# Genetic and Epigenetic Factors in Autoimmune Reactions Toward Cytochrome P4502E1 in Alcoholic Liver Disease

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**Autoimmune reactions are often associated with alcoholic liver disease; however, the mechanisms responsible are largely unknown. This study investigates the potential role of the immune response against hydroxyethyl free radical (HER)-derived antigens and of polymorphisms in immunoregulatory genes in the development of anti-cytochrome P4502E1 (CYP2E1) autoantibodies in alcohol abusers. Immunoglobulin G (IgG) recognizing human CYP2E1 and HER-derived epitopes were measured by microplate immunosorbent assay in the sera of 90 patients with alcoholic fibrosis/cirrhosis (ALD), 37 heavy drinkers without liver disease or steatosis only (HD), and 59 healthy subjects. Single nucleotide polymorphisms in the interleukin 10 (IL-10) promoter and in exon 1 of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene were genotyped by polymerase chain reaction-restriction fragment length polymorphism analysis. The titers and frequency of anti-CYP2E1 autoantibodies were significantly higher in ALD than in HD subjects or controls. ALD patients with anti-HER IgG had higher titers and a 4-fold increased risk (OR: 4.4 [1.8-10.9]) of developing anti-CYP2E1 autoantibodies than subjects without anti-HER antibodies. The mutant CTLA-4 G allele, but not the IL-10 polymorphism, was associated with an enhanced risk of developing anti-CYP2E1 IgG (OR: 3.8 [1.4-10.3]). CTLA-4 polymorphism did not influence antibody formation toward HER-antigens. ALD patients with concomitant anti-HER IgG and the CTLA-4 G allele had a 22-fold higher (OR: 22.9 [4.2-125.6]) risk of developing anti-CYP2E1 autoreactivity than subjects negative for these factors. In conclusion, antigenic stimulation by HER-modified CYP2E1 combined with an impaired control of T-cell proliferation by CTLA-4 mutation promotes the development of anti-CYP2E1 autoantibodies that might contribute to alcohol-induced liver injury.** (HEPATOLOGY 2003;37:410-419.)

Autoimmune reactions are frequently observed in patients with alcoholic liver disease (ALD).[1,2] Autoantibodies directed toward alcohol dehydrogenase, hepatic asialoglycoprotein receptor, heat shock protein 65, and phospholipids are present in 25% to 50% of patients with alcoholic hepatitis or cirrhosis.[2,3] However, little is known about the mechanisms responsible for the breaking of self-tolerance associated with alcohol-related liver damage. Some clues may come from our recent work showing that oxidative modifications of cellular phospholipids might be involved in the development of antiphospholipid antibodies seen in patients with alcoholic cirrhosis.[4] Furthermore, the immunization of animals with proteins modified by malondialdehyde-acetaldehyde adducts has been shown to promote the development of antibodies toward the native carrier proteins.[5]

Recently, Lytton et al.[6] reported that chronic intragastric ethanol-fed rats developed circulating immunoglobulin G (IgG) directed against cytochrome P450 (CYP) isoenzymes CYP2E1 and CYP3A. The titers of anti-CYP2E1 but not those of anti-CYP3A autoantibodies were associated with the severity of alcohol liver damage,

*Abbreviations: ALD, alcoholic liver disease; IgG, immunoglobulin G; CYP2E1, cytochrome P450 2E1; HER, hydroxyethyl free radical; IL-10, interleukin 10; CTLA-4, cytotoxic T lymphocyte antigen-4; Th, T-helper; HD, heavy drinkers without advanced liver damage; ELISA, enzyme-linked immunoabsorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; o.d., optical density.*

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and the inhibition of CYP2E1-mediated ethanol metabolism by chlormethiazole prevented both liver injury and anti-CYP2E1 auto-reactivity.[6] The potential role of an autoimmune response toward CYP2E1 in the pathogenesis of alcohol liver damage in humans is suggested by the detection of anti-CYP2E1 IgG in the sera of heavy drinkers and the demonstration that CYP2E1 is present on the surface of both rat and human hepatocytes.[6-8]

If the anti-CYP2E1 response might be involved in the pathogenesis of ALD, understanding the mechanisms leading to its generation assumes great importance. It has been postulated that structural modifications of self macromolecules by reactive drug metabolites can represent one of the mechanisms triggering autoimmune reactions.[9] Indeed, protein fragments modified by drug metabolites have been shown to induce T-cell clones recognizing as "nonself," short linear peptides derived from the native unmodified protein. In turn, these T lymphocytes are capable of activating B lymphocytes to produce antibodies directed against both drug-modified and nonmodified proteins.[9,10] This mechanism potentially explains the concomitant presence of antibodies recognizing trifluoroacetyl-CYP2E1 and tienilic acid-CYP2C9 adducts along with autoantibodies against conformational CYP2E1 and CYP2C9 epitopes in patients with halothane and tienilic acid-induced hepatitis, respectively.[11,12] We have recently reported that hydroxyethyl free radicals (HER), arising during CYP2E1-mediated ethanol metabolism, alkylate CYP2E1 and that HER-modified CYP2E1 is the main antigen recognized by anti-HER antibodies present in patients with alcoholic cirrhosis.[13] It therefore seems possible that an immune response to HER-CYP2E1 may be one mechanism leading to the development of autoantibodies directed toward epitopes in native CYP2E1.

Recent evidence suggests that genetic polymorphisms in a variety of immunoregulatory cytokines, including interleukin 1$\alpha$ (IL-1$\alpha$), IL-1$\beta$, IL-4, and IL-10, and their receptors might favor tolerance breakdown during autoimmune diseases and atopic reactions.[14-17] Moreover, primary biliary cirrhosis,[18,19] type 1 autoimmune hepatitis,[20] Graves' disease, and type 1 diabetes mellitus[21,22] have all been linked to the presence of an A→G base exchange at position 49 in exon 1 of the gene encoding cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), a cell surface molecule implicated in the control of T-cell responses to antigenic stimulation.[23] CTLA-4 is a CD28 homologue constitutively expressed on the recently described $CD25^+CD4^+$ regulatory cells and on activated T cells; the ligation of which results in an attenuation of T-cell-mediated immune responses.[23] This effect may be a result of CTLA-4 down-regulating the T-cell activation threshold or modulating the T-cell expansion after activation.[24] The immunosuppressor function of CTLA-4 is confirmed by observations in CTLA-4 knockout mice showing an expansion of $CD4^+$ and $CD8^+$ T lymphocytes and of B-cell clones in conjunction with a 10- to 100-fold increase in IgG polyclonal production leading to lethal autoreactive diseases.[25] Recent *in vitro* data have shown that the G allele of the exon 1 polymorphism is associated with a diminution of the normal inhibitory influence of CTLA-4 on T-cell responses.[22] We have previously reported associations between advanced ALD and this CTLA-4 polymorphism as well as with a "low activity" polymorphism in the promoter region of the IL-10 gene.[26,27] IL-10 also down-regulates immune responses by inhibiting antigen-specific activation of Th lymphocytes through the reduction of the surface expression of class II MHC and B7 molecules on antigen presenting cells.[28] The C→A substitution at position −627 in combination with an A→G transition at position −1,117 in the 5′ flanking region of the IL-10 gene identifies a haplotype associated with decreased secretion of IL-10 by $CD4^+$ T-helper (Th) lymphocytes, monocytes, and Kupffer cells.[16] The association with ALD may be explained, at least in part, by this low secretion/proimmune phenotype.

The first aim of the present study was to seek further support for a role of the anti-CYP2E1 immune response in the pathogenesis of alcohol liver injury by determining the prevalence of anti-CYP2E1 autoantibodies in heavy drinkers with and without liver disease. Our second aim was to characterize the mechanisms involved in the induction of this potentially injurious immune response. To this end, we determined whether the anti-CYP2E1 antibody response correlated with the following: (1) the antibody response against HER-derived antigens and/or (2) the presence of functional polymorphisms in the genes encoding IL-10 and CTLA-4 previously associated with advanced ALD.

## Patients and Methods

***Patients and Control Subject Recruitment.*** For this study, 127 heavy drinkers with an alcohol consumption above 8 drinks a day (80 g ethanol) for more than 10 years were recruited by the Centre for Liver Research at the University of Newcastle upon Tyne (United Kingdom). All subjects were white and originated in the Northeastern United Kingdom as did their parents and grandparents. Lifetime cumulative alcohol intake was determined in 81 patients by structured interview administered by a Specialist Nurse as described previously.[29] The presence and severity of ALD was assessed initially by standard liver blood tests. If the alanine transaminase, alkaline phospha-

tase, or bilirubin were more than twice normal on 2 separate occasions within a 6-month period, an ultrasound scan was also arranged and a liver biopsy performed in patients without biliary dilatation or coagulopathy. On the basis of these investigations, drinkers were categorized either as having advanced ALD (clinical evidence of decompensation or steatohepatitis and/or fibrosis on biopsy; ALD group) or as having no evidence of ALD or simple steatosis (HD group). For inclusion into the HD group, patients had to be actively drinking, have no clinical evidence of liver disease, and have either normal liver blood tests on two occasions or liver histology showing normal liver or steatosis only. Exclusion criteria were the following: serologic evidence of previous HBV or HCV infection, excessive liver iron staining, or homozygosity for the C282Y mutation in the HFE gene. Ninety patients were recruited into the ALD group (85 underwent biopsy, 70 cirrhotic, 15 fibrotic, 67 men; mean age 48 ± 9 years, range 31 to 73 years) and 37 into the HD group (23 underwent biopsy, 31 men; mean age 45 ± 11 years, range 33 to 69 years). Fifty-nine healthy controls (38 men, 21 women; mean age 47 ± 10 years, range 34 to 68 years) originating from the same geographic area were recruited from hospital and university staff. All the controls were drinking within the WHO guidelines for sensible limits (21 drinks per week for men and 15 drinks per week for women). All subjects gave informed consent to the analysis, and the study was planned according to the guidelines of the local ethical committee. Blood samples (5 mL) were taken after an overnight fast for the preparation of DNA and serum. All groups were abstinent from alcohol for at least 24 hours prior to the blood sample being taken.

***Determination of IL-10 and CTLA-4 Genotypes.*** Genotyping for 2 of the upstream IL-10 polymorphisms (at positions −627 and −1,117) and the CTLA-4 exon 1 polymorphism was performed on DNA extracted from peripheral blood leukocytes according to previously described methods.[20,27] Briefly, 0.6 μmol/L of the primers 5′-GGTGAGCACTACCT-3′ (sense), 5′-CCTAGGTCACAGTGACGTGG-3′ (antisense) for a 412-bp fragment containing the −627 IL-10 polymorphism; 5′-CCAGAGACTTTCCACATATCT-GAAGAAG-3′ (sense), 5′-AAGCTTCTGTGGCTGGAGTC-3′ (antisense) for a 321-bp fragment containing the −1,117 IL-10 polymorphism; and 5-CCACGGCTTCCTTTCTCGTA-3′ (sense) and 5′AGTCTCACTCACCTTTGCAG-3′ (antisense) for a 328-bp fragment of the first exon of the CTLA-4 gene containing the polymorphic site were used in polymerase chain reaction amplifications. Ten microliters of each amplicon were digested with, respectively, *Rsa*I endonuclease for the −627 C→A IL-10 promoter polymorphisms or *Bst*711 endonuclease (isochizomer of Bbv 1) (Bioline, London, United Kingdom) for the A→G CTLA-4 polymorphism as previously reported.[20,27] DNA fragments were separated by electrophoresis on a 2% agarose gel and revealed by ethidium bromide staining and ultraviolet transillumination. Positive and negative controls were included in each batch. The −1,117 genotype of IL-10 was determined using single strand conformational polymorphism analysis of polymerase chain reaction products on a 1× MDE gel as previously reported.[27]

***Measurement of Anti-CYP2E1 Autoantibodies Titers.*** Polystyrene microwell plates for enzyme-linked immunosorbent assay (ELISA) (Nunc-Immuno Poly-Sorp, Nunc, S/A, Roskilde, Denmark) were used for autoantibody detection. Half of each plate was coated by 4-hour incubation at 37°C with 300 ng of purified recombinant human CYP2E1 (Oxford Biochemicals Inc., Oxford, MI) solubilized in 0.1 mol/L phosphate buffer, pH 7.4. The remaining wells were coated with 300 ng of purified bovine serum albumin (BSA) dissolved in the same buffer. After incubation, solutions were removed, and nonspecific binding sites were blocked by 1-hour incubation with 0.3 mL of coating buffer containing 3% (vol/vol) BSA in phosphate buffered saline (PBS), pH 7.4. The coated wells were washed 3 times with PBS containing 0.25% Triton X-100. Patients sera (0.2 mL diluted 1:50 in coating buffer) were added in duplicate to the wells containing CYP2E1 or BSA, and the plates were incubated for 1 hour at 37°C. After 3 washes with PBS-0.25% Triton X-100 peroxidase-linked goat anti-human IgG serum (dilution 1:6,000; Dako S.p.A., Milano, Italy) was added and incubated for 60 minutes at 37°C. Antibody binding was revealed by a reaction mixture containing 0.4 mg/mL 1-phenylendiamine, 0.4 μl/mL hydrogen peroxide (30%), 5.1 mg/mL citric acid, and 6.1 mg/mL $Na_2HPO_4$ (anhydrous), pH 5.0, and absorbances were measured at 490 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The results were expressed as optical density (o.d.) at 490 nm after subtracting the background reactivity of the sera tested in the wells containing BSA alone.

***Determination of Immune Response Against Hydroxyethyl Radical-Derived Antigens.*** Hydroxyethyl radical adducts with BSA (HER-BSA) were prepared by reacting for 30 minutes at 25°C BSA (1 mg/mL solution in PBS, pH 7.4) with 1 mg of freshly prepared 1,1′-dihydroxyazoethane crystals, synthesized as described by Stoyanovsky et al.[30] HER-modified BSA was filtered through disposable desalting columns (Econo-Pac

10DG; Bio-Rad Laboratories Inc., Richmond, CA) and 0.3 mg of HER-conjugated or native BSA solubilized in 0.1 mol/L bicarbonate buffer, pH 9.6, was used to coat Nunc-Immuno MaxiSorp IV microwell ELISA plates (Nunc, S/A, Roskilde, Denmark). The blocking of nonspecific binding sites and the detection of antibodies recognizing HER-derived epitopes was performed as described above. The results were expressed as o.d. at 490 nm after subtracting the background reactivity in the wells containing unmodified BSA.

***Data Analysis and Statistical Calculations.*** Statistical analysis was performed using the Instat-3 statistical software (GraphPad Software Inc, San Diego, CA) using 1-way ANOVA. The degree to which the data were normally distributed in the groups was preliminarily evaluated by Kolmogorov and Smirnov test. Significance was taken at the 5% level. Odds ratio (OR) with 95% confidence intervals (CI) and $\chi^2$ tests were used for risk and frequency analysis. Difference of 2 proportion test with 95% CI was also performed.

## Results

The prevalence of autoantibodies against human recombinant cytochrome P4502E1 (CYP2E1) was investigated in 2 groups of heavy drinkers with either advanced ALD or without clinical evidence of liver damage or simple steatosis (HD). Figure 1 shows that IgG reactivity against human CYP2E1 was not different between HD (o.d.$_{490nm}$ 0.519 ± 0.119) and control groups (o.d.$_{490nm}$ 0.474 ± 0.176). Conversely, a significant increase ($P <$ .001) in the titers of anti-CYP2E1 autoantibodies was appreciable in ALD patients (o.d.$_{490nm}$ 0.773 ± 0.353). Furthermore, anti-CYP2E1 IgG titers above the 95th percentile of the controls were seen in 36 of 90 (40%) ALD but only in 4 of 37 (11%) HD subjects. Distribution analysis of anti-CYP2E1 autoreactivity confirmed these findings by showing a similar distribution profile in HD and control groups, whereas ALD patients displayed a distribution profile much broader and shifted to higher values (Fig. 1). In the 81 patients (65 ALD and 16 HD) in whom cumulative lifetime alcohol intake was available, no difference in alcohol consumption (1,540 ± 1,741 kg in ALD vs. 1,940 ± 930 kg in HD) was appreciable. Furthermore, there was no correlation between the titers of anti-CYP2E1 autoantibody and lifetime alcohol consumption. In the ALD patients, anti-CYP2E1 IgG titers were not associated with either serum bilirubin or prothrombin time, and neither of these autoantibodies were significantly different between patients with cirrhosis (n = 75) and those with fibrosis only (o.d.$_{490nm}$ 0.782 ± 0.364 vs. 0.654 ± 0.254).

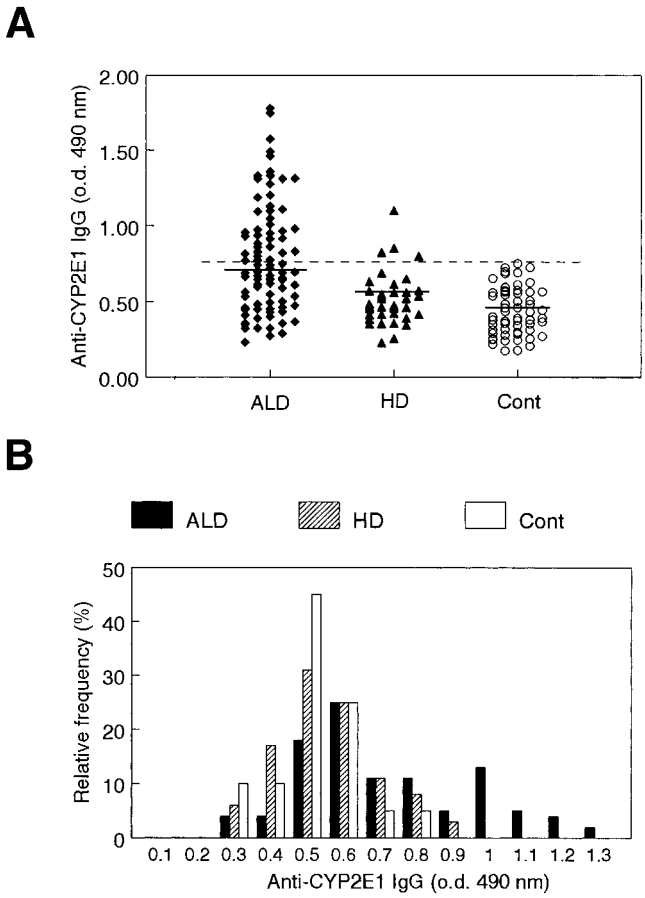



Fig. 1. Titers (A) and frequency distribution (B) of antibodies against recombinant human cytochrome P4502E1 (CYP2E1) in 90 patients with advanced alcoholic liver disease (ALD), 37 heavy drinkers without liver damage or with steatosis only (HD), and 59 healthy controls. Human sera were tested at 1:50 dilution in microplate ELISA plates coated with recombinant human CYP2E1 and revealed with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as optical density (o.d.) at 490 nm after subtracting the background reactivity of each serum. **Horizontal bars** represent the median values of each group. **Dotted lines** show the cut-off value calculated on the 95th percentile of the control population. The values were normally distributed as evaluated by Kolmogorov and Smirnov test.

Previous studies have shown that CYP2E1 is the main liver protein recognized by the antibodies directed against HER present in the sera of alcohol abusers.[13] To avoid interference by the carrier protein, the role of the immunization against HER-derived epitopes in the development of anti-CYP2E1 autoreactivity was investigated using as antigen HER adducted to bovine serum albumin. As shown in Fig. 2, the titers of anti-HER IgG were significantly higher in ALD (o.d.$_{490nm}$ 0.147 ± 0.126) and HD (o.d.$_{490nm}$ 0.132 ± 0.065) groups as compared with controls (o.d.$_{490nm}$ 0.080 ± 0.035; $P <$ .0005). Anti-HER IgG titers above the 95th percentile of the control group were detectable in 34 of 90 (38%) ALD and in 8 of

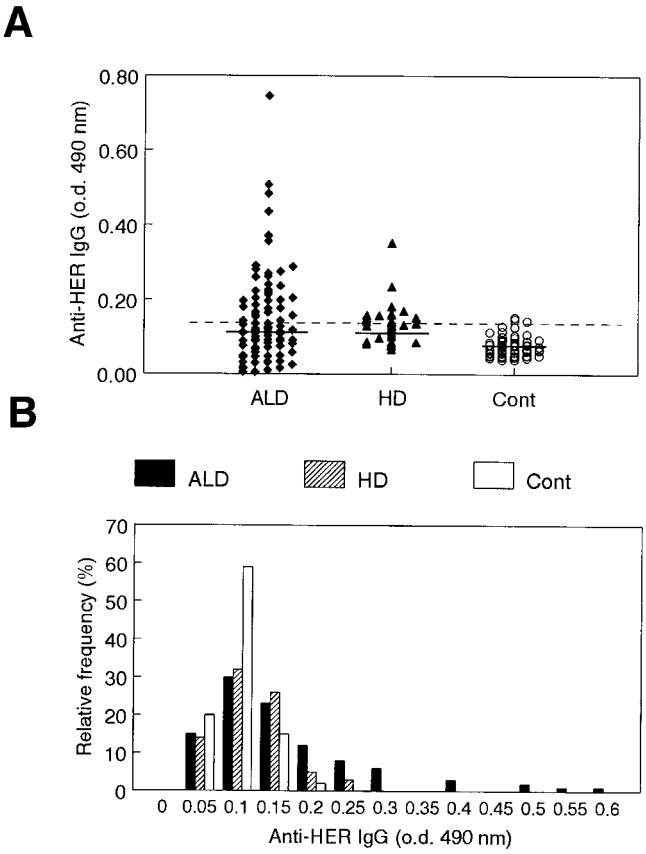
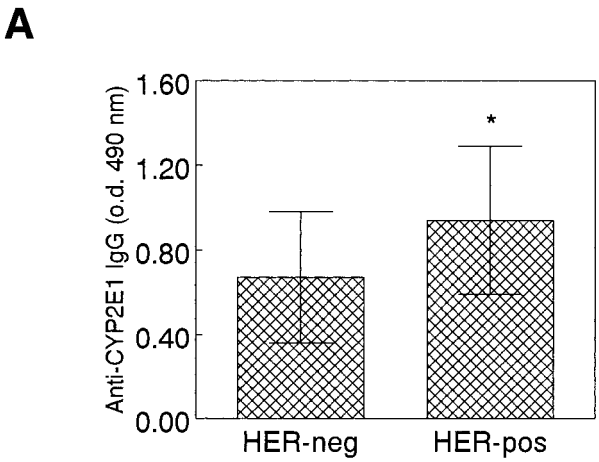



Fig. 2. Titers (A) and frequency distribution (B) of antibodies against epitopes derived from the binding of hydroxyethyl free radical (HER) to proteins in 90 patients with advanced alcoholic liver disease (ALD), 37 heavy drinkers without liver damage or with steatosis only (HD), and 59 healthy controls. Human sera were tested at 1:50 dilution in microplate ELISA plates coated with HER-modified bovine serum albumin (BSA) and revealed with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as optical density (o.d.) at 490 nm after subtracting the background reactivity of each serum against unmodified BSA. **Horizontal bars** represent the median values of each group. **Dotted lines** show the cut-off value calculated on the 95th percentile of the control population. The values were normally distributed as evaluated by Kolmogorov and Smirnov test.

37 (22%) HD subjects. When ALD patients were divided according to the presence of anti-HER antibodies, those displaying anti-HER IgG had higher titers ($P < .001$) and a 4-fold increased risk (OR 4.4; CI 1.8-10.9; $P = .002$) of developing anti-CYP2E1 autoantibodies than subjects without anti-HER immunity (Fig. 3). However, the immune response against HER epitopes did not influence the presence of autoantibodies toward CYP2E1 in heavy drinkers without liver disease ($o.d._{490nm}$ 0.573 ± 0.152 in anti-HER+ve vs. 0.473 ± 0.123 in anti-HER−ve; $P = .12$; OR 2.36; CI 0.31-17.8; $P$ = NS). This suggests that the immune response toward epitopes originating from CYP2E1 modification by HER is one of the factors contributing to the development of anti-CYP2E1 autoreactivity in patients with severe alcohol liver injury.

The possible influence of genetic factors in the development of alcohol-induced alloimmune and autoimmune responses was investigated by genotyping the patients for the −627 (C→A) and −1,117 (G→A) upstream IL-10 polymorphisms and the A→G (Thr→Ala) polymorphism in exon 1 of the CTLA-4 gene. As shown in Table 1, these polymorphisms were common in patients with ALD, and the distribution of the genotypes in the patients was in Hardy-Weinberg equilibrium. As we have previously reported,[26] the proportion of patients carrying at least 1 copy of the CTLA-4 G allele was greater in the ALD population (63%) than in the HD population (49%). By grouping the patients according to the presence or absence of each polymorphism, we observed that the titers of anti-CYP2E1 IgG (Fig. 4) and the frequency of positive for these autoantibodies (Table 2) did not dif-




B

| | HER+ | HER- | Total |
|---|---|---|---|
| CYP2E1+ | 21 (23%) | 15 (17%) | 36 (40%) |
| CYP2E1- | 13 (14%) | 41 (46%) | 54 (60%) |
| Total | 34 (37%) | 56 (63%) | 90 |

Fig. 3. Effect of immune response against epitopes derived from hydroxyethyl free radicals (HER) on the titers (A) and frequency distribution (B) of anti-CYP2E1 autoantibodies in patients with advanced alcoholic liver disease. (A) Values are expressed as mean ± SD optical density (o.d.) at 490 nm obtained after subtracting the background reactivity of each sera. Statistical significance: *$P < .001$ vs. HER negative. (B) CYP2E1+ and HER+ designate IgG titers above the 95th percentile in the control population. Odds ratio for possession of anti-CYP2E1 autoantibodies in patients with anti-HER antibodies vs. those without: 4.4 (1.8-10.9), $P = .001$.

**Table 1. Distribution of Polymorphisms in the Interleukin 10 (IL-10) and Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) Genes in Patients With Advanced Alcoholic Liver Disease**

| Polymorphisms | WW (%) | WM (%) | MM (%) |
|---|---|---|---|
| IL-10 (−627 C→A) | 42 (47) | 41 (45) | 7 (8) |
| IL-10 (−1,117 G→A) | 21 (23) | 44 (49) | 25 (28) |
| CTLA-4 (49 A→G) | 33 (37) | 47 (52) | 10 (11) |

NOTE. Figures show the number of subjects carrying 1 or 2 copies of wild-type (W) or mutant (M) alleles of the IL-10 and CTLA-4 genes.

fer according to IL-10 genotype. In contrast, anti-CYP2E1 autoreactivity was significantly ($P < .05$) increased in ALD patients possessing at least 1 copy of the CTLA-4 G allele compared with A/A homozygotes (Fig. 4). These patients were also more likely than A/A homozygotes to have anti-CYP2E1 IgG titers above the 95th percentile of the control group (OR 3.8; [1.4-10.3]; $P = .011$) (Table 2). These differences could not be accounted for by differences in circulating IgG between ALD patients with and without the CTLA-4 G allele (13.25 ± 5.67 g/L vs. 14.05 ± 5.43 g/L; $P = .71$). G/G homozygotes had higher anti-CYP2E1 reactivity than heterozygotes ($o.d._{490nm}$ 0.991 ± 0.416 vs. 0.833 ± 0.357; $P = .29$). However, this difference was not statistically significant, possibly because of the low number of homozygotes (n = 10). Furthermore, despite that CTLA polymorphism was also detectable in 49% of HD subjects, no difference in anti-CYP2E1 autoreactivity was evident between those with and those without the G allele ($o.d._{490nm}$ 0.548 ± 0.111 vs. 0.520 ± 0.245; $P = .65$). Among ALD patients included in this study, 44 of 90 (49%) possessed the CTLA-4 G allele in combination with 1 or both of the 2 IL-10 polymorphisms. However, the combined presence of these polymorphisms did not significantly influence either the titers or the frequency of anti-CYP2E1 IgG (data not shown). Interestingly, none of the genetic polymorphisms investigated had any effect on the development of IgG toward HER-derived epitopes (Table 2 and Fig. 4), suggesting a specific role for CTLA-4 in the maintenance of self tolerance with less influence on the immune response to neoantigens.

To evaluate a possible interaction between CTLA-4 genotype and the immune stimulation by HER-derived antigens in promoting autoimmune reactions toward CYP2E1, we further analyzed ALD patients possessing the mutant CTLA-4 G allele. As shown in Fig. 5, the presence of IgG against HER epitopes was associated with the development of anti-CYP2E1 autoantibodies in this group (OR for developing anti-CYP2E1 antibodies in HER+ve vs. HER−ve patients 3.7; [1.2-11.4]; $P = .031$). When the patients concomitantly displaying an anti-HER immune response and possessing at least 1 G allele of CTLA-4 were compared with subjects negative for both of these factors (Table 3), the risk of developing anti-CYP2E1 IgG was increased by 22-fold (OR 22.9; [4.2-125.6]; $P = .0001$). This strongly suggests that the presence of the CTLA-4 G allele in combination with an immune reaction against HER-derived antigens has an additive effect in promoting the breakdown of immune tolerance toward CYP2E1.

## Discussion

The development of anti-CYP autoreactivity is not uncommon in liver diseases. Anti-CYP autoantibodies have been observed in patients with hepatitis caused by dihydralazine (anti-CYP1A2), tienilic acid (anti-CYP2C9), or halothane (anti-CYP2E1) as well as during hypersensi-

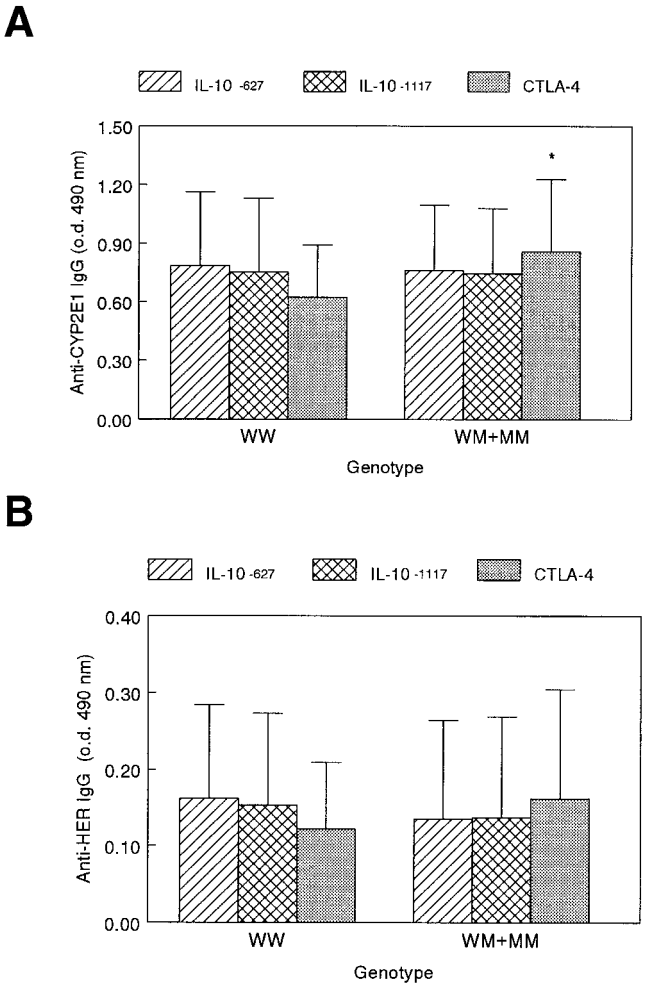


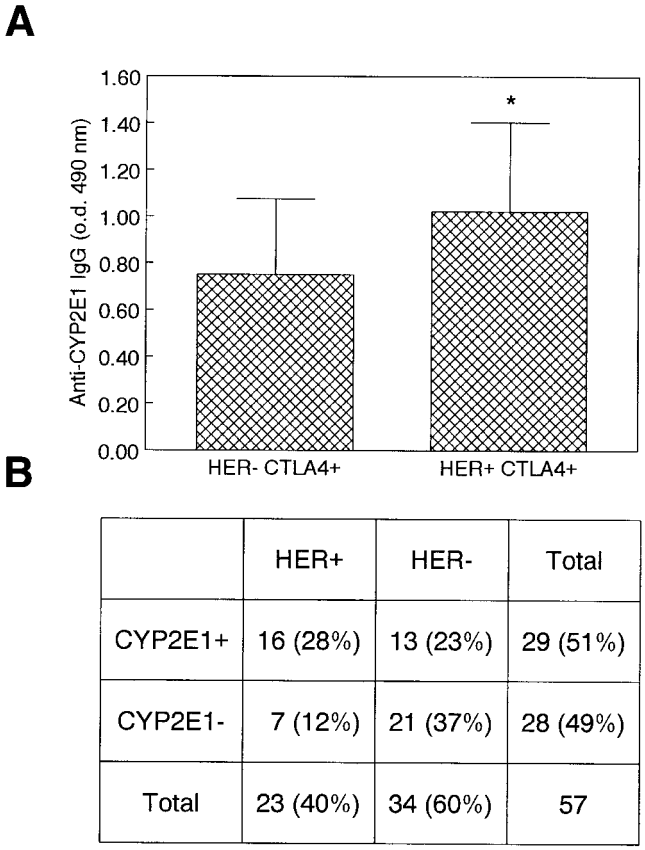

Fig. 4. Titers of antibodies against recombinant human cytochrome P4502E1 (CYP2E1) (A) and hydroxyethyl free radical (HER)-derived antigens (B) among patients with advanced alcoholic liver disease carrying 1 or 2 copies of wild-type (W) or mutant (M) alleles of the IL-10 and CTLA-4 genes. The values are means ± SD of the optical density (o.d.) at 490 nm obtained after subtracting the background reactivity of each sera. Statistical significance: $*P < .05$ vs. homozygotes for the wild-type alleles.

**Table 2. Influence of IL-10 and CTLA-4 Polymorphisms on the Antibody Responses Against CYP2E1 and Hydroxyethyl Free Radical (HER) Antigens in Patients With Advanced Alcoholic Liver Disease**

| | Anti-CYP2E1 IgG | | Anti-HER-BSA IgG | |
|---|---|---|---|---|
| Polymorphisms | WW (%) | WM + MM (%) | WW (%) | WM (%) |
| IL-10 (−627 C→A) | 17/42 (40) | 19/48 (49) | 17/42 (40) | 17/48 (35) |
| IL-10 (−1,117 G→A) | 8/20 (40) | 28/70 (40) | 8/20 (40) | 26/70 (37) |
| CTLA-4 (49 A→G) | 7/33 (21) | 29/57 (51)* | 11/33 (33) | 23/57 (40) |

NOTE. Figures show the number of the subjects with, respectively, anti-CYP2E1 and anti-HER IgG titers above the percentile in the control population and carrying 1 or 2 copies of wild-type (W) or mutant (M) alleles of the IL CTLA-4 genes.

*Odds ratio vs. WW patients 3.8 (1.3–10.3), $P = .011$.




| | HER+ | HER- | Total |
|---|---|---|---|
| CYP2E1+ | 16 (28%) | 13 (23%) | 29 (51%) |
| CYP2E1- | 7 (12%) | 21 (37%) | 28 (49%) |
| Total | 23 (40%) | 34 (60%) | 57 |

Fig. 5. Effect of immune response against epitopes derived from hydroxyethyl free radicals (HER) on the titers (A) and the frequency distribution (B) of anti-CYP2E1 autoantibodies in patients with advanced alcoholic liver disease carrying the mutant allele(s) of CTLA-4 gene. IgG against human recombinant CYP2E1 was measured in subjects homozygous or heterozygous for the CTLA-4 exon 1 G allele with titers of IgG against HER-BSA adducts above (HER+) or below (HER−) the 95th percentile in the control population. (A) The values are means ± SD of the optical density (o.d.) at 490 nm obtained after subtracting the background reactivity of each. Statistical significance: *$P < .05$ vs. HER negative CTLA-4 positive. (B) CYP2E1+ and HER+ designates that IgG titers were above the 95th percentile in the control population. Odds ratio for possession of anti-CYP2E1 autoantibodies in patients with anti-HER antibodies vs. those without: 3.7 (1.2-11.4), $P = .031$.

tivity reactions to aromatic anticonvulsants (anti-CYP3A).[10,31,32] Furthermore, CYP2D6 is a target for the antiliver-kidney microsome type I (LKM-1) antibodies present in patients with type II autoimmune hepatitis and hepatitis C.[33,34] The results of this study demonstrate that the development of anti-CYP2E1 autoantibodies during ALD is not a feature of alcohol abuse *per se*, because the heavy drinkers without liver damage of simple steatosis had similar titers to controls, but rather is associated with advanced liver disease. This observation raises 2 key questions: First, what factors determine why some patients with ALD break tolerance to CYP2E1, whereas others do not? Second, are the antibodies part of an immune response that is important in the pathogenesis and progression of ALD? Our results provide answers to the first question and some clues to the second question.

It has been proposed that CYP alkylation by reactive drug metabolites is responsible for antiself responses involving CYPs in subjects with drug-induced hepatitis.[11,12,35] ALD patients have an increased susceptibility to develop immune responses against several new or "neo" antigens produced as a result of the interaction between liver proteins and reactive ethanol metabolites, such as acetaldehyde and HER[36-38] or lipid peroxidation products.[39,40] We have previously reported that CYP2E1 is among the liver proteins alkylated by HER.[13] In both

**Table 3. Combined Effect of the Immune Response Against Hydroxyethyl Free Radical Antigens and the Presence of the CTLA-4 G Allele(s) on the Development of Autoantibodies Against CYP2E1 in Patients With Advanced Alcoholic Liver Disease**

| | Anti-HER IgG + CTLA-4 + (%) | Anti-HER IgG − CTLA-4 − (%) |
|---|---|---|
| Anti-CYP2E1 IgG + | 16/23 (70) | 2/22 (10) |
| Anti-CYP2E1 IgG − | 7/23 (30) | 20/22 (90) |

NOTE. Anti-CYP2E1 IgG+ and anti-HER IgG+ designates titers above the 95th percentile in the control population. Odds ratio for possession of anti-CYP2E1 auto antibodies in patients with the combination of anti-HER immune response and CTLA-4 G allele vs. those without both these factors: 22.9 (4.2–125.6), $P = .001$.

rodents and humans, the formation of anti-HER IgG is strictly dependent on CYP2E1 activity,[41,42] and HER complexes with CYP2E1 are the main antigens recognized by these antibodies.[13,43] By demonstrating that ALD patients with anti-HER immune response not only have titers of anti-CYP2E1 IgG higher than those without anti-HER antibodies but also a 4-fold increased risk of developing anti-CYP2E1 autoreactivity (defined as titers above 95th percentile in the control population), the present results support the hypothesis that CYP2E1 modifications by HER are a trigger for anti-CYP2E1 autoreactivity associated with alcohol-induced liver injury.

Recent evidence suggests that genetic factors influence the susceptibility to autoimmunity.[14-22] Among the patients enrolled in the present study, the IL-10 and CTLA-4 polymorphisms are widely distributed, with frequencies comparable with those of our previously published reports.[26,27] However, only the CTLA-4 polymorphism is significantly associated with autoreactivity toward CYP2E1, increasing the risk of developing anti-CYP2E1 autoantibodies by 3.8-fold. The mechanisms by which mutant CTLA-4 represent a risk factor for autoimmune responses is still poorly understood. The +49 A→G transition in the exon 1 of the CTLA-4 gene causes the substitution of threonine for alanine at position 17 in the leader peptide sequence, possibly affecting protein trafficking to the secretory portion of the endoplasmic reticulum. Accordingly, recent observations in human T lymphocytes from individuals homozygous for G and A alleles have demonstrated that the mutant G allele reduces the expression of CTLA-4 on the plasma membrane during T-cell stimulation.[44] Furthermore, upon exposure to immature, but not to mature, allogeneic dendritic cells, T lymphocytes from G/G homozygotes proliferate more than T cells from A/A homozygotes.[44] This latter observation is consistent with the hypothesis that CTLA-4 might attenuate weak T-cell activation signals mediated by the antigen receptor and CD28 and, thereby, contributes to the maintenance of immune tolerance.[24]

In the present study, the effect of the CTLA-4 polymorphism and the anti-HER immune response were synergistic, with ALD patients possessing both the G allele and anti-HER IgG having a 22-fold increased risk of developing anti-CYP2E1 autoreactivity compared with patients with neither of these factors. This suggests that in the presence of antigenic stimulation by HER-modified CYP2E1 peptides combined with an impaired Th cell regulation by the mutant CTLA-4 allele favors the expansion of autoreactive Th cell clones. In support, the exon 1 CTLA-4 polymorphism has been associated with high titers of autoantibodies against liver-specific antigens in type-1 autoimmune hepatitis and in primary biliary cirrhosis.[19,20] It would, therefore, appear that both genetic (CTLA-4) and epigenetic (immune response against CYP2E1-HER adducts) factors determine why some patients with ALD develop autoimmune reactions directed against CYP2E1 and others do not. This represents the first demonstration of how heavy drinking might lead to the breaking of self tolerance in the liver. These observations may also have wider implications for the field of autoimmunity in general, suggesting that the combination of genetic defects in the regulation of immune response and chronic allogenic stimulation can lead to the development of autoreactivity even in the context of a "nonclassical" autoimmune disease.

The most intriguing question arising from this and other studies is whether this autoimmune response plays any role in the pathogenesis of ALD or is simply an epiphenomenon associated with liver damage. A role for immune factors in disease pathogenesis is provided by our previous report (confirmed in this study) of an association between CTLA-4 polymorphism and the development of ALD.[26] Furthermore, the present demonstration of a link between the autoimmune response to CYP2E1 and advanced ALD provides evidence that CYP2E1 may be one target for a potentially injurious immune response. The presence of activated CD4 and CD8 positive T cells in the liver biopsy specimens of ALD patients, and their correlation with the degree of necrosis and inflammation,[45] offers further support for a role of immune factors in the pathogenesis of ALD and is not inconsistent with our observations because autoantibodies are often associated with cell-mediated response in many autoimmune diseases. The observation that very few heavy drinkers without liver damage or with steatosis only develop anti-CYP2E1 autoantibodies, despite possessing the CTLA-4 "at-risk" allele and/or an anti-HER response, suggests that their generation is probably downstream from the initial injury, most likely resulting from alcohol-induced oxidative stress and/or cytokine release.[46] Advanced ALD seems likely to be the result of a variety of nonmutually exclusive mechanisms, and the individual susceptibility to alcohol liver damage might result by a combination of different genetic and environmental factors, affecting either the magnitude of the initial injury or the response to, and perpetuation of, this injury. Our results suggest that genetic and epigenetic factors determining the magnitude of the autoimmune response to CYP2E1 may fall into the latter category. Clearly, prospective studies are required to dissect the precise role of the autoimmune responses in the pathogenesis of ALD. However, if supported by other studies, this work could lead to trials of immune-directed

therapy in alcohol abusers identified by genetic and serologic tests as having a prominent immunologic component to their hepatic disease.

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