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Human Microsomal Epoxide Hydrolase Is the Target of Germander-Induced Autoantibodies on the Surface of Human Hepatocytes

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ABSTRACT

Germander, a plant used in folk medicine, caused an epidemic of cytolytic hepatitis in France. In about half of these patients, a rechallenge caused early recurrence, suggesting an immunoallergic type of hepatitis. Teucrin A (TA) was found responsible for the hepatotoxicity via metabolic activation by CYP3A. In this study, we describe the presence of anti-microsomal epoxide hydrolase (EH) autoantibodies in the sera of patients who drank germander teas for a long period of time. By Western blotting and immunocytochemistry, human microsomal EH was shown to be present in purified plasma membranes of both human hepatocytes and transformed spheroplasts and to be exposed on the cell surface where affinity-purified germander autoantibodies recognized it as their autoantigen. Immunoprecipitation of EH activity by germander-induced autoantibodies confirmed this finding. These autoantibodies were not immunoinhibitory. The plasma membrane-located EH was catalytically competent and may act as target for reactive metabolites from TA. To test this hypothesis CYP3A4 and EH were expressed with human cytochrome P450 reductase and cytochrome b_5 in a "humanized" yeast strain. In the absence of EH only one metabolite was formed. In the presence of EH, two additional metabolites were formed, and a time-dependent inactivation of EH was detected, suggesting that a reactive oxide derived from TA could alkylate the enzyme and trigger an immune response. Antibodies were found to recognize TA-alkykated EH. Recognition of EH present at the surface of human hepatocytes could suggest an (auto)antibody participation in an immune cell destruction.

A recent trend in western countries was the increasing use of herbal medicines because of their supposed safety in contrast to chemical drugs. However, a broad variety of herbal remedies was shown to induce liver disease (Larrey, 1997). Wild germander (*Teuchrium chamaedrys* L.) was traditionally used as a folk medicine for its choleretic and antiseptic properties. In 1991, an advertising campaign promoted the use of germander teas or germander powder-containing capsules to treat obesity. Ingestion of tea in great quantity or capsules caused an epidemic of cytolytic hepatitis. Thirty cases of hepatotoxicity were first reported, including cases with positive rechallenge (Castot and Larrey, 1992; Larrey et al., 1992; Ben Yahia et al., 1993; Dao et al., 1993) and a fatality due to fulminant hepatic necrosis (Mostefa-Kara et al., 1992). Recently, two other cases, with one positive rechallenge were reported in Canada (Laliberte and Villeneuve, 1996). For patients with a rechallenge, an early recurrence was observed despite lack of other features of hypersensitivity (Castot and Larrey, 1992).

Investigations into the mechanism of germander hepatotoxicity began with the demonstration that a germander tea lyophilizate and the fraction containing furano neoclerodane diterpenoids were responsible for the in vivo toxicity of germander in mice. This toxicity required CYP3A-dependent metabolism (Loeper et al., 1994). These results were corroborated by Kouzi et al. (1994) who extended them to show that the metabolic activation of the furan ring of teucrin A (TA), the major germander neoclerodane diterpenoid, caused hepatotoxicity in mice. TA and teuchamaedrin A were shown to be toxic via CYP3A-generated electrophilic metabolites that were detoxified by glutathione conjugation, depleted cellular thiols, and caused apoptosis in isolated rat hepatocytes (Lekehal et al., 1996; Fau et al., 1997). However, if a toxic metabolite was able to induce hepatocyte death by necrosis or

ABBREVIATIONS: CYP, cytochrome P450; TA, teucrin A; EH, epoxide hydrolase; hmEH, human microsomal epoxide hydrolase; PM, plasma membrane; GIAA, germander-induced autoantibodies; CPR, NADPH-cytochrome P450-reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; bp, base pair; PAGE, polyacrylamide gel electrophoresis; B(*a*)P, Benzo(*a*)pyrene; MHC, major histocompatibility complex.

apoptosis, these processes did not explain the immunological type of hepatitis observed in several cases of germander-induced liver injury.

To further understand this immunological process, we analyzed the sera of patients who ingested germander tea in great quantity. By Western blot analysis, these sera were shown to contain autoantibodies directed only against human microsomal epoxide hydrolase (hmEH) (EC 3.3.2.3). In both human hepatocyte or hmEH-expressing yeast, hmEH was present in a dual location, in the endoplasmic reticulum and the plasma membrane (PM). Immunopurified germander-induced autoantibodies (GIAA) were shown to recognize hmEH on the cell surface. To establish that hmEH was implicated in TA metabolism we used a "humanized" yeast strain that coexpressed human cytochrome P450-reductase (CPR), human cytochrome b_5, CYP3A4, and hmEH. TA was oxidized by CYP3A4 into a metabolite that inactivated hmEH in microsomes and PM. This metabolite could modify the structure of the enzyme and lead to an autoantibody formation.

Materials and Methods

Human Liver Specimens. Human liver specimens were obtained by surgery under a protocol approved by the Comité Consultatif d'Ethique Médicale du Centre Hospitalier et Universitaire Bichat-Beaujon. These liver specimens were obtained from right or left hepatectomies performed in patients with metastatic colon carcinoma or benign liver tumors. Pieces of normal liver were cut at distance from the hepatic tumor. A pathological examination indicated normal liver histology in all pieces.

Patient Sera. Human sera were obtained from four patients seen at the Liver Unit of Hospital Beaujon (Clichy, France). These four patients (designated EMI, MAR, MOT, and NOU) exhibited germander-induced hepatitis after drinking large quantities of germander tea (at least 2 l/day) for several months (4–6 months). These patients had no other cause of hepatitis. Autoimmune markers as anti-mitochondrial, anti-actin, anti-nuclear, anti-soluble liver antigen autoantibodies and anti-microsomal autoantibodies were not detected in the sera (titer 1:40) by indirect immunofluorescence on rat liver and kidney sections. Serologic tests for A, B, and C viruses were negative, and these patients were not taking drugs except germander tea. The serum level of IgG was slightly over normal 16 ± 2 g/l (n = 7–14 g/l), whereas IgM and IgA were normal. One patient (NOU) was rechallenged and hepatitis occurred more rapidly than on the first onset of hepatitis. Clinical information concerning these patients has been published elsewhere (Castot and Larrey, 1992). Control sera were from healthy staff members of the Liver Unit who never took germander tea or capsules.

GIAA were affinity-purified by acidic elution of these autoantibodies bound to the microsomal protein band localized (on nitrocellulose sheets) at the level of purified human liver mEH as described previously (Loeper et al., 1993).

Purified hmEH, Anti-EH Antibodies, and TA and Preparation of Isolated Hepatocytes, PM, and Microsomal Fractions from Human Liver. Purification, characterization of human liver mEH, and corresponding polyclonal antibodies raised in rabbits were described in Beaune et al. (1988). Isolation, purification and characterization of TA were as described in Lekehal et al. (1996).

Isolated hepatocytes, PM, and microsomal fractions were prepared as previously described (Loeper et al., 1993). Microsomal contamination of this PM fraction is less than 1%, as judged from the specific activities of marker enzymes (5′ nucleotidase, sodium/potassium ATPase, and glucose 6-phosphatase) in the PM and microsomal fractions, respectively (Loeper et al., 1993).

Yeast Strains and Plasmids. pYeDP60 (V60) and pYeDP80 (V80) were previously described (Pompon et al., 1996). V80 was derived from pYeDP60 by substitution of the *ADE2* gene by *TRP1* gene, two selection markers. pCYP1A1/V60 (pCYP1A1), pCYP3A4/V60 (pCYP3A4), and phmEH/V60 (phmEH) were constructed as previously described (Gautier et al., 1993) and express human CYP1A1, CYP3A4, and hmEH, respectively. When CYP3A4 was coexpressed with hmEH, pCYP3A4/V80 (p3A4) expressing human CYP3A4 was used instead. Each plasmid (V60 and V80) possesses a different selection marker, enabling their coexpression in the same cell.

W303-1B (MATa, *leu*2-3,112, *his*3-11,15, *ade*2-1, *trp*1-1, *ura*3-1) was used. W(R) was engineered from W303-1B to express the open reading frame of yeast CPR gene under a galactose-inducible promoter (Pompon et al., 1996). W(R) was transformed by pCYP1A1 (see above).

W(GhR, Yhb5) was engineered from W303-1B to express the open reading frame of the human CPR gene (instead of the yeast gene) under the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter. The open reading frame of the human cytochrome b_5 was substituted for the open reading frame of the yeast cytochrome b_5, in the natural locus. For this purpose, the homozygous diploid strains W(hR) (*leu*2-3,112, *his*3-11,15, *ade*2-1, *trp*1-1) (Pompon et al., 1996) and W(DB) (MATa and α, *leu*2-3, *ade*2-1, *trp*1-1, *ura*3-1) (Truan et al., 1994) were used.

Constructions of the integration vector were as follows: GADPH promoter (P_{GAPDH}) (Mutsi et al., 1983) was cloned from yeast genomic DNA with polymerase chain reaction (PCR) amplification with (5′-CCAAGCTTGAGTTTATCATTATCAATACTCG-3′) and (5′-CGGATCCTATTTATGTGTGTTTATTCGAAACTAAGTTCTTGG-3′) primers deduced from the published sequence, which included *Hin*dIII and *Bam*HI restriction sites (underlined), respectively. The amplified fragment of 691 base pairs was cloned into the *Srf*I site of pBlue-Script (Stratagene, La Jolla, CA) giving pAB1. The 3652-base pair (bp) linear fragment of *Hin*dIII digested pAB1 was end-filled, digested with *Bam*HI, and cloned between the *Eco*RV and *Bam*HI sites of pUP81 vector (Pompon et al., 1996) to give pAB2. In this vector the *URA3* gene initially present in pUP81 was disrupted by the *Eco*RV-*Bam*HI digestion and the cloning of P_{GAPDH} upstream of the human CPR open reading frame and phosphoglycerate kinase transcription terminator (T_{PGK}).

pDARG containing the PYb5:Yb5:TYb5 cassette (Truan et al., 1994) was cleaved at its unique *Cla*I site (within the yeast cytochrome b_5 open reading frame). This linear fragment and the 917-bp *Bam*HI-*Bgl*II fragment of pLIP1 containing the human cytochrome b_5 open reading frame flanked by parts of yeast cytochrome b_5 promoter (P_{Yb5}) and terminator (T_{Yb5}), were cotransformed in W303-1B for homologous recombination. This plasmid contained an extended PYb5:hb5:TYb5 cassette suitable for genomic integration.

Strains W(ΔB) MATa and W(hR) MATα were mated and diploids were selected on uracil- and histidine-free medium. After sporulation and microdissection of spores, haploid strains prototroph for uracil and histidine were selected. This selected strain, W(hR, Δb5), was disrupted for the endogenous cytochrome b_5 and CPR genes but contained a galactose-inducible human CPR gene. Because the simultaneous disruption of yeast microsomal cytochrome b_5 and CPR genes is lethal (Truan et al., 1994), W(hR, Δb5) is not viable on glucose-containing medium due to the absence of hCPR expression. W(hR, Δb5) was transformed by the linear integrative pAB2 cleaved by *Not*I. pASZ11 containing the *ADE2* gene (Stotz and Linder, 1990) was used as cotransformation marker. P_{GAPDH} genomic integration upstream of the human CPR open reading frame occurred by homologous recombination on one side at the level of the *URA3* marker flanking the GAL10-CYC1 promoter ($P_{GAL10\text{-}CYC1}$) in W(hR, Δb5), on the other side at the level of human CPR open reading frame. This led to excision of P_{GAL10} and ability to grow on glucose containing medium due to constitutive expression of the human CPR after promoter exchange.

W(GhR, Δb5) was transformed by the 2022-bp fragment from *Pvu*II-cleaved pAB3. In this way human cytochrome b_5 was integrated at the original *Yb5* genomic locus (previously disrupted). This strain, called W(GhR, Yhb5), constitutively expresses human CPR and human cytochrome b_5 but not their yeast counterparts.

W(GhR, Yhb5) was transformed either by phmEH alone or pCYP3A4 alone or simultaneously by the two plasmids. Transformed strains were selected for adenine and tryptophan prototrophy after transformation by pYeDP60- and/or pYeDP80-derived plasmids (Fig. 1). Transformants were grown in SLI or YPE synthetic medium (Pompon et al., 1996).

Purification of Yeast Microsomal and PM Fractions. Yeast cells, grown in SLI synthetic medium (to a cell density of A600 1.5), were transformed with phmEH and used for PM purification. After the electrostatic attachment of spheroplasts on cationic silica microbeads (Schmidt et al., 1983), PM fraction was prepared as previously described (Loeper et al., 1998a,b). The microsomal fraction was prepared from cells grown in complete medium YPL containing galactose as described in Pompon et al. (1996).

Immunoblotting. PM and microsomal proteins from human hepatocytes or transformed yeast were subjected to SDS-polyacrylamide (9%) gel electrophoresis (PAGE), transferred onto nitrocellulose sheets, and incubated for 2 h with either GIAA (diluted 1:100), control sera (diluted 1:100), or rabbit anti-hmEH antibodies (diluted 1:800). Binding of the antibodies was detected with peroxidase-conjugated anti-human IgG or anti-rabbit IgG, diluted 1:2500. Immunoblots were developed as previously described (Loeper et al., 1990, 1993). Blots of human hepatocyte PM proteins also were incubated with affinity-immunopurified anti-hmEH GIAA. Immunoblots of yeast fractions were incubated with rabbit anti-mEH, GIAA, or secondary antibodies absorbed overnight at 4°C against homogenate of nontransformed yeasts (without plasmid).

Immunofluorescence and Electron Microscopy. To reveal the presence of epitopes expressed on the cell surface, unpermeabilized, fixed normal hepatocytes or fixed yeast spheroplasts expressing hmEH were exposed to the various antibodies. Normal human hepatocytes and transformed spheroplasts were fixed with 4% paraformaldehyde and incubated with affinity-immunopurified anti-hmEH GIAA used as previously described (Loeper et al., 1993). Before incubation with the spheroplasts, anti-hmEH antibodies, rabbit preimmune sera, normal human sera, and fluorescein isothiocyanate- or peroxidase-conjugated secondary antibodies (Pasteur Production, Marnes-la-Coquette, France) were diluted 1:200 and absorbed overnight at 4°C against control yeasts (transformed with the vector plasmid) and paraformaldehyde-fixed control spheroplasts (Loeper et al., 1998a,b). Labeled cells were analyzed for immunofluorescence. After peroxidase labeling, aliquots of human hepatocytes were postembedded, cut, and examined by electron microscopy, as previously described (Loeper et al., 1993).

Immunoprecipitation and Immunoinhibition of hmEH by GIAA and Rabbit Anti-hmEH Antibodies. Yeast microsomes expressing hmEH were solubilized in 1% sodium cholate for 1 h at 0°C and centrifuged at 100,000*g* for 1 h. Aliquots of the supernatant corresponding to a final concentration of 125 nM hmEH were incubated with equal volumes of rabbit anti-hmEH antibodies, GIAA, or control sera complemented in 100 mM phosphate buffer, pH 8, overnight at 4°C. Protein A-agarose beads (Sigma, St. Louis, MO) suspended in the same buffer (50% v/v) were added and incubated 2 h at 4°C. After centrifugation, benzo(*a*)pyrene-4,5 oxide [B(*a*)P-4,5 oxide] (10 mM at final concentration) was added to each supernatant and incubated at 37°C for 15 min (Dansette and Jerina, 1974). Each supernatant was analyzed for hmEH residual activity based on the conversion of B(*a*)P-4,5 oxide into B(*a*)P-4,5 dihydrodiol with an optimized HPLC fluorescence procedure (Gautier et al., 1996). To determine whether the autoantibodies cause immunoinhibition of hmEH, solubilized microsomes (containing 125 nM hmEH, final concentration) were incubated with different volumes of autoantibodies (10, 30, 50 μl), or the same volumes of control human sera, for 2 h at 4°C. Then B(*a*)P-4,5 oxide (10 μM at final concentration) was added and incubated for 15 min at 37°C. The reaction products were analyzed with the HPLC fluorescence procedure as described below.

Enzymatic Assays. Total CYP content was calculated from the reduced carbon monoxide difference spectra (Omura and Sato, 1964). Protein concentration was determined with the Pierce bicinchoninic acid procedure, according to the manufacturer's instructions.

To study TA metabolite formation, yeast microsomes expressing in the same membranes, CYP3A4 (0.5 μM P450), human cytochrome b_5 (0.5 μM) with or without coexpressed hmEH (1.25 μM), were incubated for 1 h at 37°C with 100 μM TA and an NADPH-regenerating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, 3 U/ml glucose-

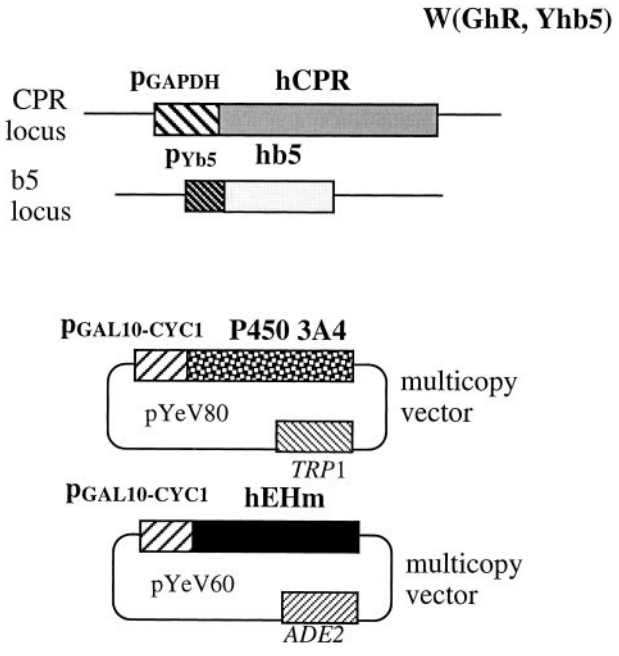

Fig. 1. Engineered yeast strain W(GhR, Yhb5) and plasmids used for single transformation or cotransformation. In this strain, the yeast CPR open reading frame is substituted by its human counterpart (hCPR) under the control of the constitutive GAPDH promotor. Yeast cytochrome b_5 open reading frame is substituted by its human counterpart (hb5) under the control of the endogenous yeast promotor. CYP3A4 and hmEH expression cassettes are under the control of the inducible promotor pGAL10-CYC1 in two plasmids containing, respectively, *TRP1* or *ADE2* as selection markers.

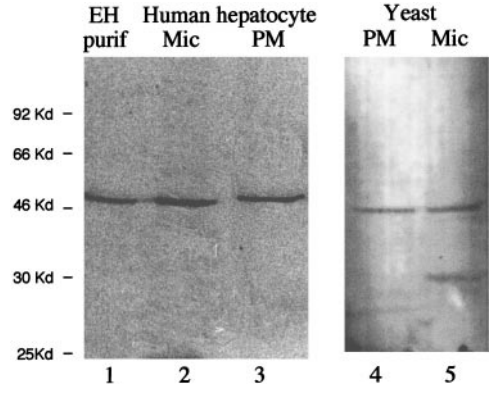

Fig. 2. SDS-PAGE immunoblots of microsomal and PM fractions from human liver and hmEH-expressing yeast. Polyacrylamide gels (9%) were loaded with 1 pmol of purified hmEH (lane 1), 30 μg of human liver microsomal proteins (lane 2), 60 μg of human hepatocyte PM proteins (lane 3), 25 μg of yeast PM proteins (lane 4), and 10 μg of yeast microsomal proteins (lane 5). After nitrocellulose transfer, blots were incubated with rabbit anti-hmEH antibodies diluted 1:800.

6-phosphate dehydrogenase) in 100 mM phosphate buffer, pH 8. The reaction was stopped in ice. Extraction was performed with an equal volume of dichloromethane. The organic extracts were collected, air dried, and products were analyzed by HPLC. Then 20-μl aliquots were injected onto a 10-mm octyldecylsilyl column. Elution was performed at a flow rate of 1 ml/min, with a linear gradient of 0 to 40% (by volume) acetonitrile in water (containing 0.02% trifluoroacetic acid) for 12 min, followed by a 1-min gradient to pure acetonitrile. TA and the TA metabolites I, II, and III were detected at 215 nm with retention times of 13, 12.5, 9.5, and 8.2 min, respectively. TA metabolites were quantified by measuring peak areas on chromatograms. Microsomal and PM EH activities were determined with styrene oxide as substrate; products were separated by HPLC as previously described (Gautier et al., 1993).

The TA-mediated inactivation hmEH activity toward B(a)P metabolites was performed in two steps as follows: samples containing 0.5 μM CYP3A4, 0.5 μM human cytochrome b_5, and 250 nM microsome-bound hmEH or silica bead-bound 100 nM PM hmEH were incubated at 37°C for 0, 15, 30, 45, and 60 min with or without an NADPH-regenerating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, 3 U/ml glucose-6-phosphate dehydrogenase). Experiments were performed either with or without 100 μM TA in 100 mM phosphate buffer, pH 8. In the latter case, 100 μM TA was added at the end of the incubation. To test the residual hmEH activity, each sample was diluted 1:100 in the same buffer containing a final concentration of 0.16 μM CYP1A1 (yeast microsomes), 15 μM B(a)P, and 0.15 mM NADPH and incubated for 10 min at 37°C. The reaction was stopped on ice and products were extracted and analyzed by HPLC as described (Gautier et al., 1996). Supernatants of PM-silica beads also were tested for hmEH residual activity as described above.

Recognition of TA-alkylated hmEH by patient serum:incubations were performed as described above to study TA metabolite formation. Experiments were performed with or without TA and with or without an NADPH-regenerating system. Aliquots (15 μl from 200 μl) were analyzed by SDS-PAGE and immunoblotting with patient's sera (MOT or NOU) diluted 1:250 incubated overnight at room temperature. Binding of the antibodies was detected with peroxidase-conjugated anti-human IgG, diluted 1:10000. Immunoblot detection was performed by enhanced chemiluminescence. The primary and secondary antibodies were absorbed overnight at 4°C against microsomes of control yeasts (with a void plasmid) before use.

Results

Presence of hmEH in PM of Human Hepatocyte and Humanized Yeast. Yeast expressing various human proteins represents a useful tool to determine the targets of human autoantibodies. Furthermore, the expression of human CYP2D6 in yeast was shown to reproduce the dual

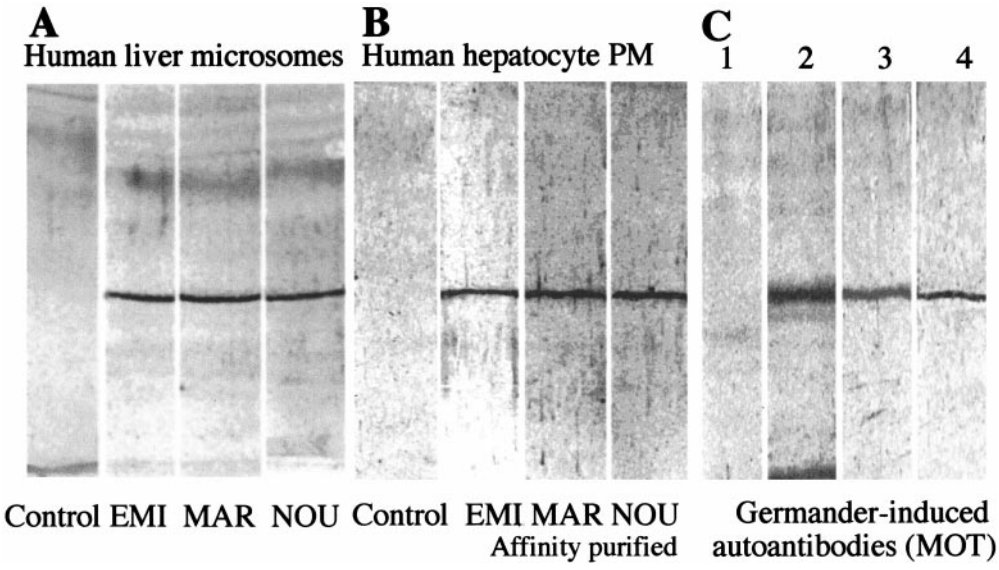

Fig. 3. Western blot analysis of hmEH recognition by GIAA. Polyacrylamide gels (9%) were loaded with 50 μg of human liver microsomal proteins (A); 90 μg of human hepatocyte PM proteins (B); 40 μg of control (lane 1) or transformed (lane 2) yeast microsomal proteins, 3 pmol of purified hmEH (lane 3), and 80 μg of transformed yeast PM proteins (lane 4) (C). Immunoblots were incubated either with whole anti-germander sera (EMI, MAR, and NOU) diluted 1:100 (A and C), with affinity-purified autoantibodies from these sera prepared as described under *Materials and Methods* (B) or with adsorbed germander serum (MOT) against homogenate of nontransformed yeasts (C).

TABLE 1

TA-mediated inactivation of hmEH activity towards B(a)P metabolites in yeast microsomal and PM fractions

A first incubation was performed for different times with 500 nM microsomal CYP3A4, 500 nM human cytochrome b5 and 250 nM microsomal hmEH or 100 nM PM hmEH on the silica beads, with or without an NADPH-regenerating system and with or without 100 μM TA in 100 mM phosphate buffer, pH 8. Then, for a second incubation of 10 min, each sample was diluted 1:100 in the same buffer containing 160 nM yeast microsomal CYP1A1, 15 μM B(a)P, and 0.15 mM NADPH. For samples without TA during the first incubation, 1 μM TA was added in the second incubation. The reaction products were analyzed by HPLC as described under *Materials and Methods*. Hydrolysis of B(a)P oxides formed in situ by CYP1A1 were quantified and reported to a calibration curve of hmEH activity. All experimental results were replicated twice in three separate experiments.

	Residual Functional hmEH (%)[a]				
Time (min)	0	15	30	45	60
Microsomes					
With TA	95 ± 8	56 ± 6	48 ± 7	40 ± 7	37 ± 7
Without TA	100 ± 5	95 ± 7	95 ± 5	95 ± 5	95 ± 5
NADPH-free	95 ± 5	95 ± 5	94 ± 5	94 ± 6	94 ± 5
PM					
With TA	90 ± 8	65 ± 6	50 ± 7	36 ± 6	34 ± 5
Without TA	95 ± 6	95 ± 5	95 ± 3	95 ± 4	95 ± 4
NADPH-free	100 ± 3	95 ± 3	95 ± 5	95 ± 5	95 ± 5

[a] 100% corresponds to 2.5 nM microsomal hmEH or 1 nM PM hmEH.

location (endoplasmic reticulum and PM) of this CYP observed in human hepatocytes (Loeper et al., 1993, 1998a,b). In the present study, the *Saccharomyces cerevisiae* strain W(GhR, Yhb5) was transformed with a galactose-inducible form of hmEH (phmEH/V60) and used to assess both the reactivity of GIAA toward hmEH and the localization of this enzyme on the PM.

The two methods, used to prepare human hepatocytes or yeast PM, were based on the electrostatic attachment of the external face of the cell PM to polyethyleneimmine-polyacrylamide beads or to cationic silica microbeads, respectively, before cell lysis. These procedures allowed PM preparations that were practically devoid of microsomal contamination (Loeper et al., 1993, 1998a). Western blot experiments performed with rabbit anti-hmEH antibodies revealed that hmEH was present not only in human liver microsomes but also in hepatocyte PM (Fig. 2, lanes 2 and 3) and in both yeast microsomal and PM fractions (Fig. 2, lanes 4 and 5). Thus, in both human hepatocytes and transformed yeast, hmEH was present in both microsomes and PM.

hmEH Is the Specific Target of GIAA. Sera from patients with germander-induced hepatitis (EMI, MAR, MOT, and NOU) were screened for the presence of anti-microsomal autoantibodies by Western blotting against human liver microsomes. The sera recognized a single band in human liver microsomes where no protein was recognized by human control sera (Fig. 3A). The molecular mass of the recognized protein was in the 50-kDa range expected for xenobiotic-metabolizing enzymes, such as CYPs or hmEH. GIAA only recognized chromatographically purified hmEH (Fig. 3C, lane 3) but none of the tested CYPs (1A2, 2C, 2D6, 2E1, and 3A4) (data not shown).

To certify their specificity, affinity-purified GIAA were used to test hepatocyte PM fractions. The autoantibodies purified from all sera recognized only one protein, hmEH, in human hepatocyte PM proteins tested by Western blot analysis, whereas human control sera recognized no protein (Fig. 3B). This recognition was confirmed with yeast microsomal and PM fractions. All patient sera behaved similarly with a positive signal toward expressed hmEH (Fig. 3C, lanes 2 and 4). No signal was obtained with control yeast microsomes (Fig. 3C, lane 1).

Immunoprecipitation and Immunoinhibition of hmEH by GIAA. To further confirm this recognition, we tested the ability of GIAA to immunoprecipitate hmEH expressed in yeast. Solubilized microsomes were incubated with different concentrations of GIAA. The immune complexes were precipitated by adding protein A-agarose beads that bind the Fc fragment of the autoantibodies, whereas the uncombined antigen (hmEH) remained in solution. Each supernatant was tested for hmEH residual activity based on conversion of B(a)P-4,5 oxide to B(a)P-4,5 dihydrodiol. Positive and negative controls were performed under the same conditions with rabbit anti-mEH antibodies or control sera (preimmune serum or normal human serum), respectively. Immunoprecipitation of hmEH activity was observed after incubation of hmEH with GIAA (MOT) (Fig. 4). hmEH activity was immunoprecipitated to the same order of magnitude by the three other sera (EMI, MAR, and NOU) (data not shown). Half-inhibition of hmEH activity was obtained with 10 μl of GIAA, whereas 1 μl of rabbit anti-hmEH antibodies sufficed to cause the same effect (Fig. 4). This lower immunoprecipitation efficiency of patient sera reflected their lower affinity, as also shown in Western blot analysis (Figs. 2 and 3). Usually, rabbit anti-mEH antibodies, in contrast to anti-CYP antibodies, were not immunoinhibitory. Similarly, GIAA did not inhibit hmEH. This was shown by measuring hmEH activities on the protein A-agarose bead-immune complexes (Fig. 4) and by the absence of direct inhibition when solubilized microsomes were incubated with different volumes of serum (data not shown). Thus, GIAA immunoprecipitate hmEH but do not inhibit it. It can be postulated that these autoantibodies are not directed against the catalytic site of hmEH.

hmEH Is Located on the External Face of Human Hepatocyte or Transformed Yeast and Recognized by GIAA. A bright fluorescence staining on the outside of the PM of fixed unpermeabilized human hepatocytes was observed after exposure to rabbit anti-hmEH antibodies (Fig. 5A). In other experiments, to remove all staining against other PM proteins, fixed human hepatocytes were exposed to affinity-purified GIAA. Fluorescence labeling of the PM also was clearly observed with these purified autoantibodies (Fig. 5B). In contrast, no hepatocyte staining was observed with human control serum (Fig. 5C) or preimmune serum (data not shown). The same type of bright fluorescence staining on the cell surface was observed with unpermeabilized fixed spheroplasts expressing hmEH after incubation with rabbit anti-hmEH antibodies (Fig. 5D). The fluorescence pattern was weaker with affinity-purified GIAA because of the low concentrations of eluted autoantibodies (Fig. 5E). No PM fluorescence was observed when control spheroplasts (containing a void plasmid) were exposed to affinity-purified GIAA (Fig. 5F) or rabbit anti-hmEH antibodies (data not shown). These results demonstrated that hmEH was located on the outside of the PM of both human hepatocytes and transformed spheroplasts, in addition to its regular microsomal location. Moreover, GIAA recognized PM-exposed

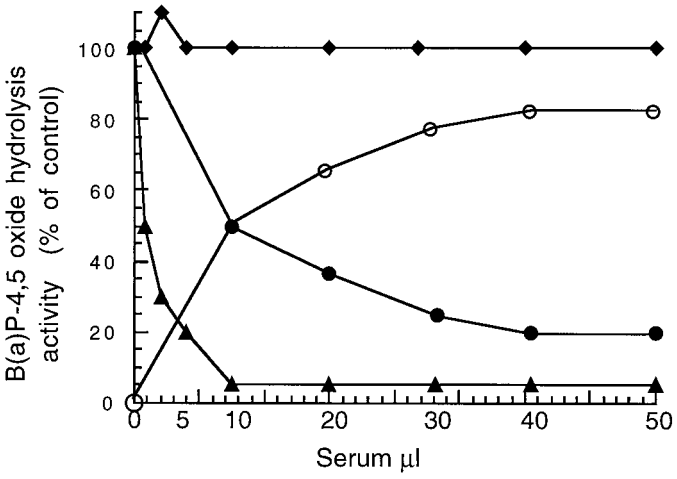

Fig. 4. Immunoprecipitation of hmEH by rabbit anti-hmEH antibodies and GIAA. Cholate-solubilized hmEH-expressing yeast microsomes were incubated with rabbit anti-hmEH antibodies (▲–▲), normal human sera (♦–♦), or GIAA (MOT) (●–●), and then with Protein A-agarose beads as described under *Materials and Methods*. Supernatants from immunoprecipitates were used to determine hmEH activity toward B(a)P-4,5 oxide as substrate. Activity (100%) refers to the control activity with control human serum. hmEH activities also were determined on the Protein A-immunoprecipitates obtained with GIAA (○–○). Each point is the mean of two incubations. The incubations were run twice with similar results each time.

hmEH, and not another PM protein, as their autoantigen at the cell surface.

Electron microscopy was used to further demonstrate that hmEH was exposed on the surface of human hepatocyte and was recognized by GIAA. Immunolabeling was performed on uncut, unpermeabilized, fixed human hepatocytes that were then postembedded and cut. When incubated with anti-hmEH antibodies, human hepatocytes exhibited a clear immunostaining only at the cell surface, along linear parts of PM and its microvilli (Fig. 6A). With affinity-purified GIAA, a similar positive peroxidase-labeling of PM was observed, albeit weaker because of the low concentrations of these eluted autoantibodies (Fig. 6B). No PM staining was detected with normal human sera (Fig. 6C) or with preimmune rabbit sera (data not shown).

Metabolic Activation of TA. Because yeast-expressed CYP3A4 was optimally functional in the presence of both human CPR and human cytochrome b_5, the "humanized"

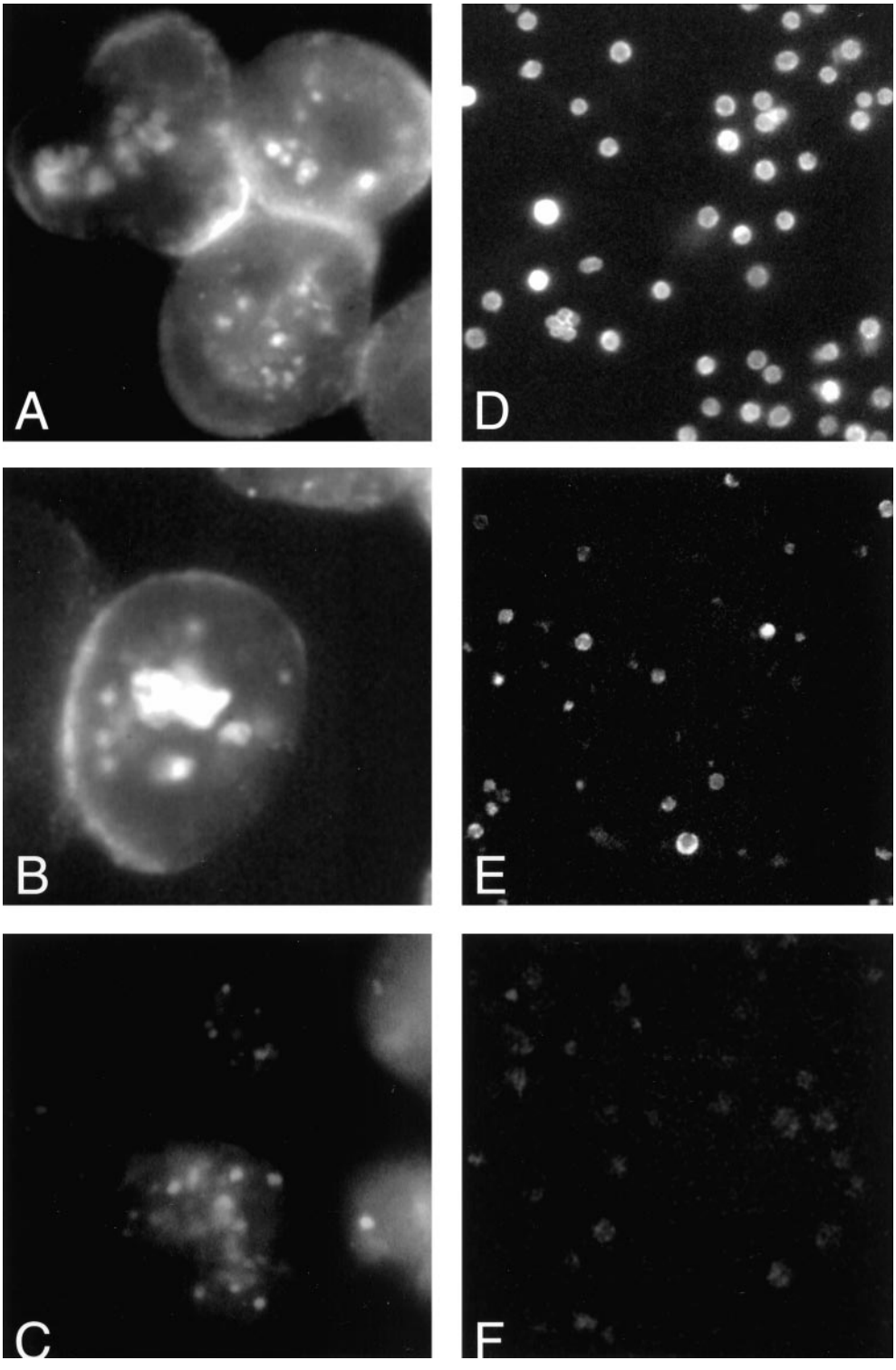

Fig. 5. Immunofluorescence PM localization of hmEH on human hepatocyte and transformed yeast and its recognition by GIAA. Unpermeabilized human hepatocytes (A–C) and transformed spheroplasts (D–F) were fixed and incubated with rabbit anti-hmEH antibodies (A and D), affinity-purified GIAA (B and E), or human control sera (C and F). Cells were processed for immunofluorescence as described under *Materials and Methods*. The immunofluorescence staining at the PM (colored in green on the original photographs) shows the presence of hmEH epitopes exposed on the outer cell surface. Intracellular bright fluorescence spots inside human hepatocytes are colored in yellow (and not green) on the original colored photograhs and correspond to the endogenous fluorescence of lipofuscins.

yeast strain W(GhR, Yb5) was used in this study. In this strain, the molar ratios of CYP3A4, CPR, and human cytochrome b_5 (10:1:10) were close to the ratios in human liver. This strain, W(GhR, Yb5), was doubly transformed, either with galactose-inducible expression vectors pCYP3A4/V80 (expressing CYP3A4) and pV60 (control plasmid), or with pCYP3A4/V80 (expressing CYP3A4) and phmEH/V60 (expressing hmEH) (Fig. 1).

In the presence of TA, NADPH, and yeast microsomes expressing CYP3A4 alone, the HPLC metabolite profile at 215 nm showed formation of a TA metabolite (metabolite I) (Fig. 7A) that was absent in the NADPH-free control (Fig. 7B) or with microsomes from yeast transformed with vector controls (data not shown). TA and metabolite I retention times were 13 and 12.5 min, respectively (Fig. 7A). In contrast, in the presence of hmEH metabolite I decreased, whereas metabolites II and III appeared (Fig. 7C). Metabolites II and III were not generated in the presence of TA with microsomes containing hmEH alone (Fig. 7D). In all experiments, the relative peak areas of metabolite II and III versus metabolite I were 0.45 ± 0.10 and 0.32 ± 0.08, respectively. The hmEH-mediated decrease in metabolite I could be consistent with an epoxide and the hmEH-dependent accumulation of metabolites II and III is expected for diol derivatives (Fig. 7C). However, the structure of the three metabolites has not been determined.

Inactivation of hmEH by TA in Transformed Yeast Microsomal and PM Fractions. To evaluate hmEH functionality in transformed yeast PM, its specific activity was determined from styrene oxide hydrolase activity. PM and microsomal hmEH specific activities at 37°C were 60 ± 6 and 42 ± 8 nmol of styrene glycol formed/min/mg of proteins, respectively. These two activities were of the same order of magnitude as the activity found in human liver microsomes (58 nmol of styrene glycol formed/min/mg of proteins) (Skoda et al., 1988). The activity found in human hepatocyte PM was 40 ± 7 nmol of styrene glycol formed/min/mg of proteins.

Yeast microsomes or yeast PMs containing CY3A4 and hmEH were incubated with or without TA at 37°C for various times (0, 15, 30, 45, and 60 min). After a 1:100 dilution, these fractions were tested for their ability to hydrolyze the B(a)P oxides formed in situ by CYP1A1-mediated B(a)P. In microsomes, hmEH was inactivated by 44 ± 6% after 15 min of incubation in the presence of TA, based on B(a)P-7,8 and -9,10 dihydrodiol formations (Table 1). With PMs, TA-mediated hmEH inactivation was 35 ± 6% in the same conditions (Table 1). In both, microsomes and PM, hmEH inactivation was no longer observed after a 45-min incubation (Table 1), implying either inactivation of CYP3A4 by TA or by self-inactivation (usually observed with CYP3A4 in yeast microsomes), or perhaps, hmEH modification by TA did not completely inhibit it. However, this hmEH inactivation suggested that some TA reactive metabolite, probably an epoxide (metabolite I) formed by CYP3A4 on the furan ring, alkylated, modified, and inactivated hmEH, in the microsomes and the PM.

Recognition of TA-Alkylated hmEH by Patient's Sera. To verify that hmEH-TA metabolite adducts generated in vitro included the antigen that elicited immune response in patients with germander hepatitis, experiments were performed with or without TA by using yeast microsomes expressing CYP3A4 and hmEH incubated for 1 h in the presence or in the absence of an NADPH-generating system. Samples were then analyzed by SDS-PAGE and immunoblotting for expression of protein antigen recognized by patient's sera (MOT and NOU). Recognition of hmEH protein band was significantly higher with hmEH

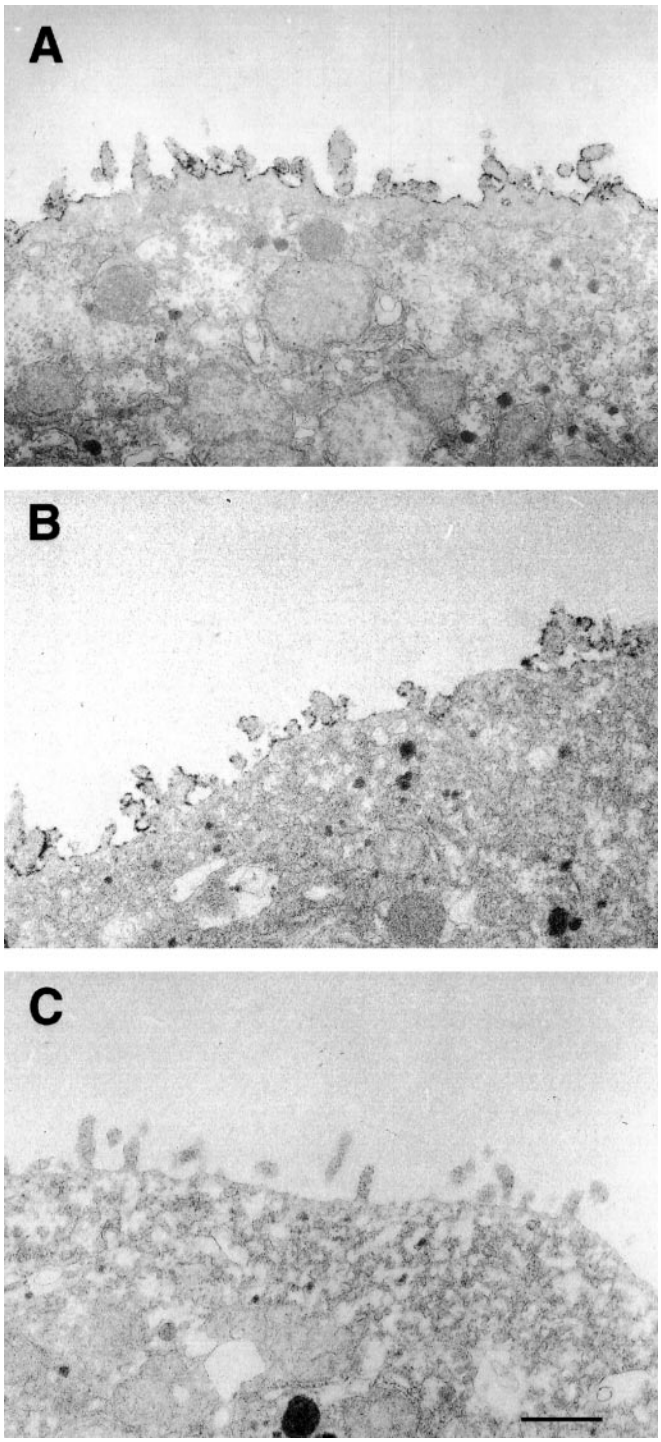

Fig. 6. Electron micrographs show the localization of hmEH on the surface of human hepatocytes. Unpermeabilized normal hepatocytes were fixed and incubated with rabbit anti-hmEH antibodies (A), affinity-purified GIAA (B), or normal control sera as control (C), and with horseradish peroxidase-conjugated anti-rabbit or anti-human IgG. The cells were processed for diaminobenzidine cytochemistry and cut for electron microscopy. The electron-dense peroxidase reaction product, showing hmEH presence, resides on the PM both along its linear parts and its microvilli (A and B). No labeling is observed with human control sera (C). Scale bar, 1 μm.

microsomes incubated with TA and an NADPH-regenerating system (Fig. 8, lane 1) than either without TA (Fig. 8, lane 2) or with an NADPH-free system (Fig. 8, lane 3). Immunoblotting studies revealed that TA-alkylated hmEH was expressed in vitro when microsomes were incubated in the presence of TA and an NADPH-generating system and was recognized by antibodies present in the patient's sera (Fig. 8, lane 1), in addition to the anti-hmEH-autoantibodies.

Discussion

This study demonstrates for the first time that a plant substance can induce autoantibody formation in some patients. Moreover, the autoantibodies are directed against hmEH, a phase II microsomal enzyme, and are not inhibitory. In contrast, immunoinhibitory anti-CYP autoantibodies are present in both idiopathic autoimmune hepatitis type 2 and autoimmune hepatitis induced by several drugs. In idiopathic autoimmune hepatitis the autoantobodies are directed against CYP2D6 (Zanger et al., 1988; Kiffel et al., 1989). In tienilic acid-, dihydralazine-, anticonvulsant-, and halothane-induced hepatitis, autoantibodies are directed against CYP2C9, 1A2, 3A, and 2E1, respectively (Beaune et al., 1987; Bourdi et al., 1990, 1996; Leeder et al., 1992).

In this study, four patients with germander tea-induced hepatitis were shown to develop autoantibodies that reacted with hmEH in the microsomes or PM of both human hepatocytes and hmEH-expressing yeasts. Dose-dependent immunoprecipitation of hmEH by these human sera confirmed hmEH as their autoantigenic target. As frequently observed with the anti-mEH antibodies raised in rabbits, these human autoantibodies caused no direct immunoinhibition, in contrast to all known anti-CYP autoantibodies. This suggests that anti-hmEH (auto)antibodies are not directed against the active site of the enzyme.

To develop an immune response, the immune system must be stimulated by a nonself protein or a modified self-protein (neoantigen). Covalent binding was demonstrated in isolated hepatocytes incubated with TA (Lekehal et al., 1996), suggesting the formation of metabolite-modified neoantigen(s). To better understand the possible involvement of hmEH, we used a humanized yeast strain expressing CYP3A4 with or without hmEH. CYP3A4 alone only formed TA metabolite I. An epoxide might be formed by metabolic activation of the furan ring of TA (Kouzi et al., 1994). In the presence of hmEH, metabolite I decreased and two other metabolites appeared, probably diol derivatives. However, the structures of these metabolites were not investigated. Aggregates and oligomerization of CYP and mEH were reported (Oesch and Danley, 1972; Guengerich and Davidson, 1982). Due to this proximity, a reactive epoxide formed by CYP could contact hmEH during its lifetime and alkylate it. Indeed, preincubation of yeast microsomes containing CYP3A4 and hmEH with TA caused time-dependent inactivation of hmEH, suggesting that the CYP3A4-generated reactive epoxide could alkylate and modify hmEH. This might trigger anti-hmEH autoantibody formation in germander-consuming patients.

Peptides derived from foreign or modified proteins must be

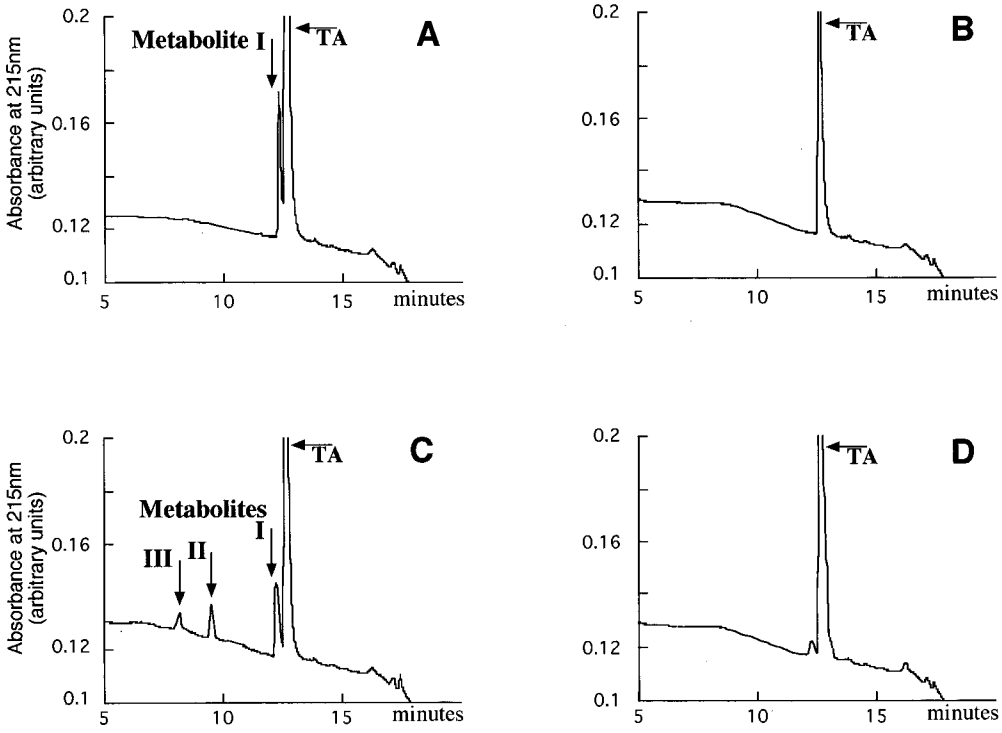

Fig. 7. HPLC profiles obtained by TA metabolic activation with CYP3A4 alone or both CYP3A4 and hmEH. A, microsomes of the yeast strain W(GhR, Yb5), transformed with both pCYP3A4/V80 and pV60 (thus expressing only CYP3A4), were incubated with 100 mM TA and with an NADPH-regenerating system as described under *Materials and Methods*. Metabolite I formation by CYP3A4 is observed. B, microsomes from the same strain transformed with both expression vectors pCYP3A4/V80 and phmEH/V60 were incubated with TA and without an NADPH-regenerating system. There is no detectable metabolite. C, microsomes from the same strain transformed with both pCYP3A4/V80 and phmEH/V60 (thus expressing both CYP3A4 and hmEH) were incubated with TA and an NADPH-regenerating system. Metabolite I formation is decreased while two new metabolites (II and III) are formed. Chromatograms were normalized with phenacetin as internal standard (data not shown). D, microsomes of the yeast strain W(GhR, Yb5), transformed with both pV80 and phmEH/V60 (thus expressing only hmEH), were incubated with TA and an NADPH-regenerating system. No metabolite was detected.

presented by major histocompability complex (MHC) class II molecules to interact with T helper lymphocytes and start the immunization process. MHC class II molecules are not normally expressed by hepatocytes, which may be initially unable to trigger immunization. However, whole apoptotic cells and/or the cellular contents of disrupted, necrotic hepatocytes can be taken up and endocytosed by macrophages and other antigen-presenting cells. These cells express MHC class II molecules and can thus start immunization. Germander furanoditerpenoids exhibit direct toxicity causing glutathione and protein thiol depletion and either necrosis (Loeper et al., 1994) or apoptosis (Fau et al., 1997). This direct toxicity could start the immunization process, leading to the appearance of both anti-hmEH autoantibodies and possibly also anti-hmEH-adduct antibodies. The latter possibility was observed by the higher recognition of hmEH by the human sera, with microsomes preincubated with TA than with microsomes not exposed to TA. Both anti-CYP autoantibodies and anti-CYP adduct antibodies were described in halothane- (Vergani et al., 1980; Pohl, 1990), tienilic acid- (Robin et al., 1996), and ethanol-induced liver injury (Clot et al., 1997).

To participate in the immune destruction of hepatocytes, autoantibodies should be able to recognize protein epitopes exposed on the outer surface of the PM. mEH was reported to be present in the PM of rat hepatocytes or transfected Madin-Darby canine kidney cells (Alves et al., 1993; von Dippe et al., 1996), although immunofluorescence studies with monoclonal antibodies did not clearly detect mEH on the surface of transfectred BHK21 fibroblasts (Honscha et al., 1995). In the present study, Western blots of PM fractions and measurement of epoxide hydrolase activity showed that hmEH was both present and functional in the PM of human hepatocytes and also in hmEH-expressing yeast. To determine whether hmEH was present on the outside of the PM, unpermeabilized, fixed human hepatocytes or hmEH-expressing yeast spheroplasts were exposed to rabbit anti-hmEH antibodies or to the affinity-purified human anti-hmEH autoantibodies. Immunofluorescence and immunoperoxidase labeling of the PM showed that hmEH was indeed exposed on the outer surface of the PM. In this respect, germander tea-induced hepatitis resembled tienilic acid- or dihydralazine-induced hepatitis, in which antimicrosomal autoantibodies were present that could recognize their antigenic CYP targets on the outer surface of human hepatocytes (Loeper et al., 1993).

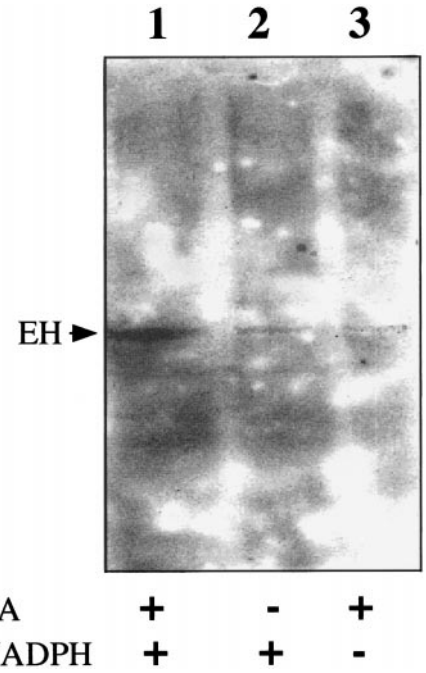

Fig. 8. Immunoblotting assessment of the presence of hmEH-TA metabolite adducts recognized by a germander patient serum. TA-metabolite adducts on hmEH formation in the presence of CYP3A4 and hmEH after incubation as described under *Materials and Methods*, with TA (lanes 1 and 3) or without TA (lane 2). An NADPH-regenerating system was present for lanes 1 and 2. Aliquots (15 μl of 200 μl/lane) were recognized by germander patient serum (MOT) diluted 1:250. Antibody reactivity was visualized with peroxidase-conjugated anti-human IgG antibodies diluted 1:10.000, with enhanced chemiluminescence detection. This experiment was repeated three times.

The presence of the antigenic molecule at the cell surface makes it theoretically possible for the anti-hmEH (auto)antibodies to participate in the immune destruction of hepatocytes, through complement-induced cytotoxicity and/or antibody-dependent cell-mediated cytotoxicity. Although anti-adduct antibodies rather than autoantibodies may be involved, antibody-dependent cell-mediated cytotoxicity was demonstrated with sera containing both anti-CYP autoantibodies and anti-CYP adduct antibodies in halothane hepatitis (Vergani et al.; 1980), tienilic acid-induced hepatitis (Neuberger and Williams, 1989), and alcohol-induced liver injury (Clot et al., 1997). Although (auto)antibodies may play some role in the immune destruction of hepatocytes in these diseases, it is nevertheless likely that cytotoxic T cells may play a more important role, possibly by recognizing modified peptides expressed by MHC class I molecules on the surface of hepatocytes.

As already discussed, it is likely that germander-induced hepatitis may be due to both direct toxicity and secondary immune reactions, with probably a varying contribution of these two mechanisms in different patients. An immune reaction was suspected in several patients, based on the quick onset of the relapse caused by a rechallenge, contrasting with the late onset of hepatitis (several months) during the initial exposure (Castot and Larrey, 1992). In another patient, chronic active hepatitis with a cirrhogenic outcome (consistent with autoimmunity) occurred after a short rechallenge, months after a first episode of acute hepatitis (Dao et al., 1992). Genetic predisposition may be required to develop an immune reaction, including polymorphism in drug-metabolizing enzymes, and the polymorphism of MHC molecules (Berson et al., 1994).

In summary, we have shown for the first time that anti-

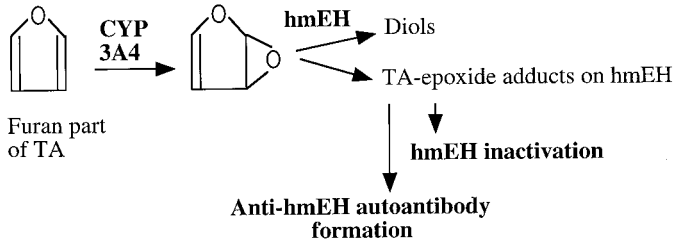

Fig. 9. Proposed mechanism for anti-hmEH autoantibody formation in germander-induced hepatitis. CYP3A4 oxidizes the furan ring of TA into an epoxide by CYP3A4 that is in part hydrolyzed by hmEH while another part of this reactive metabolite would alkylate hmEH to form an adduct. This alkylation leads to the inactivation and structural modification of the enzyme. Such modification can induce anti-hmEH autoantibody formation observed in the patient sera with germander-induced hepatitis.

hmEH autoantibodies can develop in plant-induced hepatitis. hmEH is present and functional in the human hepatocyte PM and hmEH epitopes are exposed on the outer surface of the PM, allowing the autoantibodies to possibly participate in the immune destruction of hepatocytes. CYP3A4-mediated metabolic activation of TA into a reactive epoxide could modify and inactivate hmEH. This altered protein may bypass immune tolerance and trigger the immune response against hmEH (Fig. 9). This first demonstration that plant substances may induce autoantibody formation and immune hepatitis may have implications for the etiology of some cases of autoimmune hepatitis that are currently classified as "idiopathic", in the absence of a recognized cause. Reactive metabolites from fruit or vegetable substances might modify self-proteins and trigger immune reactions in a few susceptible patients.

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