature. Target strips were then washed once with PBS containing 2% (w/v) dry milk powder and 0.1% (w/v) thimerosal and four times with PBS; visualization of TFA-adducts on target strips was by peroxidase-dependent enhanced chemiluminescence detection. In control experiments, coincubation of source disks with target strips was done in the presence of 1 mM *N*-ε-TFA-L-lysine; also, naked nitrocellulose disks with no immobilized cross-reactive polypeptides were incubated with anti-TFA antibody and used as source disks.

Indirect peroxidase ELISA. ELISA plates (96 wells) were prewashed two times, for 5 min, with 200 μl of PBS/well. Wells were coated with 100 ng/well TFA-RSA in 200 μl of 0.1 M sodium carbonate buffer, pH 9.6, for 16 hr at 4°. Wells were then washed once with 200 μl of PBS containing 0.05% (v/v) Tween 20 and two times with PBS alone. Blocking was for 90 min at 37° with PBS containing 2% (w/v) dry milk powder. After four washes with PBS containing 0.05% (v/v) Tween 20, first antibody (as indicated in the figure legends), adequately diluted in 200 μl of PBS, was added and incubated for 90 min at 37°. Four washes with PBS containing 0.05% (v/v) Tween 20 were followed by addition (200 μl) of HRP-labeled goat anti-rabbit IgG (1/1000 dilution in PBS) as a second antibody and further incubation for 90 min at 37°. Wells were washed with PBS containing 0.05% (v/v) Tween 20; finally, 100 μl of the peroxidase substrate solution (i.e., 0.1 M citric acid, pH 5.0, containing 0.4 mg of 1,2-phenylendiamine/ml and 0.1%, v/v, H_2O_2) were added to each well. Color development was stopped by addition of 100 μl of 2 M H_2SO_4, and the plates were read at 492 nm.

Other methods. Protein concentrations were estimated by the Bio-Rad assay procedure, using BSA as a standard. The extent of modification of free amine residues in the TFA-RSA preparation used throughout this study was estimated according to the method of Habeeb (26); when compared with unreacted control RSA, about 90% of these residues were modified in TFA-RSA. Cleavage of the trifluoroacetyl group from TFA-adducts present in hepatocyte homogenates from halothane-treated rats was done with piperidine (1 M) according to the method of Goldberger and Anfinsen (22); in some experiments, hepatocyte homogenates obtained from rats not treated with halothane were also treated with piperidine (1 M).

Results and Discussion

Ample evidence suggests that halothane-induced hepatitis, a rare but severe form of an idiosyncratic drug reaction associated with the anesthetic, has a immunological basis (1, 2). Most convincingly, when assayed on Western blots, sera obtained from patients suffering from halothane-induced hepatitis, but not those from healthy control individuals who had received halothane, contain antibodies directed towards a number of heterologous liver microsomal polypeptides (i.e., neoantigens)

[2]The term "52-kDa cross-reactive polypeptide" designates the cross-reactive polypeptide with apparent molecular mass of 52 kDa on SDS-PAGE that was first identified by anti-TFA antibody on Western blots of hepatocyte homogenates from rats not treated with halothane. The term is used also to designate the cross-reactive polypeptide with an identical apparent molecular mass of 52 kDa present in different tissues of the rat and in the liver of different species. It has not yet been established that the 52-kDa cross-reactive polypeptide represents the same polypeptide in each case. The same applies to the term "64-kDa cross-reactive polypeptide," which designates the cross-reactive polypeptide with apparent molecular mass of 64 kDa on SDS-PAGE that was initially identified by anti-TFA antibody on Western blots of hepatocyte homogenates from rats not treated with halothane.

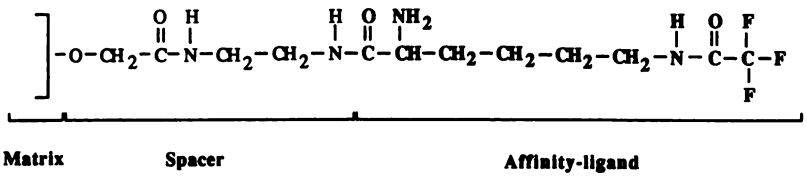

Fig. 1. Scheme of the affinity matrix used to purify anti-TFA antibody. *N*-ε-TFA-L-lysine was coupled to Affi-Gel 102 amino-terminal agarose by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chloride-facilitated chemistry, according to the manufacturers' guidelines (Bio-Rad). The affinity ligand is shown in *bold type*.

of distinct molecular mass (100, 76, 59, and 54 kDa) that were trifluoroacetylated upon oxidative metabolism of halothane (3, 4, 27). Some of these neoantigens have been isolated and cloned (28, 29). Limited data suggest that these sera also recognize neoantigenic determinants in liver biopsies of halothane hepatitis patients (7, 9). Moreover, earlier studies had revealed that halothane hepatitis patients carried a population of sensitized lymphocytes that were cytotoxic against hepatocytes obtained from rabbits previously treated with halothane (30, 31). So far, a true animal model of halothane hepatitis is still lacking (16), although a transient production of anti-TFA antibodies (IgG) in response to halothane treatment of rabbits (17) or guinea pigs (32) was observed in at least some animals.

In the course of our own studies, we have found that all rats treated with halothane do carry TFA-adducts; however, these animals have no indications of the development of hepatitis.[3] These findings might suggest that normal experimental animals are naturally tolerant against TFA-adducts; that is, polypeptides modified through trifluoroacetylation are generally not recognized by the immune system as non-self. The immunological self/non-self discrimination is thought to involve, in the thymus, major histocompatibility complex-restricted elimination (clonal deletion) or functional silencing (clonal anergy) of maturing T cells expressing the potential for reactivity against self-peptides (10–14). In addition, ectotopic presentation of antigen by "nonprofessional antigen-presenting cells" may lead, probably due to lack of appropriate second signals, to an anergic state of unprimed T cells refractory to subsequent stimulation by normal antigen-presenting cells (15). If, for any reason, animals might carry constitutive self-peptides that structurally mimic those motifs on TFA-adducts that are formed in the course of metabolism of halothane, one might speculate that these animals would most likely be tolerant against TFA-adducts; consequently, they would not mount an immune response towards TFA-adducts.

One strategy to identify such putative, constitutively expressed, self-peptides involves the generation of monoclonal or polyclonal monoform antibodies directed against the major haptenic determinant arising upon drug-protein adduct formation. Using such an approach, we have obtained a monoform anti-TFA antibody through affinity chromatography on a *N*-ε-TFA-L-lysine matrix of a rabbit polyclonal anti-TFA-RSA serum. This anti-TFA antibody recognized, in a highly specific manner, TFA-adducts on Western blots of hepatocyte homogenates obtained from rats pretreated with halothane. It also recognized a cross-reactive polypeptide of ~52 kDa and, to a much lesser degree, a cross-reactive polypeptide of ~64 kDa on Western blots of hepatocyte homogenates from rats that had not been treated with halothane or its metabolites. At least two pieces of evidence strongly suggest that these two cross-reactive polypeptides, indeed, do carry epitopes that immunochemically mimic epitopes on TFA-adducts elicited upon metabolism of halothane. First, inhibition studies on Western blots revealed a strong competition by *N*-ε-TFA-L-lysine of binding of anti-TFA antibody to the 52-kDa and the 64-kDa cross-reactive polypeptides; in the presence of the hapten derivative, half-maximal binding was observed at ~10 μM. In comparison, the recognition by anti-TFA antibody of TFA-adducts was slightly less sensitive to competition by *N*-ε-TFA-L-lysine (apparent IC_{50} of ~100 μM for the most sensitive TFA-adducts). Second, in antibody-exchange experiments, anti-TFA antibody, affinity-adsorbed on Western blots to epitopes of the 52-kDa or the 64-kDa cross-reactive polypeptide, did recognize as targets many of the TFA-adducts present on Western blots of microsomal membranes obtained from halothane-treated rats. The recognition of the target TFA-adducts was abolished in the presence of *N*-ε-TFA-L-lysine, and no reactivity was found in microsomal membranes of rats not treated with halothane.

Affinity purification and characterization of anti-TFA antibody. TFA-RSA is commonly used as a model immunogen for TFA-adducts (5), which are thought to be generated *in vivo* upon oxidative metabolism of halothane in the liver. Polyclonal antisera raised in rabbits against TFA-RSA showed considerable nonspecific cross-reactivity on Western blots towards constituents of hepatocytes from rats not treated with halothane (Fig. 2B, *lane 2*). In order to obtain from those sera the population of antibodies highly specific for *N*-ε-TFA-L-lysine residues, we synthesized an affinity matrix (Fig. 1) by coupling the hapten-derivative *N*-ε-TFA-L-lysine to Affi-Gel 102 amino-terminal agarose. The total IgG fraction of anti-TFA-RSA serum was adsorbed to this matrix, and anti-TFA antibody was then eluted through competitive displacement by the free ligand *N*-ε-TFA-L-lysine (100 mM); a typical elution pattern is shown in Fig. 2A, *top*. Fractions reactive against TFA-RSA on Western blots (Fig. 2A, *bottom*) were pooled. The specificity of anti-TFA antibody towards TFA-RSA was then compared with that of anti-TFA-RSA serum in an ELISA. Using TFA-RSA as the coating antigen as well as the competitor, IC_{50} values of ~0.8 and ~10.0 nM were observed for anti-TFA-RSA serum and anti-TFA antibody, respectively (Fig. 3A). IC_{50} values of ~0.2 and ~0.8 mM for anti-TFA antibody and anti-TFA-RSA serum, respectively, were obtained with *N*-ε-TFA-L-lysine as the inhibitor (Fig. 3B). These data suggested that anti-TFA antibody and anti-TFA-RSA serum did not differ considerably in their affinity towards the antigen TFA-RSA. However, when compared with anti-TFA-RSA serum, anti-TFA antibody showed a clearly increased affinity towards the hapten-derivative *N*-ε-TFA-L-lysine but a decreased affinity towards TFA-RSA. While this study was in progress, a report by Hubbard *et al.* (33) further confirmed the usefulness of synthetic antigenic matrices in obtaining antibodies directed in a highly specific manner against protein adducts.

The patterns of polypeptides recognized by anti-TFA-RSA serum or anti-TFA antibody were very different when Western blots of hepatocyte homogenates obtained from halothane-treated or untreated rats were probed. Anti-TFA-RSA serum did recognize a number of polypeptides on Western blots of hepatocyte homogenates from both halothane-treated and untreated rats (Fig. 2B, *lanes 1* and *2*, respectively); the recognition of only a few polypeptides was due to their trifuoroacetylation (data not shown). In contrast, anti-TFA antibody recognized several polypeptides of distinct apparent molecular mass on Western blots of hepatocyte homogenates from halothane-treated rats (Fig. 2B, *lane 3*). In this particular experiment, a single cross-reactive polypeptide with an apparent molecular mass of 52 kDa (Fig. 2B, *lane 4*) was recognized on Western blots of hepatocyte homogenates obtained from rats not treated with halothane. Except for this 52-kDa cross-reactive polypeptide, and in clear contrast to anti-TFA-RSA serum, no further cross-reactive material was detected on West-

[3] J. Gut, unpublished observations.

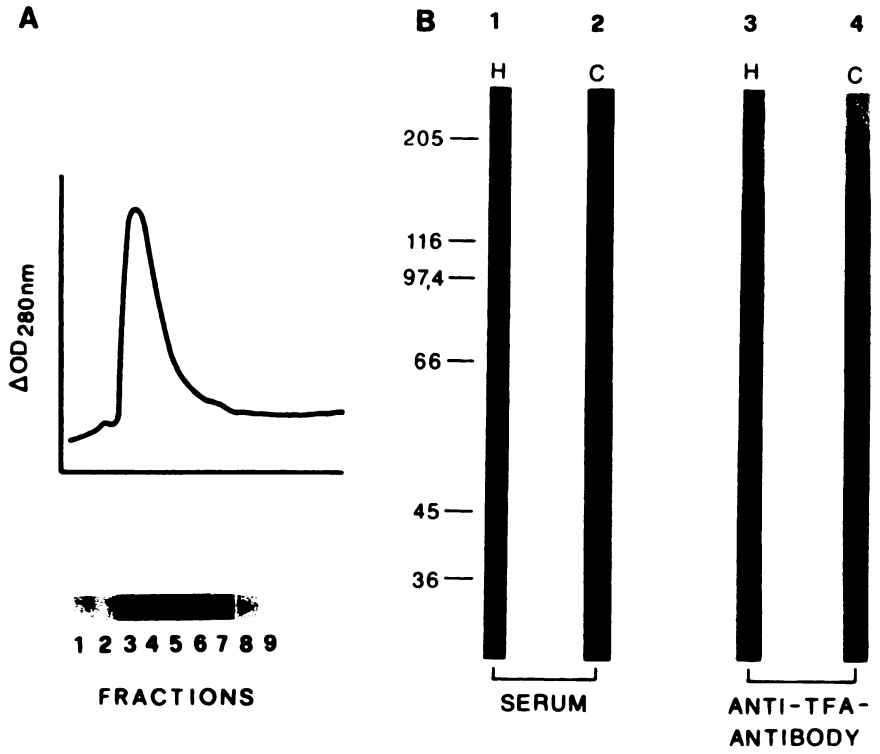

Fig. 2. Affinity purification of anti-TFA antibody on a *N*-ε-TFA-L-lysine matrix. The experiment shown here is representative of four experiments performed. A, The total IgG fraction obtained by ammonium sulfate precipitation (40%, w/v) from polyclonal rabbit anti-TFA-RSA serum (10 ml) was applied to the affinity column (*N*-ε-TFA-L-lysine coupled to Affi-Gel 102 amino-terminal agarose; 1.0 × 10 cm; 4 ml/hr), as described in Experimental Procedures. After extensive washing, elution of anti-TFA antibody was mediated through addition of 100 mM *N*-ε-TFA-L-lysine to the wash buffer. $\Delta A_{280\,nm}$ was recorded, and fractions 3–7 (~1.5 ml each), reactive on Western blots towards TFA-RSA, were pooled. B, The specificity of anti-TFA-RSA serum and of affinity-purified anti-TFA antibody, respectively, was tested on Western blots of rat hepatocyte homogenates obtained from rats 18 hr after exposure to a single dose of halothane (*H, lanes 1* and *3*) or from untreated control rats (*C, lanes 2* and *4*). Visualization of TFA-adducts was by peroxidase staining using 4-chloro-1-naphthol as a substrate. Apparent molecular masses of TFA-adducts in this and all subsequent experiments were estimated by comparison of their relative migration distances on SDS-PAGE with those of marker proteins of known molecular mass, as indicated [i.e., myosin, rabbit muscle subunit (205 kDa); β-galactosidase, *Escherichia coli* (116 kDa); phosphorylase *b*, rabbit muscle subunit (97.4 kDa); albumin, bovine plasma (66 kDa); albumin, egg ovalbumin (45 kDa); and glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36 kDa)].

ern blots of hepatocyte homogenates obtained from rats not treated with halothane. Piperidine has previously been shown to cleave acyl groups from primary amines (3, 22). Thus, in order to confirm that polypeptides recognized by anti-TFA antibody were in fact TFA-adducts, hepatocyte homogenates obtained from halothane-treated animals were treated with piperidine (1 M), followed by Western blot analysis. Under these conditions, removal of reactivity of anti-TFA antibody against TFA-adducts was observed (Fig. 4, *lane 2*), with the exception of the reactivity towards the 52-kDa cross-reactive polypeptide (Fig. 4, *lane 2*). Additionally, the very faint recognition of a cross-reactive polypeptide with an apparent molecular mass of ~64 kDa was detected, whose recognition also resisted the treatment with piperidine (Fig. 4, *lane 2*).

Characterization of constitutive polypeptides cross-reactive with anti-TFA-antibody. The recognition by anti-TFA antibody of cross-reactive polypeptides of ~52 kDa (Fig. 2B, *lane 4*) and ~64 kDa (Fig. 4, *lanes 2, 3,* and *4*) did suggest for the first time that, barring trivial reasons for their recognition, these polypeptides might bear constitutive epitopes that share similarity to epitopes elicited on TFA-adducts upon metabolism of halothane. The reactivity, on Western blots, of anti-TFA antibody against the 52-kDa and the 64-kDa cross-reactive polypeptides was not abolished by piperidine treatment of hepatocyte homogenates obtained from untreated rats (Fig. 4, *lane 4*); however, piperidine treatment did derange, in some way, the interaction of anti-TFA antibody with both cross-reactive polypeptides. Regardless, the data suggested that the 52-kDa and the 64-kDa cross-reactive polypeptides might carry epitopes that do not contain amino acid residues modified through trifluoroacetylation; alternatively, binding of trifluoroacetyl residues might not be reversible by piperidine, possibly due to chemical modification of the amide bond. A likely source of compounds covalently modifying the two distinct polypeptides present in rat hepatocytes with trifluoroacetyl groups recognized by anti-TFA antibody remains obscure at present. One should note that trivial reasons for detection of the two cross-reactive polypeptides have been ruled out. Preimmune serum does not recognize these polypeptides, nor was there any peroxidase reaction when Western blots of hepatocytes from both halothane-treated and untreated rats were incubated with either HRP-goat anti-rabbit second antibody or peroxidase substrate alone (data not shown).

In an attempt to further characterize the relatedness of the 52-kDa and 64-kDa cross-reactive polypeptides to TFA-adducts, we probed Western blots of hepatocytes from both halothane-treated and untreated rats with anti-TFA antibody, in the presence of the hapten-derivatives *N*-ε-TFA-L-lysine and

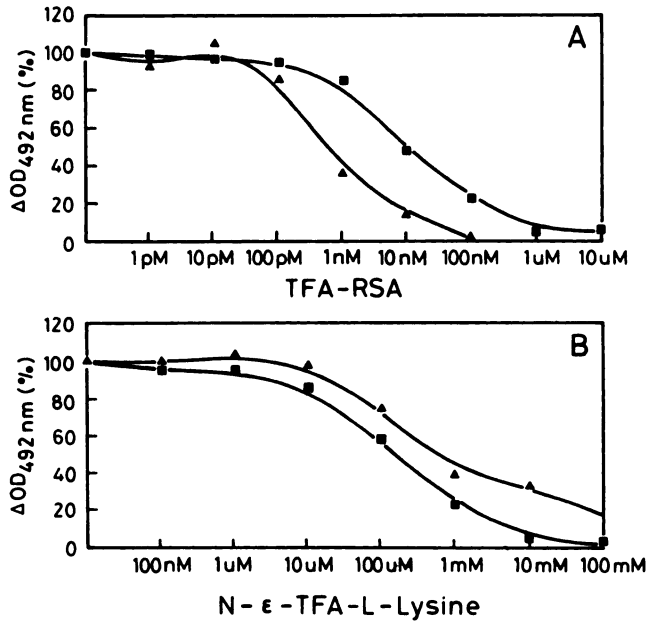

Fig. 3. Indirect peroxidase ELISA. Microtiter plates (96 wells) were coated with TFA-RSA as described in Experimental Procedures. Anti-TFA-RSA serum (▲) and anti-TFA antibody (■) were appropriately diluted into PBS and incubated together with increasing concentrations of TFA-RSA (A) or *N*-ε-TFA-L-lysine (B). Plates were then developed by addition of goat anti-rabbit IgG, followed by the substrate mixture, as described in Experimental Procedures. The results are expressed as percentage of $\Delta A_{492\,nm}$ obtained in the presence of inhibitor, relative to that obtained in the absence of inhibitor.

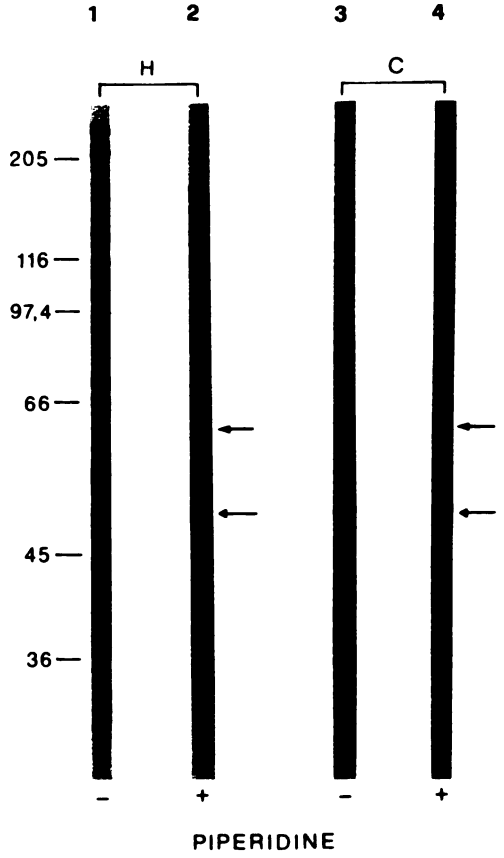

Fig. 4. Influence of piperidine treatment on recognition of TFA-adducts by anti-TFA antibody on Western blots. Hepatocyte homogenates obtained from rats 18 hr after a single dose of halothane (*H, lanes 1* and *2*) or from untreated rats (*C, lanes 3* and *4*) were treated (+) or not treated (−) with piperidine (1 M) and subsequently were subjected to SDS-PAGE, followed by Western blot analysis using anti-TFA antibody, as described in Experimental Procedures. Development of blots was by peroxidase staining with 4-chloro-1-naphthol as a substrate. *Arrows*, relative locations of the 52-kDa and the 64-kDa cross-reactive polypeptides. Note that, compared with the original blot, the reproduction processes diminished the intensity of the staining of the 52-kDa and the 64-kDa cross-reactive polypeptides.

N-ε-acetyl-L-lysine. A *N*-ε-TFA-L-lysine concentration-dependent decrease in recognition of polypeptides by anti-TFA antibody was observed; a mean IC_{50} of ~100 μM was found for TFA-adducts on blots obtained from halothane-treated rats (Fig. 5A, *top*). Surprisingly, the cross-reactive 52-kDa polypeptide found on blots from untreated rats was more sensitive to competition with *N*-ε-TFA-L-lysine (IC_{50} ~ 10 μM) (Fig. 5A, *bottom*) than were most of the TFA-adducts. Using the same approach, *N*-ε-acetyl-L-lysine competed with anti-TFA antibody staining in a similar fashion, with a mean IC_{50} of ~10 mM both for TFA-adducts (Fig. 5B, *top*) and the 52-kDa cross-reactive polypeptide (Fig. 5B, *bottom*). Identical results were obtained with the 64-kDa cross-reactive polypeptide (data not shown). L-Lysine at 1 and 100 mM did not interfere with the recognition of TFA-adducts or of the cross-reactive polypeptides on Western blots (data not shown). These data strongly suggest a close structural relatedness of epitopes on both the 52-kDa and the 64-kDa cross-reactive polypeptides to epitopes on TFA-adducts of distinct molecular mass elicited in experimental animals upon oxidative metabolism of halothane.

Here, we have excluded a general inhibition of binding of first antibodies to their antigens on Western blots through the presence of the hapten-derivatives. Western blots of hepatocytes from both halothane-treated and untreated rats were developed in the presence of, concomitantly, anti-TFA antibody and a polyclonal antibody directed against human microsomal epoxide hydrolase. Recognition of TFA-adducts by anti-TFA antibody ceased at ~100 μM *N*-ε-TFA-L-lysine; no inhibition of recognition of epoxide hydrolase by anti-epoxide hydrolase antibody was observed, even at 50 mM *N*-ε-TFA-L-lysine (data not shown).

Identification of immunochemically identical epitopes. A strong structural relatedness, expressed as immunochemical identity, of epitopes on both the 52-kDa and the 64-kDa cross-reactive polypeptides to epitopes present on TFA-adducts was demonstrated by antibody-exchange experiments (25). The finding of prominent expression in the heart (see below) of the 52-kDa and the 64-kDa polypeptides cross-reacting with anti-TFA antibody prompted us to investigate the relatedness of epitopes on these polypeptides to epitopes elicited on TFA-adducts of liver microsomal membranes. Anti-TFA antibody was affinity-adsorbed to either the 52-kDa cross-reactive polypeptide or the 64-kDa cross-reactive polypeptide present on nitrocellulose strips of Western blots of rat heart homogenates obtained from untreated rats. Such antibodies, adsorbed to their respective antigens (i.e., epitopes) on Western blots, are considered highly specific agents for heterologous detection of identical epitopes (25). Nitrocellulose chips containing antigen-antibody complexes of the 52-kDa cross-reactive polypeptide with anti-TFA antibody or the 64-kDa cross-reactive polypeptide with anti-TFA antibody, as the sole source of anti-TFA antibody, were coincubated with chips originating from West-

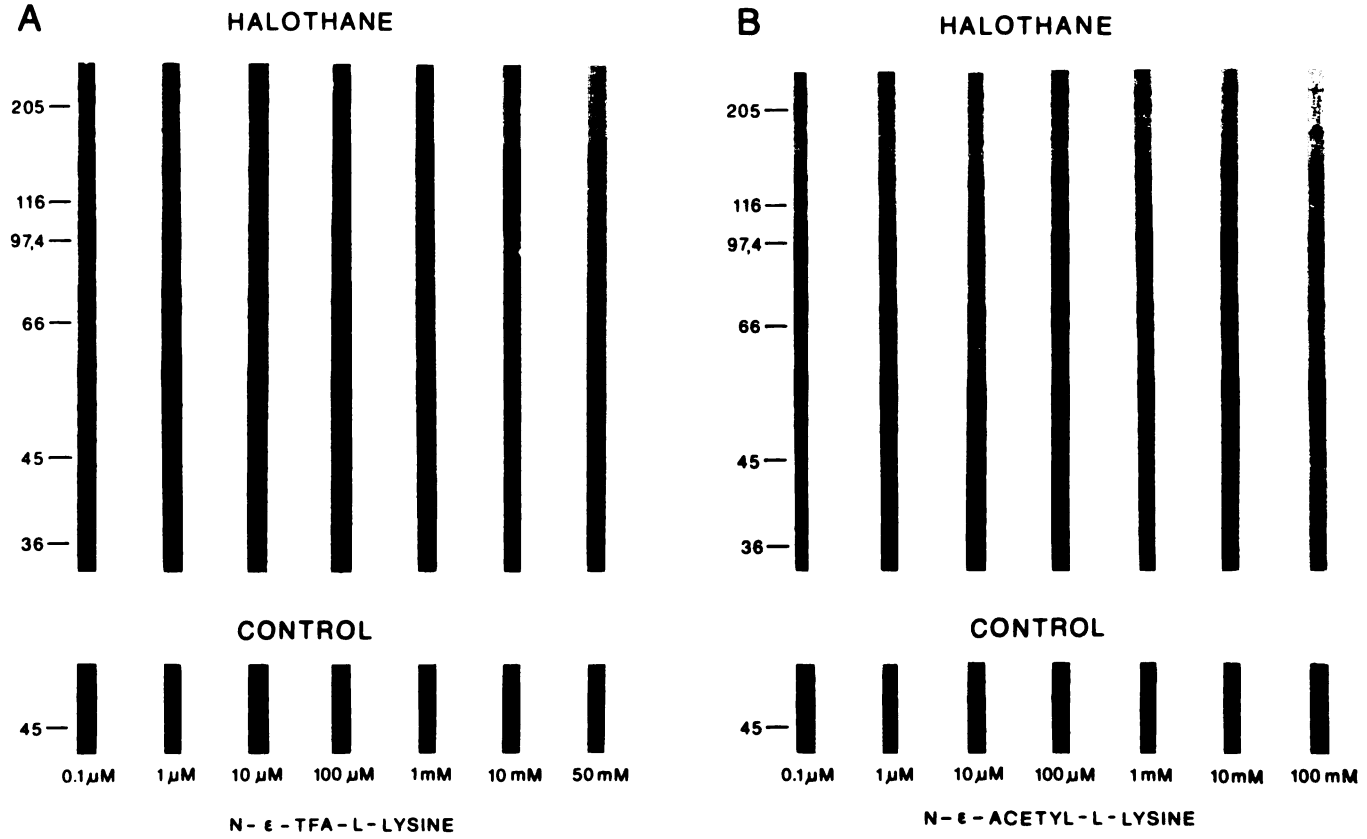

Fig. 5. Inhibition of recognition by anti-TFA antibody of TFA-adducts on Western blots. In the presence of increasing concentrations of the hapten-derivatives *N*-ε-TFA-L-lysine (A) and *N*-ε-acetyl-L-lysine (B), binding of anti-TFA antibody to TFA-adducts was tested on Western blots of hepatocyte homogenates obtained from rats 18 hr after a single dose of halothane (*top* of respective panel) or from untreated rats (*bottom* of respective panel). The hapten-derivatives were coincubated, at the concentrations indicated, with anti-TFA antibody (9.4 μg of IgG/500-μl final volume), in PBS containing 2% (w/v) dry milk powder and 0.02% (w/v) thimerosal, for 18 hr at room temperature, as described in Experimental Procedures. Development of blots was by peroxidase staining with 4-chloro-1-naphthol as a substrate.

ern blots of liver microsomes obtained from rats 18 hr after exposure to a single dose of halothane, as targets for TFA-adducts (Fig. 6A, *lane 5*). Visualization of anti-TFA antibody that had spontaneously transferred to and recognized the target TFA-adducts was done with HRP-conjugated second antibody and peroxidase-dependent enhanced chemiluminescence detection. Many of the TFA-adducts present on target strips were recognized by spontaneously exchanging anti-TFA antibodies that had been affinity-adsorbed to epitopes on the 52-kDa cross-reactive polypeptide (Fig. 6A, *lane 1*) or the 64-kDa cross-reactive polypeptide (Fig. 6B, *lane 1*) of rat heart, as shown in Fig. 6A, *lane 2*, and Fig. 6B, *lane 2*, respectively. A certain preference for recognition of a target TFA-adduct with apparent molecular mass of ~140 kDa was noted in these antibody-exchange experiments. In keeping with our earlier observations (Fig. 5A), 1 mM *N*-ε-TFA-L-lysine completely abolished recognition of target TFA-adducts by anti-TFA antibody affinity-adsorbed to either the 52-kDa (Fig. 6A, *lane 3*) or the 64-kDa cross-reactive polypeptide (Fig. 6B, *lane 3*). No reactivity of anti-TFA antibody exchanged from the 52-kDa cross-reactive polypeptide (Fig. 6A, *lane 4*) or the 64-kDa cross-reactive polypeptide (data not shown) was observed on Western blots of microsomes obtained from untreated rat liver. This finding is in keeping with the observation that no cross-reactive polypeptides are found in microsomes obtained from untreated rats (Fig. 6A, *lane 6*, and Fig. 7B, *lane 4*). In control experiments, no reactivity towards target TFA-adducts was observed when naked nitrocellulose disks were incubated with anti-TFA antibody and subsequently used as source disks (data not shown).

Tissue-specific expression and subcellular localization of cross-reactive polypeptides. In a separate set of experiments, the tissue-specific expression and the subcellular localization of polypeptides cross-reactive with anti-TFA antibody were examined. Western blots of homogenates of the heart, lung, spleen, thymus, kidney, and muscle obtained from rats 6 hr after exposure to a single dose of halothane were probed for TFA-adducts and/or cross-reactive polypeptides with anti-TFA antibody. Three major observations were made. First, upon re-examination of Western blots of hepatocytes obtained from control rats, using HRP-goat anti-rabbit second antibody and the highly sensitive peroxidase-dependent enhanced chemiluminescence detection method, we confirmed the recognition by anti-TFA antibody of the cross-reactive polypeptide of ~64 kDa (Fig. 7A, *lane 7*). This cross-reactive polypeptide was only faintly recognized before, presumably due to too low a sensitivity of peroxidase-dependent staining of Western blots with 4-chloro-1-naphthol used in those earlier experiments. Second, we identified prominent polypeptides with apparent molecular masses of ~52 and ~64 kDa, cross-reacting with anti-TFA antibody, in the heart (Fig. 7A, *lane 3*), the kidney (Fig. 7A, *lane 8*), and, to a much lesser degree, the lung (Fig. 7A, *lane 7*), the spleen (Fig. 7A, *lane 4*), the thymus (Fig. 7A, *lane 6*), and

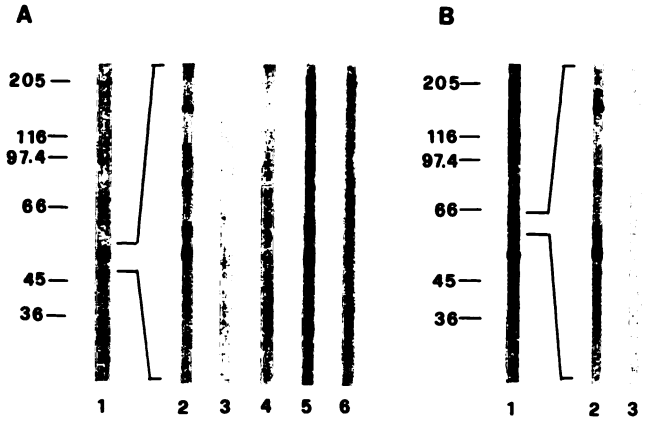

Fig. 6. Antibody-exchange immunochemistry. Rat heart tissue homogenate was subjected to SDS-PAGE (375 μg of protein/cm slot width), followed by Western blotting. After whole blots were cut into strips, they were incubated with anti-TFA antibody (18 hr, room temperature). The locations of the 52-kDa and the 64-kDa cross-reactive polypeptides were determined on analogous control strips by peroxidase staining, and the respective areas (disks of ~3 × 10 mm) were cut from the corresponding source strips. In the antibody-exchange step, five source disks were coincubated with one target strip for 18 hr at room temperature. Target strips only were then incubated for 2 hr with HRP-goat anti-rabbit second antibody (diluted 1/100). Throughout this experiment, TFA-adducts were visualized by peroxidase-dependent enhanced chemiluminescence detection. A, *Lane 1*, disks containing the 52-kDa polypeptide/anti-TFA antibody complex were used as sources. The following targets were used: *lane 2*, microsomes obtained from phenobarbital-pretreated rats 18 hr after a single dose of halothane; *lane 3*, same as *lane 2*, but 1 mM *N*-ε-TFA-L-lysine was included in the antibody-exchange step; *lane 4*, microsomes obtained from rats not treated with halothane; *lane 5*, microsomes obtained from rats 18 hr after a single dose of halothane; in this experiment, detection of TFA-adducts was by using anti-TFA antibody without prior affinity adsorption to the 52-kDa cross-reactive polypeptide; *lane 6*, same as *lane 5*, but microsomes were obtained from rats not treated with halothane. B, *lane 1*, disks containing the 64-kDa polypeptide-anti-TFA antibody complex were used as sources. Targets were as follows: *lane 2*, microsomes obtained from rats 18 hr after a single dose of halothane; *lane 3*, same as *lane 2*, but 1 mM *N*-ε-TFA-L-lysine was included in the antibody-exchange step.

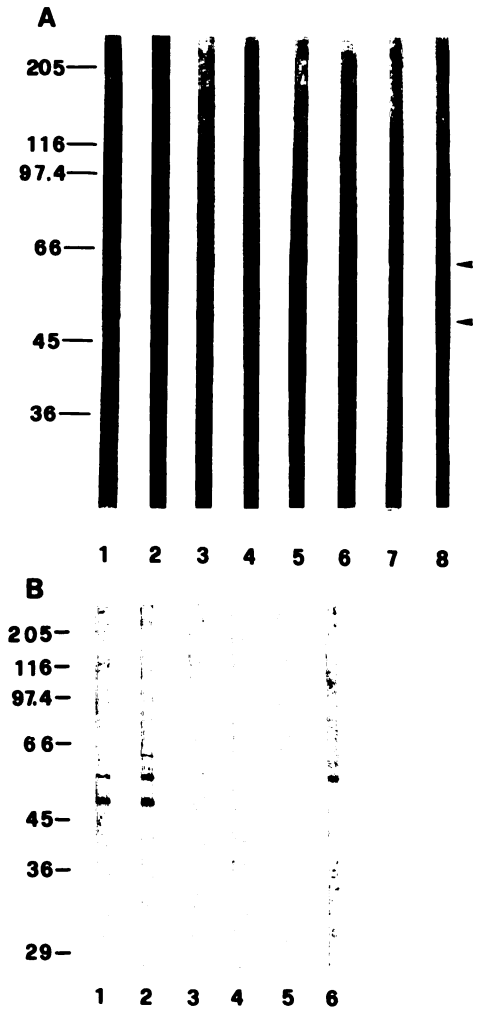

Fig. 7. Tissue-specific expression (A) and subcellular localization (B) of the 52-kDa and the 64-kDa cross-reactive polypeptides. A, Tissues were obtained from rats 6 hr after exposure to a single dose of halothane. Tissue homogenates were subjected to SDS-PAGE (250 μg of protein/cm slot width), followed by Western blot analysis using anti-TFA antibody, as described in Experimental Procedures. Visualization of the cross-reactive polypeptides was by peroxidase-dependent enhanced chemiluminescence detection. *Lane 1*, hepatocytes from untreated rats; *lane 2*, hepatocytes obtained from rats 6 hr after exposure to halothane; *lane 3*, heart; *lane 4*, spleen; *lane 5*, skeletal muscle; *lane 6*, thymus; *lane 7*, lung; *lane 8*, kidney. B, Subcellular fractions of livers from untreated rats were obtained through differential centrifugation, as described in Experimental Procedures. Visualization of the cross-reactive polypeptides was by peroxidase-dependent enhanced chemiluminescence detection. *Lane 1*, total liver homogenate; *lane 2*, 3,000 × *g* pellet; *lane 3*, 20,000 × *g* pellet; *lane 4*, 105,000 × *g* pellet; *lane 5*, cytosolic fraction; *lane 6*, 3,000 × *g* pellet with 1 mM *N*-ε-TFA-L-lysine.

muscle (Fig. 7A, *lane 5*). The relative ratio of expression of these two polypeptides did vary considerably among the different tissues. Recognition of both the 52-kDa and the 64-kDa cross-reactive polypeptides was sensitive to *N*-ε-TFA-L-lysine (i.e., 50 mM hapten-derivative abolished recognition) (data not shown). Note here that, except for their being recognized by anti-TFA antibody and their apparently identical electrophoretic mobility on SDS-PAGE, it is not known whether the 52-kDa and the 64-kDa cross-reactive polypeptides of the distinct tissues are proteins identical to those found in hepatocytes of untreated rats (Fig. 7A, *lane 1*). For the sake of this report, they have been considered as identical. Finally, in addition to the 52-kDa and the 64-kDa cross-reactive polypeptides, the kidney, but none of the other tissues examined, exhibited extensive reactivity of anti-TFA antibody towards TFA-adducts of distinct molecular mass. A comparison, based on apparent molecular mass, of the pattern of TFA-adducts recognized in hepatocytes obtained from halothane-treated rats (Fig. 7A, *lane 2*) with that of TFA-adducts found in kidney (Fig. 7A, *lane 8*) suggested that proteins specific for the respective tissue were modified by trifluoroacetylation. Here, one should note that, in kidney obtained from rats not treated with halothane, only the 52-kDa and the 64-kDa cross-reactive polypeptides were recognized by anti-TFA antibody (data not shown). These findings extend data of recent experiments, which showed, by immunochemical detection, that TFA-adducts do not occur only in the liver but also in testes (34). However, mechanisms leading to trifluoroacetylation of kidney proteins have not yet been elucidated; metabolism of halothane within the kidney or, alternatively, bioactivation in the kidney of reactive conjugates of halothane metabolites generated within and transported from the liver may be considered (35, 36).

The recognition of the 52-kDa and the 64-kDa cross-reactive polypeptides went unnoticed by other investigators (3, 4, 6, 7)

for probably two reasons. First, most studies were performed using anti-TFA-RSA sera without prior affinity purification on a *N*-ε-TFA-L-lysine matrix. In our hands, the specific recognition of the 52-kDa and the 64-kDa cross-reactive polypeptides by anti-TFA antibody became evident only after affinity purification of anti-TFA-RSA serum and was discernible from nonspecific recognition of cross-reactive polypeptides by anti-TFA-RSA serum on Western blots of hepatocyte homogenates from rats not treated with halothane (Fig. 2B, *lane 2*). This is in keeping with our findings in ELISA experiments (Fig. 3B), indicating that the specificity of anti-TFA antibody was decreased towards TFA-RSA as an antigen but increased towards the hapten-derivative *N*-ε-TFA-L-lysine, compared with anti-TFA-RSA serum. Second, many studies (3, 4, 6, 7, 34) on TFA-adduct formation have been carried out with microsomal fractions of the liver or of other tissues. In fact, the most extensive labeling of polypeptides with halothane-derived trifluoroacetyl groups was attributed to the microsomal compartment (6). Because, at the outset of our studies, nothing was known about the subcellular localization of putative cross-reactive polypeptides, we have examined, throughout the study presented here, Western blots of homogenates of hepatocytes or of tissues rather than subcellular fractions thereof. However, experiments involving subfractionation of total liver homogenates obtained from rats not treated with halothane (Fig. 7B, *lane 1*) revealed that both the 52-kDa and the 64-kDa cross-reactive polypeptides are exclusively found in the 3,000 × *g* pellet (Fig. 7B, *lane 2*). None of the cross-reactive polypeptides was found in the 20,000 × *g* pellet (Fig. 7B, *lane 3*), in the 105,000 × *g* pellet (Fig. 7B, *lane 4*), or the cytosolic fraction (Fig. 7B, *lane 5*). Note here that a third cross-reactive polypeptide, of ~58 kDa, was found in homogenates (Fig. 7B, *lane 1*) and the 3,000 × *g* pellet (Fig. 7B, *lane 2*) of total liver but not in homogenates of hepatocytes (Fig. 8). The recognition of this polypeptide is not related to immunochemical mimicry, in that its recognition by anti-TFA antibody is not sensitive (Fig. 7B, *lane 6*) to competition with *N*-ε-TFA-L-lysine (1 mM) but is solely due to cross-reactivity of the HRP-goat anti-rabbit second antibody with a liver homogenate antigenic determinant (data not shown).

Persistence of the 52-kDa and the 64-kDa cross-reactive polypeptides. Experiments in other laboratories have indicated that TFA-adducts are not persistent over time (9, 34). We wondered whether the cross-reactive polypeptides of 52 and 64 kDa are affected, with respect to persistence, after halothane metabolism. Examination of Western blots of rat hepatocyte homogenates obtained 18 hr, 42 hr, 90 hr, and 10 days after a single dose of halothane revealed the most intense staining of TFA-adducts after 18 hr. The intensity of recognition of TFA-adducts decreased over time periods of 42 and 90 hr; on Western blots of hepatocytes obtained 10 days after exposure of rats to a single dose of halothane, only the 52-kDa and the 64-kDa cross-reactive polypeptides were still recognized by anti-TFA antibody (Fig. 8). Slight variations were observed in the time after which TFA-adducts of distinct molecular mass were still recognized by the anti-TFA antibody. Most prominently, the recognition of a TFA-adduct with an apparent molecular mass of ~48 kDa, which was expressed in considerable amounts only in heptocytes obtained from phenobarbital-pretreated rats, was already abolished after 42 hr, whereas the other TFA-adducts were persistent for at least 90 hr but no longer than 10 days. These data are consistent with at least

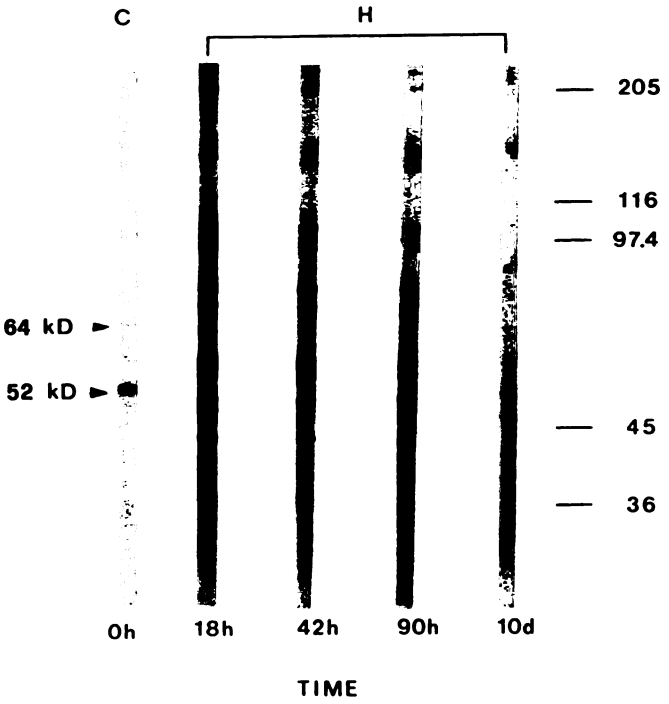

Fig. 8. Persistence of TFA-adducts in rat hepatocyte homogenates. Before administration of a single dose of halothane (10 mmol/kg of body weight), rats were pretreated with three daily injections (intraperitoneally) of 80 mg/kg of body weight sodium phenobarbital in PBS. Hepatocytes were obtained 18 hr, 42 hr, 90 hr, and 10 days later through *in situ* collagenase/dispase perfusion of the liver. SDS-PAGE (250 μg/cm slot width) was followed by visualization of TFA-adducts on Western blots using, sequentially, anti-TFA antibody, HRP-goat anti-rabbit second antibody, and development of peroxidase activity with 4-chloro-1-naphthol as a substrate. *C*, Hepatocytes prepared from a control rat not exposed to halothane; *H*, hepatocytes prepared from rats treated with halothane. *Arrowheads*, positions of the 52-kDa and the 64-kDa cross-reactive polypeptides.

three feasible mechanisms for the decline in TFA-adducts over time; (i) endogenous deacetylase activity might remove the trifluoroacetyl groups from labeled polypeptides, (ii) TFA-adducts might be removed due to normal protein turnover with $t_{1/2}$ of >90 hr but <10 days, except for the aforementioned 48-kDa TFA-adduct, which seemed to have a shorter half-life (<48 hr), or (iii) proteases specific for chemically modified proteins may act on TFA-adducts in a manner similar to that described for macroxyproteinase, a 670-kDa proteinase complex acting on oxidatively altered proteins in red blood cells (37). Among distinct types of liver cells, Kupffer cells also have recently been shown to process TFA-adducts; the half-life of some TFA-adducts was <24 hr when cells were kept in primary culture (38). Regardless, the persistent 52-kDa and 64-kDa cross-reactive polypeptides seem not to be subjected to any of these mechanisms (Fig. 8H, 10 days). As exemplified semiquantitatively by the staining intensity of the 52-kDa cross-reactive polypeptide (Fig. 8), treatment of rats with halothane had no effects on the transient levels of expression of these constitutive polypeptides.

Expression of the 52-kDa and the 64-kDa cross-reactive polypeptides in humans. The expression of the 52-kDa and the 64-kDa cross-reactive polypeptides was not species specific. Both cross-reactive polypeptides were identified in livers of rabbits and guinea pigs (data not shown). Most interestingly, however, both the 52-kDa and the 64-kDa cross-

reactive polypeptides were present on Western blots of liver tissue from all six human individuals (i.e., kidney donors) tested so far (Fig. 9). The level of expression of each polypeptide in different individuals, as well as the relative ratio of expression within a single individual, varied considerably. This finding is in keeping with the notion that, among human individuals, extensive variation in the level of expression of a given polypeptide occurs; this is extensively documented for polypeptides associated with polymorphic drug oxidation (39). Of course, many more individuals will have to be examined in order to obtain a reliable measure of the true degree of variation of expression of these two polypeptides. Whether the absence in the liver of constitutive polypeptides, immunochemically mimicking at the molecular level the structure of epitopes on TFA-adducts, renders individuals more susceptible to halothane-induced immune-mediated hepatotoxicity remains unknown. Nevertheless, if such tissue becomes available to us, it would be most interesting to learn whether the 52-kDa and 64-kDa cross-reactive polypeptides are expressed in the liver tissue of halothane hepatitis patients.

In summary, the data presented here strongly support the novel concept that in a number of tissues there exists a repertoire of constitutive polypeptides comprising epitopes that confer molecular mimicry of epitopes on TFA-adducts that are elicited on target polypeptides upon metabolism of halothane. The nature and the true function of the 52-kDa and the 64-kDa cross-reactive polypeptides remain elusive. Only their isolation, molecular cloning, and functional expression might reveal whether their intrinsic function is different from their presumed role as immunochemical mimics of trifluoroacetyl-related protein modifications. It might be interesting to note that, in the future, organisms may increasingly encounter and have to cope with such trifluoro-related modifications of macromolecules, due to occupational or environmental exposure to hydrochlorofluorocarbons, which will replace ozone-depleting chlorofluorocarbons on a large scale (40) and which can be biotransformed by cytochrome P450-dependent reactions (41) to presumably reactive intermediates. In this regard, halothane may be considered a prototypical substrate for hydrochlorofluorocarbons. Regardless, the current findings will provide the basis for additional experiments proving or disproving a possible general role for such self-peptides in conferring immunotolerance towards polypeptides chemically modified upon metabolism of xenobiotics.

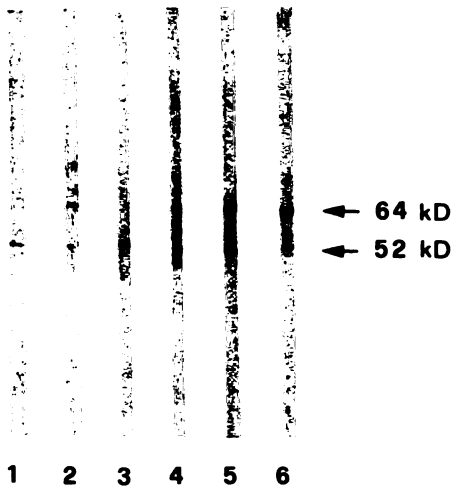

Fig. 9. Expression in human liver tissue of the 52-kDa and 64-kDa cross-reactive polypeptides. Homogenates of human liver, obtained from kidney donor individuals, were examined on Western blots for expression of the 52-kDa and 64-kDa cross-reactive polypeptides. Visualization of the cross-reactive polypeptides was by peroxidase-dependent enhanced chemiluminescence detection. *Lane 1*, patient KDL 25; *lane 2*, patient KDL 27; *lane 3*, patient KDL 28; *lane 4*, patient KDL 31; *lane 5*, patient KDL 36; *lane 6*, patient KDL 34. The coding of patients is as it appears in the human liver bank (21).

Acknowledgments

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