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Autoantibodies against Cytochromes P-4502E1 and P-4503A in Alcoholics

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ABSTRACT

Autoantibodies against soluble liver enzymes have been reported among alcoholics, but the targets of self-reactivity toward membrane proteins of the liver have not been characterized. Previously, among alcoholics, we found antibodies against ethanol-derived radical protein adducts that are dependent on cytochrome P-4502E1 (CYP2E1) for their formation. To further investigate autoantibodies against cytochrome P-450s during alcohol abuse, sera of rats chronically treated with ethanol in the total enteral nutrition model and sera from alcoholics with or without alcohol liver disease and from control subjects were analyzed by enzyme-linked immunosorbent assay and Western blotting for the presence of IgG against rat and human CYP2E1, rat CYP3A1, and human CYP3A4. A time-dependent appearance of IgG against rat CYP3A1 and CYP2E1 was evident during chronic ethanol feeding of rats. Anti-CYP2E1 reactivity showed positive correlation with the levels of hepatic CYP2E1 and was inhibited by the CYP2E1 transcriptional inhibitor chlormethiazole. Screening of the human sera by enzyme-linked immunosorbent assay revealed reactivity against CYP3A4 and CYP2E1 in about 20 to 30% and 10 to 20% of the alcoholic sera, respectively. No difference were noted between sera from alcoholics with or without hepatitis C virus infection, and only very little reactivity was seen in sera from control subjects. Western blotting analysis revealed anti-human CYP2E1 reactivity in 8 of 85 alcoholic sera and 3 of 58 control sera, whereas anti-CYP3A4 reactivity was detected in 18 of 85 alcoholic sera and 4 of 58 control sera, which were different from the sera reactive with CYP2E1. Immunoblot reactivity of CYP3A4-positive alcoholic sera was found against glutathione-*S*-transferase fusion proteins containing truncated forms of CYP3A4, and such sera were also able to immunoprecipitate in vitro translated CYP3A4. Seven of eight sera showed reactivity toward domains C-terminal of position Ser281, and 1 of 8 sera recognized autoepitopes within the region Thr207-Ser281. These findings indicate that alcoholics develop autoantibodies against CYP2E1 and CYP3A4 that the CYP3A4 C-terminal domain is a target for the autoantibody reactions among a subset of alcoholics. The novel finding of CYP3A4 autoantibodies and their significant expression among alcoholics warrants further investigation. Attention should be given to immune toxicity associated with CYP3A4 autoantibodies and cases of alcohol abuse that are accompanied by exposure to drugs and substances that are CYP3A substrates.

Cytochrome P-450s (CYPs) are responsible for oxidative metabolism of a vast number of structurally diverse foreign substrates such as drugs, food additives, carcinogens and environmental pollutants as well as endogenous molecules or steroids, prostaglandins, and fatty acids. These thio-hemoproteins are classified in families and subfamilies according to their similarities in amino acid sequences, but each member exhibits a distinct substrate specificity, catalytic activity, and immunological characteristics (Ronis et al., 1996). Certain CYPs may become altered in the line of service because of the binding of reactive metabolites produced during drug or chemical biotransformation to the protein as well as to the heme moiety (Beaune et al., 1996; Uetrecht, 1997). In most incidences, these events lead to the inactivation of CYP enzymatic activity, but because of the rapid protein turnover, there are minor perturbation in the normal function of the host, and the modified proteins escape detection. Special situations have been found in which various CYP enzymes become the target of immune response triggered by the binding of reactive intermediates. For instance, antibodies recognizing trifluoroacetyl-CYP2E1 and tienilic acid-CYP2C9 ad-

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ABBREVIATIONS: ALD, alcohol liver disease; CYP, cytochrome P-450; LKM, liver kidney microsome; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; PBS, phosphate-buffered saline; GST, glutathione-*S*-transferase; HCV, hepatitis C virus.

ducts have been detected in the sera of patients with halothane-induced (Eliasson and Kenna, 1996) and tienilic acid-induced (Beaune et al., 1994; Robin et al., 1996 hepatitus).

Formation of reactive intermediates during drug metabolism is also able to stimulate autoimmune reactions that involve native CYPs as in the case of hepatitis associated with dihydralazine (anti-CYP1A2) (Bourdi et al., 1990; Belloc et al., 1997), tienilic acid (anti-CYP2C9) (Lecoeur et al., 1996), or halothane (anti-CYP2E1) (Eliasson and Kenna, 1996) treatment, as well as hypersensitivity reactions to the aromatic anticonvulsants (anti-CYP3A) (Riley et al., 1993; Leeder et al., 1996). CYP2D6 is a target in anti-liver kidney microsome type I (LKM-1), in type II chronic autoimmune hepatitis, and in hepatitis virus C (HVC) (Manns et al., 1989; Choudhuri et al., 1997); CYP11A1 is a target in autoimmune polyendocrine syndrome type I (Winqvist et al., 1993); and CYP17 and CYP21 are targets in Addison's disease (Krohn et al., 1992; Winqvist et al., 1992). These examples suggest that human antibody reactivity toward CYPs serves as a sensitive indicator of CYP structural modifications and may play a role in the induction of immunotoxicity in drug dosage and disease conditions.

The link between expression of anti-CYP autoantibodies and the immune effector mechanisms that lead to the pathological state is not established. However, in the case of hepatocyte injury, the fact that several CYP isoforms are expressed on the outer layer of hepatocyte plasma membrane (Loeper et al., 1990, 1993) makes possible the development of antibody-mediated cytotoxicity toward liver parenchymal cells. Even more elusive are questions regarding pathways for processing and presentation of membrane-bound enzymes to immune cells. What epitope triggers autoantibody formation in the first place, and how is a covalently modified self-protein such as CYP perceived as "nonself" by the immune system? Interestingly, the sites of modification do not map to the major epitopes recognized by the autoantibodies in patient antisera (Lecoeur et al., 1996); rather, other regions of the CYP protein appear to be immunodominant and separate from those that would be predicted from the hapten-carrier hypothesis. Drug metabolite-modified protein fragments or antibodies against them (idiotype) could activate T cells that recognize short linear peptides derived from the native unmodified protein as nonself. In turn, the activated B cells produce antibodies (anti-Id) against both drug-modified and nonmodified CYPs, the latter "native epitopes" being the predominant target of patient antibodies (Robin et al., 1997). This principle could explain conformational epitopes recognized by anti-CYP2C9 antibodies of tienilic acid hepatitis and epitopes that map distant from the substrate-binding site.

The possible involvement of immune reactions in the pathogenesis of alcoholic liver disease (ALD) is suggested by the observations that on chronic exposure to ethanol, alcoholic patients and experimental animals form antibodies directed against alcohol-modified proteins (Paronetto, 1993) and lymphocytes from ALD patients are cytotoxic toward autologous hepatocytes (Izumi et al., 1983). Interestingly, heavy drinkers showing antibodies against alcohol-altered hepatocyte plasma membranes have been reported to have a significantly higher risk of developing liver cirrhosis than do subjects negative for these antibodies (Takase et al., 1993).

Considerable evidence indicates that proteins modified by acetaldehyde are antigenic, and antibodies directed against acetaldehyde-derived epitopes have been detected in alcohol-treated rats as well as in the serum of patients with alcohol abuse (Tuma and Klassen, 1992; Viitala et al., 1997). Recently, the presence of antibodies reacting with protein adducts of hydroxyethyl free radicals have been reported in rats developing ALD, after chronic intragastric ethanol feeding, and in patients with alcoholic cirrhosis (Clot et al., 1995; Albano et al., 1996). These antibodies are unrelated to those directed against acetaldehyde-derived adducts and preferentially recognize ethanol-inducible CYP2E1 complexed with hydroxyethyl radicals (Clot et al., 1996). In analogy to the fact that autoimmune response causing formation of antibodies directed against the intact P-450 molecule are seen in drug-induced hepatitis, as a possible consequence of the reaction of reactive intermediates of the drugs to cause covalent adducts to the enzymes, the current study was undertaken to analyze whether similar antibodies were formed against intact CYP2E1 in alcoholics because of protein-hydroxyethyl radical adduct formation and whether such antibodies also were formed against CYP3A that is induced by ethanol in rats and in primary cultures of human hepatocytes exposed to ethanol (Louis et al., 1994; Kostrubsky et al., 1995).

Materials and Methods

Patients and Control Subject Recruitment. In this study, the sera of three distinct groups of Swedish patients were used. The first group consisted of 85 patients (67 men and 18 women; age range, 30–65 years; mean age, 48 years) admitted for alcohol detoxification at the Center for Dependence Disorders at St. Görans Hospital in Stockholm. A venous blood samples was drawn within 24 h of admission, and serum was prepared through centrifugation. Alcohol abuse was confirmed by clinical anamnesis and a highly elevated concentration (>50 U/liter) of serum carbohydrate-deficient transferrin, a biochemical marker of prolonged heavy drinking. Chronic exposure to ethanol increases the formation of serum transferrin isoforms with a low sialic acid content, hence the name "carbohydrate-deficient" transferrin, or CDT. To render CDT levels abnormal, a regular daily intake of at least 50 to 80 g of ethanol for 1 week or longer is required. During abstention, the CDT concentration normalizes with a half-life of ~1.5 to 2 weeks (Stibler, 1991). The major advantage compared with the measurement of γ-glutamyltransferase, which is mainly a test of liver function, is that CDT produces considerably fewer false-positive test results (i.e., a superior diagnostic specificity for alcohol). CDT in serum was determined with the CDTect assay (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden).

A second group of 18 ALD patients (nine men and nine women; age range, 43–72 years; mean age, 55 years) were diagnosed on the basis of clinical and biochemical examinations, and, in 50% of the cases, liver biopsies performed at the Gastroenterology Department, Karolinska Hospital, in Stockholm. These patients tested negative for the presence of antibodies against hepatitis C virus (HCV) and liver autoimmune hepatitis markers: anti-liver kidney microsome (LKM) and antimitochondrial antibodies. A third group of 58 social drinkers (39 men and 19 women; age range, 30–65 years; mean age, 42 years) and 14 individuals abstaining from alcohol ("tea totalers": 4 men and 10 women; age range, 40–72 years; mean age, 47 years) were used as control subjects. This study was approved by the Ethics Committee at Karolinska Institutet.

The Italian patients included 25 alcohol abusers (19 men and 6 women; age range, 36–68 years, mean age, 52 years) with ultrasound examination and biochemical parameters indicating alcoholic steato-

hepatitis or alcoholic liver cirrhosis and 12 heavy drinkers (10 men and 2 women; age range, 38–63 years; mean age, 50 years) without clinical evidence of liver disease admitted to the Division of Gastroenterology of a general district hospital in Turin, Italy. All of these patients have an estimated mean daily intake of ethanol of more than 100 g (mean, 228 ± 83 g/day) and were negative for serum markers for hepatitis B virus and for the presence of antibodies versus HCV, measured with a second-generation enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, Chicago, IL). The group of healthy control subjects consisted of 14 subjects (8 men and 6 women; age range, 30–59, mean age, 44 years) abstaining from alcohol who were recruited within and through contacts of the research staff. In all patients and control subjects, alcohol abuse was assessed by a standardized questionnaire and confirmed by positivity at CDT testing. All subjects gave informed consensus to the analysis, and study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Human Microsomes. Human liver microsomes were taken from frozen stocks prepared as described previously (Ekström et al., 1989). The protein content was determined by Lowry, and the samples were diluted in 50 mM sodium phosphate, pH 7.4, buffer and adjusted to concentration of 5 mg/ml. For ELISA, the microsomes were solubilized in 0.15% sodium cholate and coated in 0.075% sodium cholate-phoshate-buffred saline (PBS), pH 7.4. To remove IgG contamination for Western blotting experiments, the liver microsomes were incubated at 4°C overnight with protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) in 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) in 50 mM Tris·HCl, pH 7.8.

Human Proteins. The human recombinant CYP3A4 (Δ3–12) and CYP2E1 (Δ1–7Trp) were produced from overexpressing plasmid in *Escherichia coli* at Oxford Biomedical Research Inc. (Oxford, MI) and Panvera Ltd. (Madison, WI). The N-terminal modifications of CYP3A4 and CYP2E1 were as described previously (Gillam et al., 1994; Guo et al., 1994).

Plasmid Constructs. For cloning into the pGEX-5X-3 bacterial vector, various 5′ deletions of CYP3A4 cDNA were generated with 3′ hexa his tag (His_6) by amplification of reverse transcription-polymerase chain reaction (Clontech) human liver mRNA using the following CYP3A4-specific synthetic oligonucleotide primers: reverse primer *Not*I His_6CYP3A4 5′-TT TTCCTTTTGCGGCCGCTCAATGATGATGATGATGATGATGATGGGCTCCACTTACGGTGCCATC-3′, forward primers: F*Bgl*II Asp123CYP3A4 5′-CTGAGATCTCTGATGAAGAATGGAAGAGATTA-3′, F*Bgl*II Thr207CYP3A4 5′-CTGAGATCTCTACCAAGAAGCTTTTAAGATTT-3′, and F*Bgl*II Ser286CYP3A4 5′-CTGAGATCTCTTCAAAAGAAACTGAGTCCCACAAA-3′. After 28 thermocycles (45 s at 94°C, 20 s at 56°C, and 8.5 min at 72°C in a Perkin-Elmer 2400), the DNA was separated on low temperature-melting agarose gel and specific band excised for purification with Wizard PCR kit (Promega, Madison, WI). The fragments were digested with restriction enzymes *Bgl*II/*Not*I and then repurified for direct ligation to factor Xa pGEX-5X-3 vector (Pharmacia Biotech, Uppsala, Sweden) cut with *Not*I/*Bam*HI. Ligated plasmids were transformed into *E. coli* strain TG1. The identity of inserts was confirmed by both fragment size after restriction digests and sequencing with pGEX-5X-3 primer 5′-GGGCTGGCAAGCCACGTTTGGTG-3′ with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (Amersham Life Science, Cleveland, OH).

For cloning into the bacterial vector pGEM-4Z, the full-length CYP3A4 cDNA with 3′ hexa his tag (His_6) was generated as described above using CYP3A4-specific synthetic oligonucleotide primers: reverse primer 5′-GTCGGTACCTCAATGATGATGATGATGATGGGCTCCACTTACGGTGCC-3′ and forward primer 5′-CTGAGATCTATGGCTCTCATCCCAGACTTG-3′. The fragments were digested with restriction enzymes *Bgl*II/*Kpn*I and then repurified for direct ligation to pGEM-4Z vector (Promega) cut with *Kpn*I/*Bam*HI. Ligated plasmids were transformed into *E. coli* strain TG1.

Fusion Protein Expression and Purification. Plasmid preparations were made with JETprep miniprep kit (Gemomed GmbH, Bad Oeynhausen, Germany) and transformed into *E. coli* strain BL21. A single colony was inoculated into Luria's broth containing 100 μg/ml ampicillin and incubated at 37°C under continuous shaking. To induce expression of heterologous proteins as fusion proteins with the glutathione-*S*-transferase (GST) of *Schistosoma japonicum* isopropyl β-D-thiogalactopyranoside was added to cultures of A_{690} = 0.8 to 1 at a final concentration of 2 mM for 1.5 h. Bacterial suspensions of 50-ml volume were then centrifuged at 5000*g* for 5 min, and pellets were immediately transferred to −70°C for storage. Single-step purification of his-tagged fusion protein was achieved under nondenaturing conditions from sonicates of lysed bacteria. Briefly, the frozen pellets were lysed in 5 ml of 1× binding buffer: 5 mM imidazole in 20 mM Tris-0.5 M NaCl, pH 8.0, containing 2 mM PMSF and 12 μg/ml leupeptin and subjected to ultrasonication (eight times 20-s pulses) on an ethanol-ice bath using 23-kHz Soni Prep 150 fitted with 9.5-mm probe adjusted to 20-μm amplitude. After centrifugation at 5000*g* for 10 min, the pellet of unbroken cells was subjected to a second sonication. Supernatants were then pooled and transferred to sterile tubes for centrifugation at 12,000*g* for 30 min before loading onto column of ni-nitriloacetate-Sepharose resin (Qiagen GmbH, Hilden, Germany) preequilibrated with binding buffer. Column washings in 60 mM imidazole and elution of protein in 1 M imidazole were carried out as described previously (Novagene Technical Bulletin, 1993). For factor Xa cleavage, the eluted fractions were dialyzed overnight at 4°C against 5 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris, pH 8.0, and incubated 16 h at room temperature with 1.2 μg of bovine factor Xa (Promega) per 100-μg fusion protein.

Treatment of Rats. Male Wistar rats weighing about 200 g were purchased from Charles River Laboratories (Hollister, CA). The intragastric cannula was implanted under phenobarbital sodium (30–50 mg/kg i.p.) anesthesia. The animals were then allowed to recover for 6 to 10 days before the experiment was started. The rats were maintained according to the guidelines of animal care as described by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985, Bethesda, MD). Animals were pair-fed ethanol or isocaloric dextrose continuously infused via a permanent intragastric cannula for 2 months in the amount necessary to maintain a constant high blood ethanol level of greater than 200 mg/100 ml as described previously (Tsukamoto et al., 1985). Two other groups were treated with dextrose plus chlormethiazole (80 mg/kg/day in the diet or in a bolus 80–120 mg/kg/day) or with ethanol plus chlormethiazole (as above). Blood samples for serum preparation were taken from the tail vein before and after 1 and 2 months of treatment. At the end of the experiment, livers were removed and microsomes were prepared as described previously (Johansson et al., 1994). The relative amounts of CYP2E1, CYP3A, and CYP4A in the different microsomal preparations were assessed in ELISA using rabbit anti-CYP3A1 (a gift from Prof. James R Halpert, University of Arizona, Tucson, AZ) (Halpert, 1988), rabbit anti-CYP2E1 (Gillam et al., 1994), and rabbit anti-CYP4A (a gift from Dr. Gordon Gibson, University of Surrey, Guildford, U.K.) antibodies.

ELISA. Polystyrene microwell plates (Sigma, St. Louis, MO) for ELISA were coated with 100 μl of microsomal preparations (50 μg/ml), purified CYPs (2.8 μg/ml), or serum albumin (50 μg/ml) (Sigma) in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), by incubation overnight at 4°C. After incubation, the antigen solutions were removed and replaced by 0.2 ml of 5% milk in 0.1 M PBS, pH 7.4. The plates were further incubated for 4 h at room temperature to block nonspecific binding sites. Rabbit sera against CYP3A1 or CYP2E1 diluted 1:5000 in duplicate or human sera (dilution from 1:50 to 1:600) were added as aliquots of 0.1 ml/well in blocking buffer, covered with parafilm, and incubated for 2 to 3 h at 37°C. The wells were washed three times with PBS in 0.05% Tween 20 and once with PBS. The secondary antibodies consisting of 1:10,000 diluted peroxidase-linked goat anti-rabbit IgG or 1:25,000 diluted peroxidase-linked goat anti-human IgG Fc domain were incubated for 2 h at room temperature in PBS. After two washings in PBS-Tween 20 and

two washing in PBS, 0.15 ml of a reaction mixture containing 0.4 mg/ml *o*-phenylenedizmine, 0.4 μl/ml 30% hydrogen peroxide, 5.1 mg/ml citric acid, and 6.1 mg/ml Na_2HPO_4 anhydrous, pH 5.0, was added to each well. After 20 min, the reaction was stopped by the addition of 50 μl of 2 N H_2SO_4, and absorbances were measured at 492 nm using a microplate reader (Titertek Multiskan Plus MkII). The results were expressed as difference between the absorbances in the well containing CYPs and those containing human serum albumin.

In Vitro-Coupled Transcription-Translation of Human CYP3A4his$_6$. Full-length human CYP3A4 was produced in vitro from the cDNA in pGEM-4Z using a TnT T7-Quick-coupled reticulocyte lysate system (Promega). Plasmid DNA (1.5 μg) was incubated for 90 min at 30°C in 40 μl of rabbit reticulocyte lysate, 2 μl of 10 mCi/ml [^{35}S]methionine, and nuclease-free water added to a final volume of a 50-μl reaction mixture. The reaction was stored at −70°C until needed. To determine the percentage of incorporation of [^{35}S]methionine, 2 μl of reaction mixture was added to 98 μl of a 2% solution of H_2O_2 in 1 M NaOH and incubated at 37°C for 15 min. Next, 900 μl of 25% trichloroacetic acid (TCA) was added and incubated on ice for 30 min. To determine total counts per minute present in the reaction, a 10-μl aliquot of the TCA reaction mix was spotted directly onto a filter and dried for 10 min before counting. To collect the precipitated translation products, the TCA reaction mix was vacuum filtered onto a Whatman GF/B glass fiber filter (Whatman International, Maidstone, England) prewetted with cold 5% TCA. The filter was rinsed five times with 4 ml of 5% TCA and once with 3 ml of acetone. The percentage of incorporation of [^{35}S]methionine was determined as counts per minute of washed filter/cpm of unwashed filter.

Immunoprecipitations of [^{35}S]Methionine-Labeled CYP3A4. For each immunoprecipitation assay, a 5-μl aliquot of in vitro translation reaction mixture (equivalent to 780,000 cpm of TCA precipitable material) was suspended in 100 μl of immunoprecipitation buffer containing 50 mM Tris·HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and 2 mM PMSF. Preclearing of the reaction mix was done by the addition of 50 μl of protein A-Sepharose (Pharmacia Biotech) prepared according to the manufacturer and incubated for 2 h under gentle shaking at 4°C. After centrifugation at 2000*g* for 3 min, the supernatant was transferred to clean Eppendorf tubes. Human serum was then added to the precleared reaction mix at a final dilution of 1:100. After 4-h incubation with shaking at 4°C, 50 μl of protein A-Sepharose was added as described previously and incubated at 4°C for 12 h with shaking. The protein A-Sepharose-antibody complexes were then collected by centrifugation and washed five times in 1.5-ml volumes of immunoprecipitation buffer at 4°C. Immunoprecipitated radioactivity was evaluated in a liquid scintillation analyzer. For analysis by SDS-polyacrylamide gel electrophoresis (PAGE), the protein A-Sepharose-antibody complexes were resuspended in 50 μl of SDS sample buffer, boiled, and centrifuged, and 30 μl of the recovered supernatant was loaded for electrophoresis.

SDS-PAGE and Immunoblotting. SDS-PAGE was performed according to the method of Laemmli (1970) using 4% stacking gel and 8.7% separating gels. Human liver microsomal preparations and rat and human proteins were solubilized in SDS gel loading buffer [3% SDS (w/v), 0.2 M Tris·HCl, pH 6.8, 26% glycerol (w/v) with 2 M 2-mercaptoethanol) and heated for 2 min at 100°C. After electrophoresis in Mini-Protean apparatus (Bio-Rad, Hercules, CA) for 40 min × 200 V, the proteins were electrotransferred at 50 mA × 15 h to H-Bond Extra (Amersham) nitrocellulose in transfer buffer of 20 mM Tris, 154 mM glycine, and 20% v/v methanol. Blots were washed in 0.1 M phosphate buffer (pH 7.4), air dried on Whatman filter paper, and then placed in 50 mM Tris·HCl (pH 7.4), 200 mM NaCl, and 0.05% Tween 20 in a 5% solution of baker's powdered milk for 4 h to block the free binding sites. Blots were washed three times for 5 min in 10-ml volumes of TBS-Tween 20 buffer and then probed with 1:5000 diluted rabbit anti-CYP2E1 or rabbit anti-CYP3A1 sera or different human sera diluted at either 1:300, 1:600, or 1:2500 in blocking buffer for 4 h with shaking. After repeated washes, immunoblots were performed for 2 h with shaking using peroxidase-linked goat anti-rabbit IgG heavy and light chains diluted 1:2,000 or goat anti- human IgG Fc diluted 1:20,000. The blots were developed on photographic film (Kodak X-Omat; Kodak, Rochester, NY) with luminol substrate and hydrogen peroxide of enhanced chemiluminescence (ECL) reagent (Amersham).

Statistical Analysis. The graphed and tabulated values presented in this work are the mean ± S.E.M. Unless mentioned otherwise, all statistical analysis was performed with StatMost program using parametric unpaired Student's *t* test:

$$t = (\overline{X}_1 = \overline{X}_2) / \text{S.D.} \sqrt{1/N2 + 1/N2}$$

Results

Detection of Antibodies to CYP3A1 and CYP2E1 in Rats Chronically Exposed to Ethanol. Rats receiving an ethanol-containing diet by intragastric feeding to maintain high blood alcohol levels develop changes that resemble those seen in ALD, including fatty change, focal inflammation, necrosis, and, after 2 months of feeding, fibrosis (Tsukamoto et al., 1985). We previously reported that in this rat model, the induction of CYP2E1 by ethanol is associated with the formation of hydroxyethyl-free radicals as well as the development of antibodies against hydroxyethyl radical protein adducts (Clot et al., 1995). Analysis by ELISA for the expression of autoimmune reactivity against CYPs demonstrated that by 1 month after ethanol administration, rats fed ethanol had an appreciable increase of IgG reactivity against either CYP3A1 and CYP2E1 compared with baseline values. (Fig. 1). This effect was specific for anti-CYP autoantibodies because ELISAs performed using either serum albumin or purified rat CYP reductase as antigen did not show increased immune reactivity against these proteins (Fig. 1C). When anti-CYP reactivity was expressed as percent of the baseline values, ethanol-fed rats exhibited a 2- to 3-fold increase over control animals not receiving ethanol (Fig. 1D). The administration of chlormethiazole, which has been shown to decrease *CYP2E1* transcription (Hu et al., 1994), caused a significant inhibition of the formation of autoantibodies against CYP2E1 (Fig. 1, B and D). A higher level of anti-CYP3A1 autoreactivity relative to control is maintained in the presence of chlormethiazole, indicating that the drug did not significantly affect autoantibody formation against CYP3A1 (Fig. 1, A and D).

There was a significant correlation between the individual levels of CYP2E1 in the different livers and the immunoreactivity of the corresponding sera against intact CYP2E1 (Fig. 2B), whereas a similar relationship was not found in the case of CYP3A1 (Fig. 2A). Liver specimens of each rat were examined at the end of experiment, and the pathological score was recorded in accordance with established method (Tsukamoto et al., 1985). There was a significant correlation between the anti-CYP antibody reactivity and severity of liver injury at 2 months of ethanol exposure (Fig. 3).

Detection of Anti-CYP Autoantibodies in Patients with Alcohol Abuse. The presence of autoantibodies against CYP2E1 and CYP3A1 in rats exposed to alcohol suggests the possibility that similar autoantibodies might arise in human alcohol abuse. In a first study in a group of 52 unselected Swedish alcoholics, sera from 18 alcohol liver disease patients negative for HCV and from 50 social drinkers selected as control subjects were evaluated for the pres-

ence of IgG reacting with CYP2E1 and CYP3A4 (the human equivalent of rat CYP3A1) by ELISA, using purified recombinant human CYP2E1 and CYP3A4. The CDT values were 15 ± 4 U/liter (range, 7–25 U/liter) for control subjects and 63 ± 9 U/liter (range, 40–103 U/liter) among alcoholics. Positive ELISA reactions against human CYP3A4 (Fig. 4, top) and CYP2E1 (Fig. 4, bottom) were found at higher frequency among the alcoholics than control subjects. The mean anti-CYP3A4 reactivity among Swedish alcoholics was A_{490} = 0.38 ± 0.28 (n = 52), and among alcoholics without HCV infection, the value (0.4 ± 0.24; n = 25) was similar, whereas among control subjects, A_{490} = 0.27 ± 0.13 (n = 50) (Fig. 4, left). The similar reaction with CYP2E1 was less intense in the sera, and the corresponding mean values were 0.12 ± 0.07 among alcoholics, 0.19 ± 0.02 in alcoholics without HCV, and 0.09 ± 0.05 in control subjects.

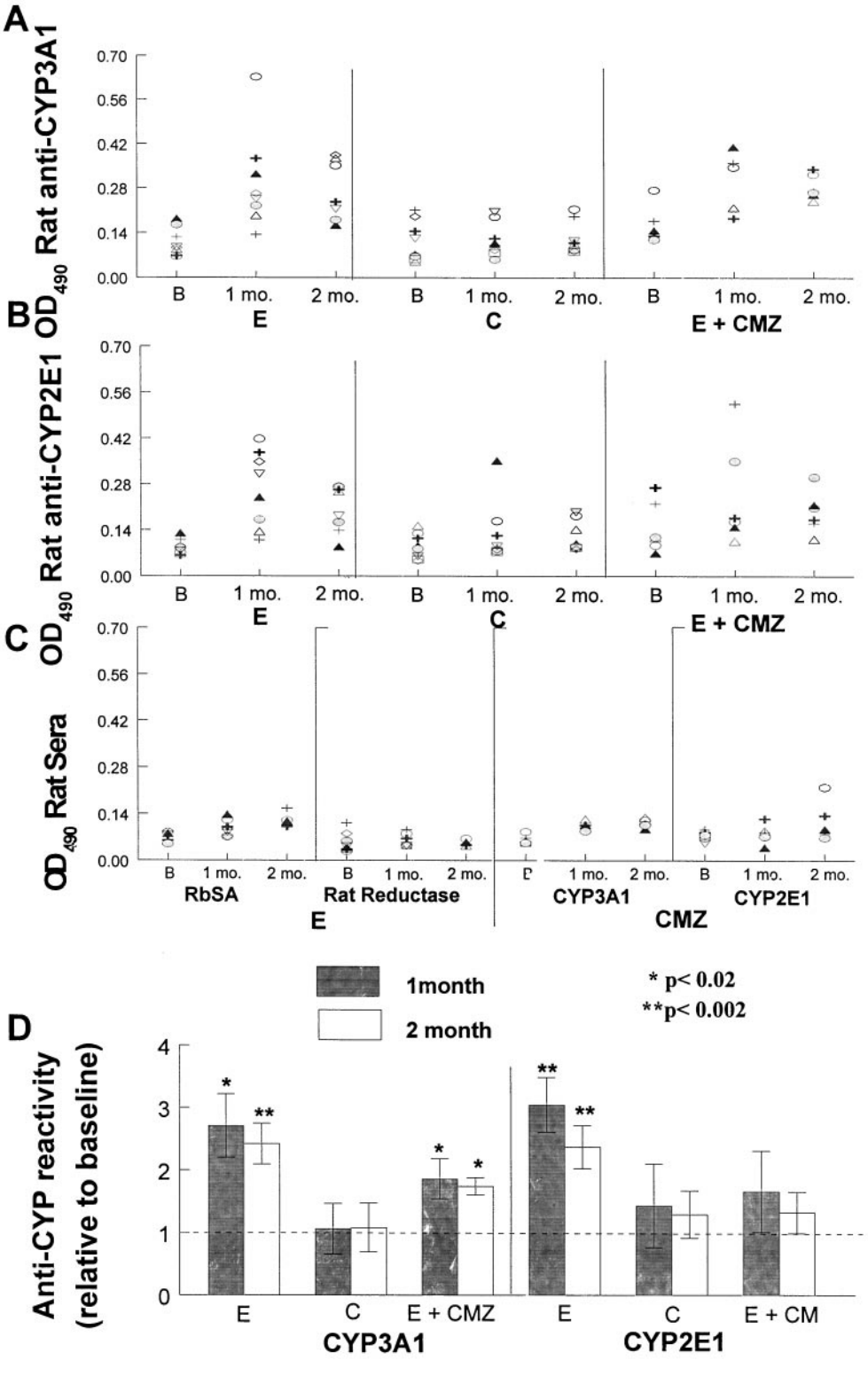

Fig. 1. ELISA of rat antibodies against CYP3A1 and CYP2E1. Rats intragastrically fed with ethanol (n = 8) or control fed with carbohydrate-protein diet (n = 9) were examined for anti-CYP3A1 or anti-CYP2E1 antibodies. The individual observations shown are ELISA of rat sera taken from ethanol-fed animals (1:300 dilution) and secondary antibody HRP-conjugated goat anti-rat (1:2000 dilution) against CYP3A1 (A) and CYP2E1 (B) versus control antigens rabbit serum albumin (RbSA) and rat reductase (1 and 2, C). Control experiments of anti-CYP3A1 and anti-CYP2E1 reactivity in sera from dextrose-chlormethiazole (DEX-CMZ) fed (3 and 4, C). The calculated mean and S.D. of the different treatment groups are corrected for baseline (the measurements made before treatment), and statistical significance is indicated by asterisks (D). ELISA plates were coated with 5 pmol/well CYP3A1 and CYP2E1, 170 pmol/well rat reductase, and 73 pmol/well rabbit serum albumin (RbSA).

Any relationship between the presence of anti-CYP2E1 and anti-CYP3A4 reactivity and that of ALD was further examined in an Italian population (Fig. 4, right) by studying sera from 12 alcohol abusers without clinical signs of liver disease and from 25 patients with ALD. Because infection with HCV can result in the development of anti-CYP2D6 autoantibodies (Greillier and Dusheiko, 1997), the alcohol abusers investigated were chosen among those negative for the presence of antibodies against HCV, as measured by a second-generation ELISA. Twelve healthy subjects abstaining from alcohol were used as control subjects. The mean IgG autoreactivity against recombinant human CYP3A4 was A_{490} = 0.43 ± 0.22 among Italian alcoholics and A_{490} = 0.36 ± 0.21 among ALD patients without HCV infection versus A_{490} = 0.21± 0.07 for the control subjects (Fig. 4, top). IgG autoreactivity against CYP2E1 was elevated among the Italian alcoholic groups to similar levels: A_{490} = 0.43± 0.3 alcoholics and A_{490} = 0.28± 0.13 ALD/HCV-negative versus A_{490} = 0.24± 0.07 for the control subjects (Fig. 4, top).

To determine whether the significant IgG reactions in ELISA against CYP3A4 were in fact representative of the presence of autoantibodies, the sera of alcoholics and control subjects were tested for their capacity to form immune complexes with [^{35}S]methionine-labeled CYP3A4 produced by in vitro translation (Fig. 5). An alcoholic sera of high reactivity (A_{490} < 0.9) gave strong band intensity in immunoprecipitation experiments (Fig. 4, lane 3), whereas the control sera of low ELISA reactivity (A_{490} < 0.2) gave only weak immunoprecipitable bands (Fig. 5, lanes 4 and 5). Alcoholic sera of weaker ELISA reactivity were in general only slightly effective in the immunoprecipitation experiments.

Characterization of Reactivity of Alcoholic Sera against Human CYPs. The reactivity of sera from alcoholics against recombinant human CYP2E1 and CYP3A4 was also investigated by immunoblotting. We observed that 18 of 85 sera (21%) from heavy drinkers and 3 of 18 sera (20%) from ALD patients recognized CYP3A4 (Fig. 6A), whereas 8 of 85 (10%) alcoholic sera and 2 of 18 ALD sera (11%) tested gave positive reactions against human CYP2E1 (Fig. 6B). Densitometric evaluation of the band intensities revealed stronger reactions against CYP3A4 [mean, 35 arbitrary units (a.u.)/pmol of CYP; range, 5–97 a.u./pmol of CYP] than that of CYP2E1 (mean, 23 a.u./pmol of CYP; range, 11–55 a.u./pmol of CYP) among alcoholics but not among the group of ALD patients: CYP3A4 (mean, 20 a.u./pmol; range, 8–42) versus CYP2E1 (mean, 44 a.u./pmol; values 39 and 50) (Fig. 6). Among the 58 control sera tested, 4 showed some reactivity with CYP3A4 and 3 gave weak reactions against CYP2E1. None of the 14 sera among the alcohol abstinence group were

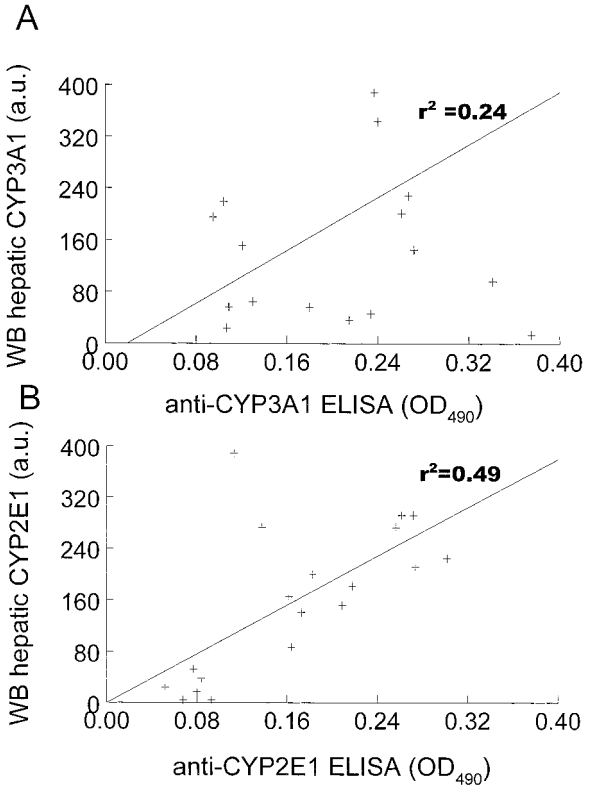

Fig. 2. The immunoreactivity of the rat sera against purified CYP3A1 (top) or CYP2E1 (bottom) as the function of the hepatic expression of the proteins as revealed by Western blotting. The amount of CYP3A1 and CYP2E1 was monitored by Western blotting of liver microsomes with polyclonal rabbit antisera. The immunoreactivity was determined by ELISA as outlined in *Materials and Methods*. The indicated values for r^2 are derived from Pearson's coefficient of correlation and significant at p < .002.

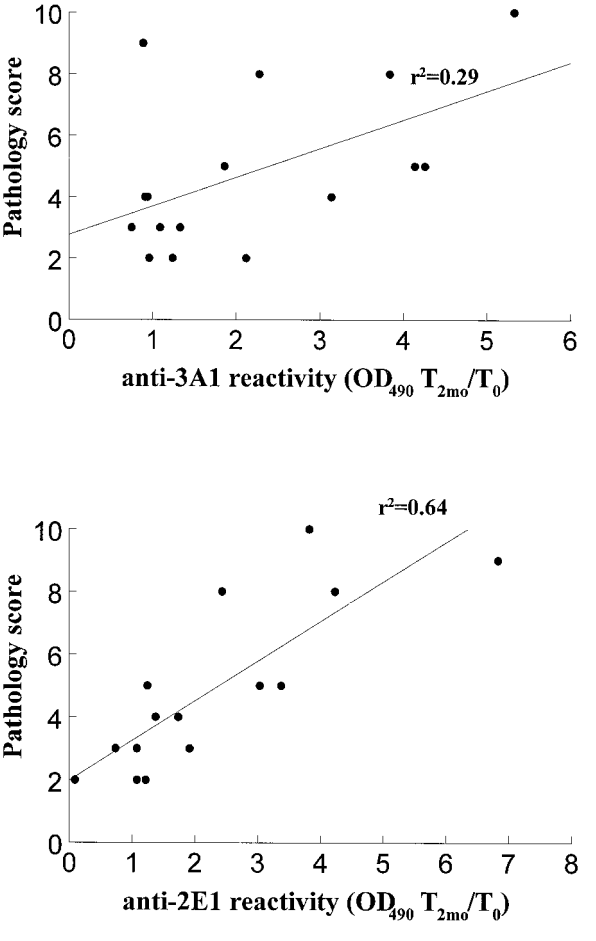

Fig. 3. Immunoreactivity of the rat sera against CYP3A1 and CYP2E1 as a function of the liver pathology. The pathology score of liver was quantified as extent of liver cells containing fat, inflammation, necrosis and fibrosis (French et al., 1993). The immunoreactivity shown are ratios of ELISA A_{490} of sera obtained at 2 months and at start of ethanol treatment as described in *Materials and Methods*. The indicated values of r^2 are derived from Pearson's coefficient of correlation and significant at p < .05 for anti-CYP3A1 and p < .001 for anti-CYP2E1.

positive (Fig. 6B). The pattern of reactivity against CYP2E1 and CYP3A4 are represented by the sera samples shown in Fig. 7.

With the exception of two patients, the antibody response seen with CYP3A4 was specific in the sense that the reactivity was exclusively directed against CYP3A4 with no reaction against CYP2E1. Moreover, sera reactive with CYP3A4 displayed a null (Fig. 7A, immunoblot Alc 683) or weak (Fig. 7B, Alc 0095) reaction against the rat analog CYP3A1. This CYP3A4 specificity is underscored by the fact that there is 73% structural homology between the isoforms. The high specificity of CYP3A4 autoantibodies is further supported by our finding that none of the 18 CYP3A4-positive sera recognized recombinant human CYP1A2 and CYP2C9 in Western blotting experiments (results not shown).

Recognition of CYP3A4 in Human Microsomes. To verify whether sera reactive with purified recombinant CYP3A4 were able to recognize the microsomal form of this cytochrome, immunoblotting was carried out using human liver microsomes. The CYP3A4 content in human liver microsomes was screened in ELISA using microsomes solubilized in 0.075% cholate as antigen and rabbit anti-CYP3A1 antibody (1:2000 serum dilution). Three microsomal preparations HL36, HL29, and HL20 showing A_{490} = 0.7, A_{490} = 0.83, and A_{490} = 1.4, respectively, were selected for probing with alcoholic sera in immunoblotting. To eliminate nonspecific antibody reactions during immunoblotting with patient sera, the human microsomal preparations were incubated with protein A-Sepharose before SDS-PAGE analysis. Specific immunoblot signals for CYP3A4 were detected in 4 of the 11 patients positive for expression of anti-CYP3A4 antibodies. Figure 7D shows an immunoblot of patient serum 0095, which is representative of this immunoreactivity against human liver microsomal preparations.

Reactivity of Rabbit Polyclonal Antibody and Alcoholic Sera against N-Terminal Deletions of CYP3A4 Cloned into pGEX-5X-3. Truncated his-tagged CYP3A4 fragments were constructed and expressed as GST fusion proteins in bacteria and purified: GST-Asp123CYP3A4his$_6$, GST-Thr207CYP3A4his$_6$, and GST-Ser281CYP3A4 his$_6$. The proteins were eluted from nickel-ni-nitriloacetate Sepharose columns at greater than 85% purity, according to SDS-PAGE Coomassie staining (Fig. 8A) and immunoblotting with the polyclonal rabbit anti-CYP3A1 sera (Fig. 8B). Eight CYP3A4-positive sera showing strongest immunoblot band intensities (>28 a.u./pmol; Fig. 6A), indicative of antibodies with relatively high binding affinities, were tested for their ability to recognize these fusion proteins (Fig. 8, C and D). Seven of eight sera gave reactions against all three N-terminal truncated variants of CYP3A4 as represented by Alc 4459 (Fig. 8C). Reactivity of one serum, Alc 683, was completely abrogated against CYP3A4 N-terminal deletion to position Ser281 (Fig. 8D). Treatment of the fusion construct with factor Xa released the Ser281CYP3A4 his$_6$ moiety that showed high reactivity with sera 0095 and 4459 but not reaction with serum 683 (Fig. 9). Both the uncleaved parent fusion protein and factor Xa cleavage product were detected by Alc 0095 (Fig. 9), whereas Alc 4459 showed immunoblot reactivity against the 28-kDa band of CYP3A4Ser281his$_6$ (Fig. 9) that was greater in its intensity than reactivity against the intact fusion protein (Fig. 8C, lane c), indicating that GST sequences play a role in folding of CYP3A4 and, in this individual, may impair the binding of CYP3A4 autoantibodies.

Discussion

There are, to our knowledge, no previous reports of antibody response against intact CYPs among alcoholics and patients with ALD. Our results here presented indicate that chronic administration of alcohol to rats by intragastric feeding is associated with the development of IgG reactions against CYP isozymes CYP3A1 and CYP2E1. Autoimmune reactivity toward *E. coli* recombinant human orthologs of

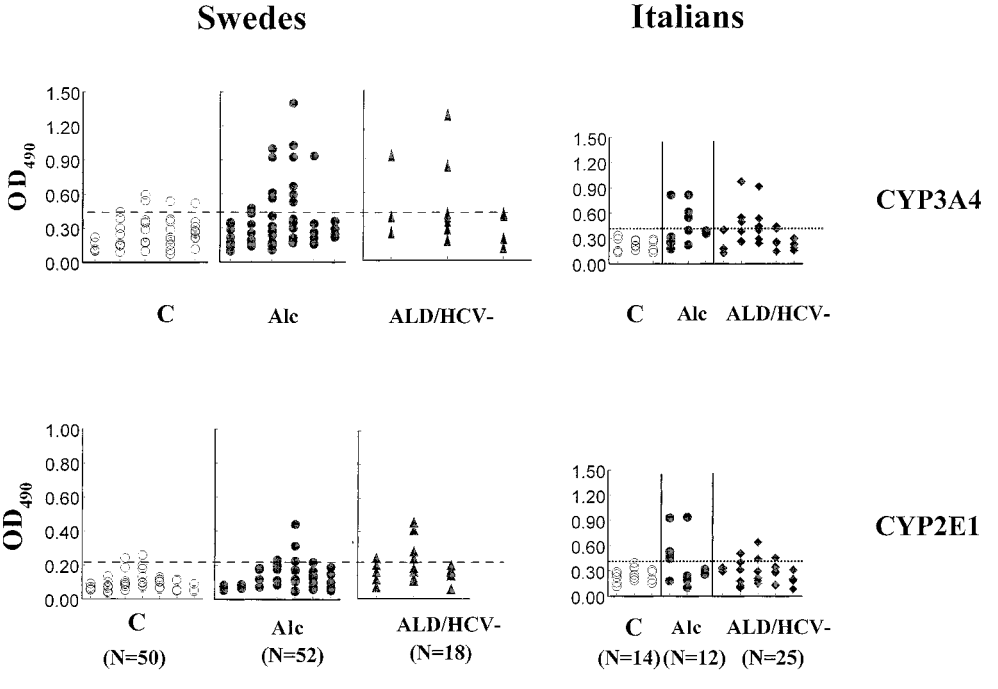

Fig. 4. Human IgG autoreactivity against recombinant human CYP3A4 (top) and CYP2E1 (bottom). Controls (C), chronic alcohol consumers (Alc), and ALD patients negative for antibodies against HCV (ALD/HCV−) from a Swedish population (left) and an Italian population (right). Sera diluted 1:300 were assayed for IgG reaction against CYP3A4 and 2E1 by microplate ELISA using human *E. coli* recombinant CYP3A4 (top) and CYP2E1 (bottom) as antigen. The values are expressed as differences in absorbances at 490 nm in ELISA plates covered with human serum albumin and, respectively, CYP3A4 and CYP2E1. The values are a.u. of means of two different determinations in duplicates. Dotted line is the cutoff taken as 2 S.D. above the mean in control group.

these CYPs was detected by both ELISA and immunoblotting using sera of subjects with alcohol abuse. It is interesting to note that although ethanol-fed rats all develop both anti-CYP3A and anti-CYP2E1 autoreactivity, the frequency of anti-CYP3A IgG is greater than that of anti-CYP2E1 IgG in humans.

It is well known that a subset of patients with HCV display immunofluorescence LKM-1 pattern that involves the presence of anti-CYP2D6 autoantibodies (Choudhuri et al., 1997) as well as other heterogeneous and not well defined microsomal antigens (Strassburg and Manns, 1995). Although HCV infection is frequent among patients with ALD, the Italian and Swedish ALD patients, showing anti-CYP3A4 and anti-CYP2E1 IgG, were negative for HCV antibodies and thus HCV-induced autoreactivity is not likely to occur. This conclusion is also relevant in the CYP autoreactivity among Swedish alcoholics in light of a recent report of absence of LKM-1 antibody reactivity in autoimmune and hepatitis C-related chronic liver disease in Sweden (Lindgren et al., 1997).

The association between alcohol intake and the development of anti-CYP antibodies is supported by the experiments with rats undergoing prolonged exposure to ethanol. These experiments demonstrate that the antibody response against CYP3A1 and CYP2E1 was highly specific because none of the sera from ethanol-fed rats showed significant binding in ELISA to rabbit serum albumin or rat CYP reductase. The absence of reactivity against CYP reductase, a membrane protein of similar topology that directly interacts with CYPs, is consistent with the hypothesis that specifically the CYPs and not other enzyme components are the physiological targets for ethanol triggering autoantibody formation against the native enzyme.

We previously demonstrated that inhibition of CYP2E1 expression during ethanol exposure strongly affected the formation of hydroxyethyl radical-derived protein adducts and decreased the levels of circulation of anti-hydroxyethyl radical IgG in the same animals (Albano et al., 1996). Consistently, anti-CYP autoreactivity in rats appears to positively correlate with the relative content of hepatic CYP2E1, whereas the treatment of ethanol-fed rats with chlormethiazole, a compound able to reduce CYP2E1 induction at the mRNA level (Hu et al. 1994), effectively prevented the antibody response to CYP2E1. The unresolved mechanistic question is how exposure to ethanol, a CYP2E1 substrate, is associated with the occurrence of anti-CYP3A autoantibodies. Sinclair and coworkers have reported that ethanol treatment is capable to induce CYP3A in both rat liver and cultured human hepatocytes (Louis et al., 1994; Kostrubsky et al., 1995). We also observed a 2-fold induction of CYP3A1 in rats undergoing intragastric ethanol feeding, but no apparent correlation was appreciable between liver CYP3A1 levels and anti-CYP3A1 autoreactivity. So far there is no evidence that CYP3A metabolizes ethanol with the formation of reactive metabolites. Interestingly, it does potentiate production of acetominophen reactive metabolites (natural autoantibodies) and liver toxicity in ethanol-fed rats (Kostrubsky et al., 1997). In addition, chlormethiazole, which inhibited the de-

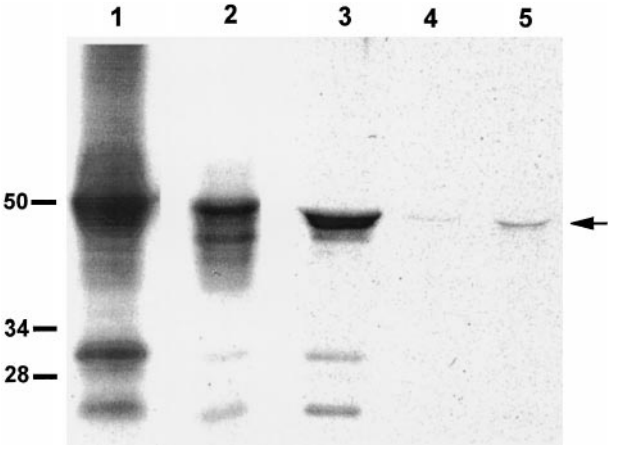

Fig. 5. SDS-PAGE and autoradiography of in vitro translated and immunoprecipitated ^{35}S-CYP3A4his$_6$. ^{35}S-CYP3A4his$_6$ was produced in vitro in a TnT T7 coupled reticulocyte lysate system, and lanes of the gel shown were loaded with 5 μl (lanes 1 and 2) or 30 μl (lanes 3–5) of sample as described in *Materials and Methods*. ^{35}S-CYP3A4his$_6$(lane 1). Immunoprecipitations of ^{35}S-CYP3A4his$_6$ with 1:200 dilution of rabbit anti-3A1 (lane 2) and 1:100 dilution of human sera: alcoholic serum 4885 (lane 3) and control sera (C 104 and C 841) (lanes 4 and 5). The autoradiographs shown are after 48 h of exposure.

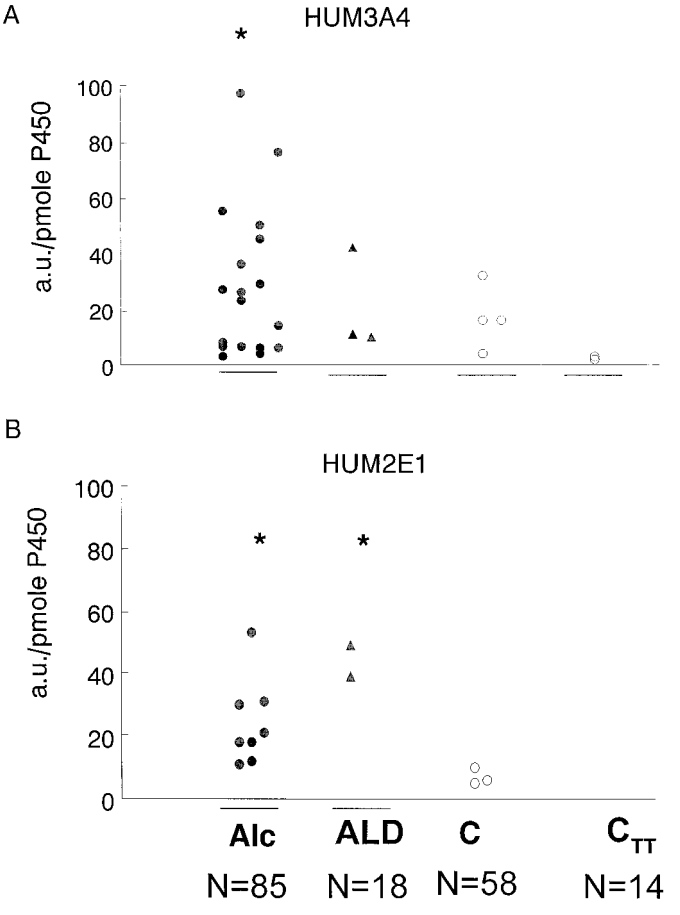

Fig. 6. Expression of anti-CYP3A4 and anti-human CYP2E1 antibodies in the sera of alcoholic patients. Binding of human antibodies (serum dilution 1:600) to 2.5 pmol of human CYP3A4 (A) and CYP2E1 (B) was investigated in ECL-Western blotting of 85 alcoholics (Alc), 18ALD patients (ALD), 58 social drinking control subjects (C), and 14 individuals of alcohol abstinence (C_{TT}) from Swedish population. The reactivity was quantified by laser scanning of the autoradiographs and densitometric analysis using Molecular Dynamics ImageQuant software version 3.2. Reactivity between control subjects (○) and alcoholics (●) is statistically significant at $*p < .05$, Student's unpaired t test. The CDT values were 15 ± 4 U/liter (range, 7–25 U/liter) for control subjects and 63 ± 9 U/liter (range, 40–103 U/liter) for alcoholics.

velopment of anti-CYP2E1 IgG in ethanol-treated rats, does not significantly affect anti-CYP3A IgG, indicating that the mechanisms leading to autoimmune response to CYP3A are different from those involving CYP2E1.

In the case of CYP3A4 autoantibodies, further studies are needed to determine whether there is a subpopulation of individuals who express natural autoantibodies against CYP3A and, if so, whether they show enhanced production or increased binding affinity as a result of chronic ethanol exposure. Natural autoantibodies, usually of low-affinity IgM but also IgG derived, circulate in sera of healthy individuals and the elderly (Dighiero, 1997) and have been found with 6.7% positivity for anti-microsomal antibodies (George and Shoenfeld, 1996). This incidence is similar to the IgG reactions against CYP3A4 among social drinking control subjects (6.9% positivity).

Autoimmune reaction against CYP3A isoenzyme has been reported by Leeder et al., (1996) in patients with drug-induced hypersensitivity. However, these patients expressed antibodies with preferential recognition of rat CYP3A1 but not human CYP3A4. Interestingly, despite the high amino acid sequence homology between CYP3A1 and CYP3A4 (72%), the sera that exhibit anti-CYP3A4 reactivity show a weak or null reaction against CYP3A1, whereas the sera that showed high anti-CYP3A1 reactivity did not recognize CYP3A4 in Western blotting or ELISA. At the moment, no explanation can be given for the high frequency of immune reactivity against rat CYP3A1, but the possibility of molecular mimicry with antigen of non-HCV infectious origin cannot be excluded.

Because the anti-CYP3A4 reactivity of alcoholics is directed almost exclusively toward CYP3A4 in SDS-PAGE

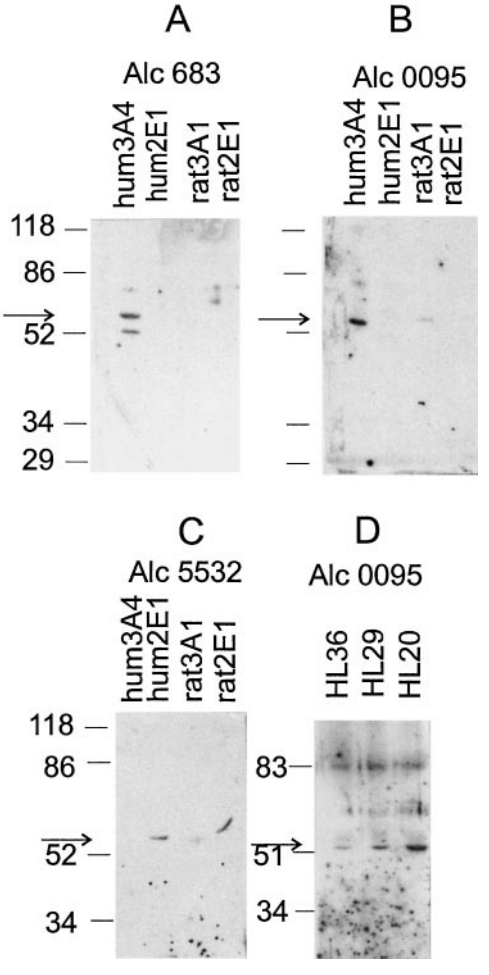

Fig. 7. Immunoblots of rat and human CYP3A and CYP2E1 with patient sera. Preparations of rat and human CYPs were loaded at 2.5 pmol/lane (A–C). Human liver microsomal preparations HL 20, HL29, and HL36 were preabsorbed with protein A-Sepharose, loaded at 12 μg/lane, and immunoblotted with alcoholic sera 0095 (D). Immunoblots were made with sera from the alcoholics indicated at 1:600 dilution in Tris-buffered saline. The ECL reactions were developed 1 min on the same strip of Kodak X-Omat photographic film (A–C) or 2 min on a separate strip (D).

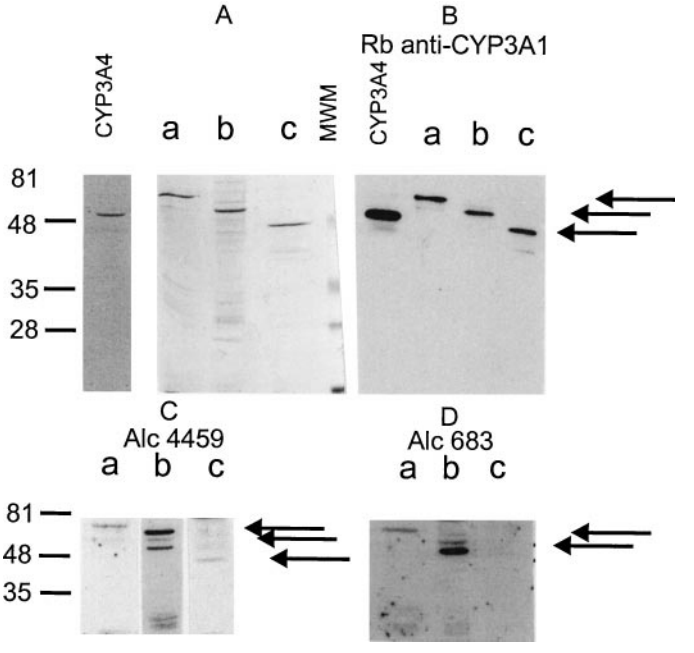

Fig. 8. Recognition of GST-CYP3A4 fusion proteins by polyclonal rabbit CYP3A4 antibodies and anti-CYP3A4 alcoholic IgG. A, SDS-PAGE Coomassie stain of purified commercial recombinant CYP3A4 at 150 μg/lane and fusion proteins GST-Asp^{123}CYP3A4his$_6$ (lane a), GST-Thr^{207}CYP3A4his$_6$ (lane b), and GST-Ser^{281}CYP3A4 his$_6$ (lane c) at 70 μg/lane. B, immunoblots reacted with anti-CYP3A1 polyclonal rabbit antibodies diluted 1:2500 and protein A. C and D, immunoblots reacted with indicated alcoholic sera (Alc) diluted 1:600 and anti-human IgG Fc diluted 1:20,000 as described in *Materials and Methods*.

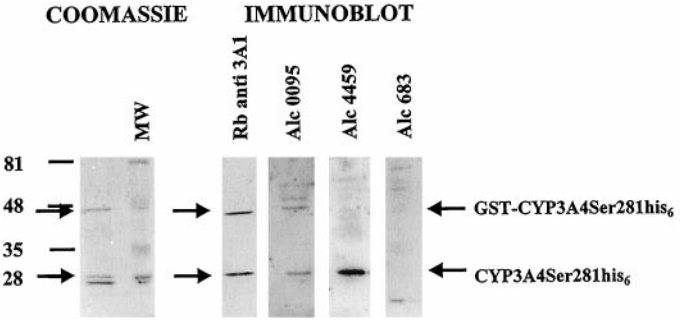

Fig. 9. Recognition of CYP3A4 C-terminal fragment by polyclonal rabbit antibodies and anti-CYP3A4 alcoholic IgG. SDS-PAGE Coomassie stain (lane 1) and immunoblots (lanes 3–6) of factor Xa cleavage of GST-CYP3A4Ser^{281} his$_6$. Position of molecular weight markers (lane 2). Immunoblots reacted with anti-CYP3A1 polyclonal rabbit antibodies (lane 3) and alcoholic patient sera: Alc 0095 (lane 4), Alc 4459 (lane 5), and Alc 683 (lane 6) as described in the legend to Fig. 8. The position of uncleaved fusion protein (top arrow) and factor Xa cleavage product (bottom arrow) are shown.

Western blots, with weak or no cross reactions to CYP3A1, it is highly likely that the dominant CYP3A4 epitopes for recognition by alcoholic autoantibodies are short linear peptide fragments derived from domains of sequence disparity. However, we cannot rule out local conformational epitopes that renature in conditions of SDS-PAGE immunoblotting as demonstrated by Beaune and colleagues in studies of CYP2C9 (Lecoeur et al., 1996) and CYP1A2 (Belloc et al., 1997) fusion proteins.

Because CYP3A4 has not previously been defined as an autoantigen and could express unique target sequences of immune recognition, we set out to identify the domains that are recognized by alcoholic anti-CYP3A4 autoantibodies using GST fusions containing N-terminal truncated forms of CYP3A4. Sera of eight CYP3A4-positive alcoholics were chosen for immunoblotting against the fusion proteins because they gave highest band intensities against the full-length recombinant protein and would be expected to retain their binding to CYP3A4 if the specific epitopes they recognized are presented within the C-terminal domains. In the current study, we report that CYP3A4 C-terminal domain harbors the major autoantigenic determinants recognized by alcoholics. One of the patient sera, Alc 683, retained immunoreactivity against GST-CYP3A4Asp123 his_6 and GST-CYP3A4Thr207 his_6 but showed complete loss of reactivity against GST-CYP3A4Ser281 his_6. However, SDS-PAGE immunoreactivity of human sera against GST sequences was controlled for by examining immunoblots against cleavage product CYP3A4Ser281 his_6, which migrated as a distinct band of 28 kDa above the position of GST.

Although no crystallographic data are available for eukaryotic CYPs, alignment of the regions concerned with bacterial P-450_{terp}, P-450_{cam}, and P-450_{BM-3} (Hasemann et al., 1995) suggests that the largest clusters of sequence disparity between CYP3A1 and CYP3A4 reside in the G and H helices, which appear to be largely solvent accessible in molecular modeling studies. Interestingly, the region Thr207-Ser281 contains three clusters of contiguous CYP3A4-specific sequences that have high disparity with CYP3A1: ^{216}LDPFLSITV, ^{243}REVTNFLRKSVK, and ^{276}IDSQNSKDET (Fig. 10) corresponding to the G helix. Basic local alignment search tool (BLAST) of the NCBI data base produced alignment of 90% positive and 8 of 10 amino acid identity between CYP3A4-specific sequence EVTNFLRKSV and a two-component sensor histidine kinase of *Bacillus subtilis*.

This cursory comparison of primary sequence information might signify a novel structure and location for epitopes recognized by alcoholic autoantibodies against CYP3A4 and molecular mimicry with sequences of bacteria and agents of infectious origin. The J, K, and L helices have been implicated as sites of conformational epitope in CYP2C11 tienilic acid-induced autoimmune hepatitis (Lecoeur et al., 1996), and the K helix of CYP3A1 was found as a major epitope recognized by autoantibodies of patients with hypersensitivity reactions to aromatic anticonvulsing drugs (Leeder et al., 1996). On this basis, it should be considered that the CYP3A4 epitopes recognized by autoantibodies from alcoholics do not necessarily correspond to highly conserved sequences in the neighborhood of the heme. Therefore, other structures unrelated to substrate binding and recruitment are potential candidates for triggering immune response of alcoholics toward CYP autoantibody formation.

In conclusion, our study indicates the formation of autoimmune reactivity against native CYP2E1 and CYP3A in alcoholics and in rats treated chronically with ethanol where the 3A4 reactivity was directed against the C-terminal region.

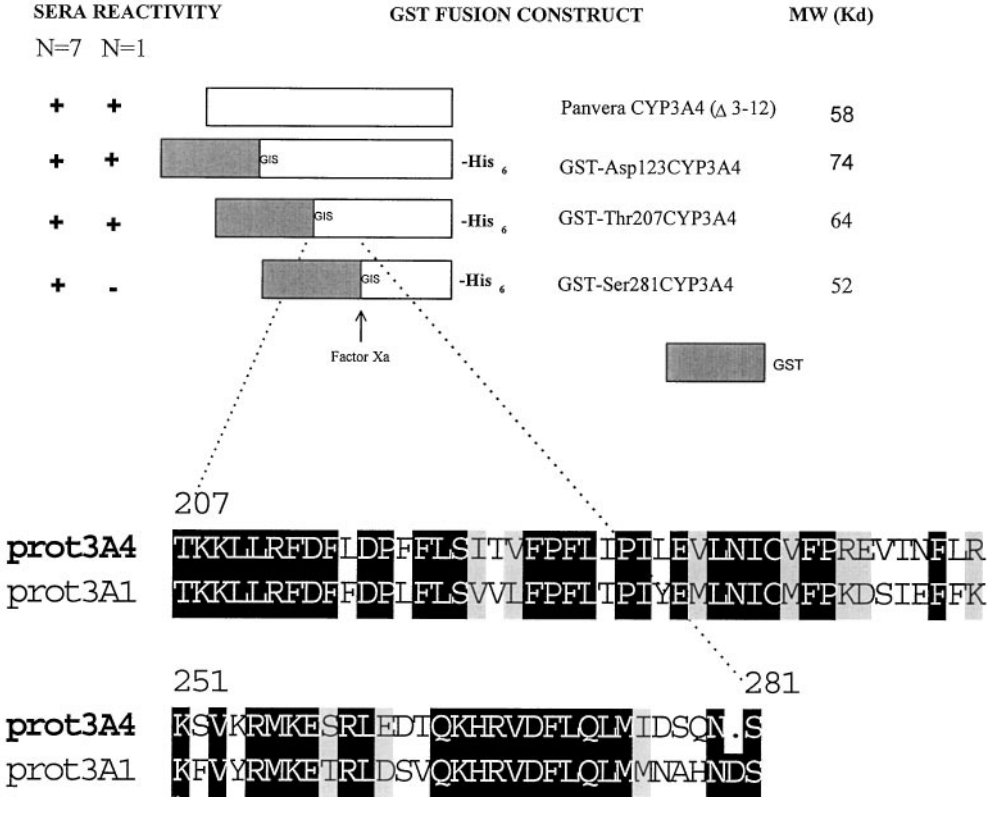

Fig. 10. Strategy for identification of CYP3A4 autoepitope domains. A, schematic representation of sera reactivity of alcoholics (n = 8) against the commercially available full-length recombinant CYP3A4 (Panvera) and fusion proteins. GST (shaded box) and hexa his-tagged CYP3A4 fragments of different length N-terminal deletions (white box). The addition of amino acids glycine-isoleucine-serine (GIS) are expressed after the factor Xa cleavage site (arrow) at N terminus of CYP3A4 sequence. B, sequence alignment of Thr^{207} to Ser^{281} with Wisconsin GCG PILEUP software, showing sequences of amino acid identity (black), homology (gray), and disparity (white).

The formation of the CYP2E1 antibodies appears to be connected with ethanol metabolism by this enzyme, whereas the etiology of anti-CYP3A autoreactivity requires further investigations.

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