Alcohol Dehydrogenase: A Target of Humoral Autoimmune Response in Liver Disease

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Background & Aims: Liver-specific membrane lipoprotein (LSP) is a heterogeneous liver preparation that has been widely used to study autoreactivity in liver disease. The aim of this study was to identify autoantigens in LSP. Methods: Guinea pig anti-LSP serum was used to screen a human liver complementary DNA (cDNA) library. Humoral immune responses to isolated potential autoantigens were investigated by immunoblotting in 91 pediatric patients with various liver diseases, 20 adult patients with alcoholic liver disease and 20 with autoimmune thyroid disease, 37 healthy children, and 20 healthy adults. Results: A 1.6-kilobase cDNA insert isolated from the cDNA library was found to encode amino acids 61–374 of the human alcohol dehydrogenase (ADH)-γ1 subunit. Antibodies to this or other ADH subunits were found significantly more frequently in autoimmune liver diseases (19 of 39 patients; 49%), Wilson’s disease (5 of 13 patients; 38%), and alcoholic liver disease (10 of 20 patients; 50%) than in normal controls (P < 0.0001, P < 0.005, and P < 0.05, respectively) and correlated with disease activity in autoimmune liver disease. Conclusions: ADH has been identified as a new antigenic component of the LSP using a xenogenic antisera to immunoprobe a human cDNA liver preparation and seems to be a target autoantigen in liver disease. This approach may be useful in identifying other potential autoantigens.

The liver-specific membrane lipoprotein (LSP) preparation was first described and partially characterized by Meyer zum Büschenfelde and Miescher.1 LSP is a large, lipoid-associated complex containing more than 20 proteins with molecular weights ranging from 5 to 220 kilodaltons,2 including species-specific and species-cross-reactive polypeptides of which at least two are known to be liver specific, one species-cross-reactive and the other human specific.3–5 The presence of cellular and humoral immune responses against LSP6–10 in patients with various types of acute and chronic liver diseases and the close relationship between serum anti-LSP titer and histological degree of disease activity in patients with autoimmune hepatitis (AIH)11 have led to the suggestion that autoimmune responses to antigens in this preparation play a role in the pathogenesis of autoimmune and virus-induced liver diseases.12

To date, the only well-characterized liver-specific component of LSP preparation is the hepatic asialoglycoprotein receptor (ASGPR),13 a target for both autoantibodies14 and autoreactive T cells15,16 in AIH. Attempts to identify and characterize additional antigenic constituents of the LSP preparation by techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or by use of monoclonal antibodies have not yielded much information. Of several hundred monoclonal anti-LSP antibodies produced in various laboratories,17–21 only one was found to react with an unequivocal liver-specific epitope expressed on the surfaces of hepatocytes, an epitope peculiar to rabbits.18

We have decided to adopt a different approach. By immunoscreening human complementary DNA (cDNA) expression libraries constructed with messenger RNA (mRNA) from a given organ such as liver, it is theoretically possible to identify targets of autoantibodies present in patients’ sera. With this approach Manns et al.,22 using sera from patients with AIH containing high-titer anti-liver-kidney microsomal antibody type 1 (LKM-1), were able to identify cytochrome P4502D6 (CYP2D6) as the target of LKM-1. We report results obtained using an anti-LSP antiserum raised in guinea pigs to screen a human liver cDNA expression library. A cDNA clone encoding an LSP component different from ASGPR was successfully isolated and characterized and shown to be a target of autoantibodies present in human liver disease.

Abbreviations used in this paper: ADH, alcohol dehydrogenase; AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ANA, antinuclear antibody; ASC, autoimmune sclerosing cholangitis; ASGPR, asialoglycoprotein receptor; LKM-1, anti-liver-kidney microsomal antibody type 1; LSP, liver-specific membrane lipoprotein; SMA, anti-smooth muscle antibody.

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Table 1. Demographic and Clinical Data of the Patients and Controls at the Time of Testing

<table>
<thead>
<tr>
<th>Disease category</th>
<th>No. of patients (M/F)</th>
<th>Age range, median (yr)</th>
<th>No. anti-LSP positive (%)</th>
<th>AST (IU/L, mean ± SD)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA/SMA + AIH</td>
<td>5/12</td>
<td>10–18 (13)</td>
<td>9 (53)</td>
<td>67 ± 62</td>
</tr>
<tr>
<td>LKM-1 + AIH</td>
<td>3/7</td>
<td>3–20 (10)</td>
<td>7 (70)</td>
<td>532 ± 831</td>
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<tr>
<td>ASC</td>
<td>3/9</td>
<td>3–17 (11.5)</td>
<td>7 (58)</td>
<td>86 ± 95</td>
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<tr>
<td>Wilson’s disease</td>
<td>10/3</td>
<td>8–16 (12)</td>
<td>5 (38)</td>
<td>114 ± 127</td>
</tr>
<tr>
<td>α1-Antitrypsin deficiency</td>
<td>9/10</td>
<td>14 days to 13 yr (1)</td>
<td>4 (21)</td>
<td>129 ± 85</td>
</tr>
<tr>
<td>HBV infection</td>
<td>13/7</td>
<td>2–20 (5)</td>
<td>11 (55)</td>
<td>109 ± 78</td>
</tr>
<tr>
<td>Autoimmune thyroiditis</td>
<td>0/15</td>
<td>22–81 (40)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>13/7</td>
<td>41–76 (55)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Healthy children</td>
<td>16/21</td>
<td>2–15 (12)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Healthy adults</td>
<td>20/0</td>
<td>21–60 (34)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.

aUpper normal limit, 50 IU/L.

Materials and Methods

Patients

Ninety-one pediatric patients with chronic liver diseases were studied (Table 1). Thirty-nine had autoimmune liver disease (AILD). Of these, 27 had AIH diagnosed according to international criteria23; 17 were antinuclear (ANA) and/or anti-smooth muscle antibody (SMA) positive and 10 anti-LKM-1 positive. Eight of the 27 were studied before immunosuppressive treatment. The remaining 12 patients with AIILD had autoimmune sclerosing cholangitis (ASC), defined by seropositivity for ANA and/or SMA with characteristic endoscopic cholangiopancreatographic and histological changes,24 and were all undergoing immunosuppressive treatment. Twenty patients with AIILD (14 AIH and 6 ASC) had a liver biopsy performed at the time serum was obtained for the present study. Eleven of them had moderate to severe liver damage, and the remaining 9 patients had mild or minimal liver damage. Of the remaining 52 pediatric patients, 19 had α1-antitrypsin deficiency (PIZZ phenotype), 13 had Wilson’s disease, and 20 had chronic hepatitis B virus (HBV) infection.

All patients with HBV were hepatitis B surface antigen (HBsAg) positive; 8 were also positive for hepatitis B e antigen (HBeAg), 2 for antibody to hepatitis B e antibody (HBeAb), and 1 for anti–hepatitis D virus (HDV). Tests for HBsAg, HBsAb, HBeAg, HBeAb, and hepatitis B core antibody (HBCcAb) were performed using commercial enzyme immunoassays (Abbott Laboratories, North Chicago, IL) and, for anti-HDV, using a kit from Incstar Ltd. (Wokingham, Berkshire, England). Liver biopsies were performed in 19 patients with chronic HBV: 10 had moderate to severe necroinflammation (chronic active hepatitis), 7 had mild inflammation (chronic persistent hepatitis), 1 had only minimal changes, and 1 had inactive cirrhosis. None of the patients in this study were anti–hepatitis C virus (HCV) antibody positive by a second-generation enzyme immunoassay (HCV EIA; United Biomedical Inc., Hauppauge, NY).25

Because autoimmunity has been implicated in the pathogenesis of alcoholic liver disease, we also studied 20 adult patients with alcoholic liver disease whose daily alcohol intake was >60 g in men and >40 g in women. Seventeen of these 20 patients had a liver biopsy performed at the time serum was obtained. Fifteen had established cirrhosis, and 2 showed increased fibrosis without cirrhosis. Twelve patients (11 with cirrhosis and 1 with fibrosis) had active necroinflammation (periportal hepatitis, with features of alcoholic hepatitis in 6), and 5 had inactive disease, 2 of whom had steatosis (1 with fibrosis and 1 with cirrhosis).

Fifteen adult patients with autoimmune thyroiditis, with anti-thyroglobulin antibodies ranging from 1/100 to 1/1600 and anti-thyroid peroxidase antibodies ranging from 1/1600 to 1/102,400, were also studied. The thyroid antibodies were detected by agglutination of thyroglobulin-coated and thyroid microsome-coated gelatin particles, respectively (Fujirebio Inc., Shinjuku-Ku, Tokyo, Japan).

Thirty-seven healthy British children and 20 healthy adults were studied as controls. The study was approved by the King’s College Hospital Ethical Committee.

Detection of Anti-LSP and Other Antibodies

Antibodies to LSP and ASGPR were measured by a staphylococcal cell radioimmunoprecipitation assay, and titers were determined as previously described.9,26 Other tissue autoantibodies (ANA, SMA, and LKM-1) were detected by indirect immunofluorescence on 5-μm cryostat sections of rat liver, kidney, and stomach27 at an initial dilution of 1:10 in phosphate-buffered saline (PBS) and then titrated out to negativity in doubling dilutions.

Preparation of Antisera

The preparation and characterization of the guinea pig anti-LSP and anti-ASGPR antisera used in this study have been described in detail elsewhere.28-30 Briefly, human and rabbit LSP were prepared by Sepharose 6B (Pharmacia, St. Albans, Herts, England) fractionation of 105,000g supernatant and characterized by immunoprecipitation of human LSP at various stages of fractionation. The human LSP employed in these experiments was prepared by this method and fractionated to batches. The antisera was then prepared by immunizing rabbits with heat-inactivated guinea pig LSP and ASGPR and characterized by immunoprecipitation of guinea pig LSP and ASGPR at various stages of fractionation and by immunoprecipitation of human LSP and ASGPR at various stages of fractionation.
nants of fresh normal liver. Rabbit ASGPR was purified by ligand-affinity chromatography from Triton X-100 extracts of acetone powder of fresh normal liver. Guinea pigs (n = 4) were immunized with either 4 × 1 mg rabbit or human LSP or 4 × 20 μg rabbit ASGPR in Freund’s adjuvant during a 6-week period. For each antigen, the antisera with the highest antibody titer (>1:10^5) was used.

Isolation of cDNA Clones

A human liver cDNA library in the phage λgt11 (Clontech, Palo Alto, CA) expression vector was screened with sera from 2 patients with AIH containing anti-LSP antibody at titers of 1/3500 and 1/3900 and with the guinea pig anti-human LSP antisera at a titer of >1/10^6 according to Sambrook et al.31 as previously described.32 Briefly, Escherichia coli was infected with bacteriophage at a density of 1 × 10^8 plaque-forming units per 90-mm Petri dish. Plaques of interest were identified by detection of antibodies bound to nitrocellulose filter overlays after incubation with the human sera or guinea pig antisera diluted 1:1000 using peroxidase-conjugated anti-human or anti-guinea pig immunoglobulin (Ig) G (Dako, Copenhagen, Denmark) and a chromogenic substrate (diaminobenzidine). This dilution of the human sera and antisera was chosen because, in a previous study32 and in our preliminary experiments, it gave high sensitivity and low background. Phage DNA from positive plaques was extracted according to Sambrook et al.31 and purified from an agarose gel using the GeneClean method (Bio 101 Inc., La Jolla, CA). The cDNA fragment was cloned into the EorI site of the pBluescript KSII vector and sequenced with a Sequenase version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH) using M13 forward and reverse primers.31

Preparation of Fusion Protein

A fusion protein, the amino terminus of which consists of β-galactosidase sequences and the carboxyl terminus of the foreign sequence, was constructed in lysogens according to the technique of Sambrook et al.31 E. coli strain Y1089 was infected with phage containing the cDNA insert at a concentration of 2 × 10^8 plaque-forming units per 90-mm Petri dish at 30°C. Fusion protein synthesis was induced at 45°C for 20 minutes by adding 10 mmol/L isopropyl β-D-thiogalactoside and then incubating the cultures for 1 hour at 37°C.

Immunoblotting

Antibodies to recombinant human alcohol dehydrogenase (ADH)-γ subunit (see below) and horse liver ADH (Sigma Chemical Co., Poole, Dorset, England) were detected and titrated by immunoblotting. Horse ADH was used as target antigen in addition to the human ADH-γ -containing fusion protein for the following reasons: (1) the isolated human fusion protein does not contain the full-length enzyme (see below) and may therefore lack relevant epitopes; (2) horse ADH has 89% identity with the human ADH-γ subunit34–36; (3) similar to human ADH-γ subunit, horse ADH contains the function-relevant serine in position 4837,38, and (4) it is commercially available in purified form. In addition, the human β subunit of ADH was used as a target antigen because its amino acid sequence is 95% identical to that of human ADH-γ.34 Recombinant human ADH-β, (produced in E. coli JM105) was kindly provided by Prof. W. F. Bosron (Indiana University School of Medicine, Indianapolis, IN). Reaction to ADH was investigated using a variety of polyclonal antisera: (1) guinea pig anti-human and anti-rabbit LSP; (2) anti-ASGPR (guinea pig anti-rabbit ASGPR and rabbit anti-human ASGPR; the latter kindly provided by Dr. U. Treichel, J. Gutenberg University of Mainz, Germany); and (3) murine anti-E. coli β-galactosidase (Sigma Chemical Co.), as a control for the fusion protein.

Antibodies to ADH were detected by probing nitrocellulose blots after electrophoresis in 7.5% (for human ADH-γ in E. coli lysates) and 12% (for human ADH-β, and horse ADH) polyacrylamide minigels (Bio-Rad Laboratories) at 115000 (Bio-Rad Laboratories, Hemel Hempstead, England) using the system of Laemmli.39 Proteins were electrophoretically transferred onto the nitrocellulose filters in a semidyry electrophoretic transfer cell (Bio-Rad Laboratories), and nonspecific binding was blocked with 1% gelatin as previously described.32 Filter strips were incubated with the patients’ sera at dilutions ranging from 1:200 to 1:3000, and polypeptides targeted by patients’ antibodies were visualized using peroxidase-conjugated rabbit anti-human IgG (Dako) at a dilution of 1:750 for 1 hour at room temperature. Titers of anti-ADH antibodies were defined as the highest dilution of serum at which a positive reaction was still detectable. To control for possible false-positive reactions caused by antibodies against β-galactosidase, which are occasionally found in human sera,40 all sera were screened by immunoblotting against E. coli β-galactosidase (Sigma Chemical Co.).

Serum Absorption

Serum absorption was performed according to Swanson et al.41 on two sera specimens positive for both anti-LSP and anti-horse ADH (one from a patient with ASC with an anti-horse ADH antibody titer of 1/500 and an anti-LSP titer of 1/750 and the other from a patient with LKM-positive 1 AIH with an anti-ADH titer of 1/3000 and an anti-LSP titer of 1/3900). In brief, 0.5 × 3 cm2 nitrocellulose strips presoaked in TNT buffer (10 mmol/L-Tris-HCl, 150 mmol/L NaCl, and 0.05% Tween 20) were individually impregnated with 50 μg horse ADH, 60 μg rabbit LSP, or 200 μg rabbit kidney homogenate. Residual nonspecific reactivities of the strips were then blocked by incubation in 1% gelatin for 1 hour. Impregnated strips were incubated in patients’ sera diluted 1:300 in 2 mL TNT buffer for 2 hours at room temperature, and the procedure was performed twice. Absorbed and nonabsorbed sera at 1:300 dilution were tested for reactivity against horse ADH by immunoblotting, and changes in intensity of the bands after absorption were quantified by densitometric scanning (Ultra-Violet Products Ltd., Cambridge, England).
Preparation of Anti-horse ADH Antibody by Affinity Purification

A purified anti-ADH antibody was prepared from the guinea pig anti-human LSP antiserum by solid-phase immunoaffinity separation. In brief, 300 μg of horse liver ADH was loaded on a 12% polyacrylamide minigel and electrophoretically transferred onto a nitrocellulose filter in a semidy electrophoretic transfer cell (procedure for the immunoblotting was as described earlier). The ADH-impregnated filter (1.5 × 4 cm) was incubated with anti-human LSP antibody diluted 1:300 in 3 mL of TNT buffer for 1.5 hours. After two 20-minute washes in 20 mL TNT buffer, the antibody was eluted with 6.3 mL 0.2 mol/L glycine, pH 2.7, for 10 minutes and neutralized by addition of 0.7 mL of 1 mol/L Tris buffer, pH 8.0 (until the protein solution reached pH 7.3). The anti-ADH antibody–containing solution was concentrated 20-fold by dialysis against high-molecular-weight polyethylene glycol (PEG 6000).

Statistical Analysis

Autoantibody prevalence in the different patient groups was compared using the χ² test. Spearman’s rank test was used to correlate the titer of the anti-LSP and anti-ADH antibodies and the titer of anti-human and anti-horse ADH antibodies. Autoantibody titer differences between groups were analyzed by Wilcoxon’s rank sum test. Correlation coefficients (r) and levels of significance (P) are given. P values of < 0.05 are considered significant.

Results

Immunoscreening of a Human Liver cDNA Library

Initial screening of 2 × 10⁶ plaques from the human liver cDNA library with the two patient sera specimens containing anti-LSP antibody failed to identify any immunopositive clones. However, by screening of 8 × 10⁵ plaques from the same cDNA library with the guinea pig anti-human LSP antiserum, we identified a positive recombinant clone carrying a 1.6-kilobase cDNA insert in the EcoRI cloning site of the lacZ gene in the λgt11 vector. This clone will be referred to as YM1. The cDNA from YM1 was purified and fully sequenced in both orientations and found to be identical to the published sequence of the γ₂ subunit of human liver ADH. The clone lacked the first 180 bases of the coding sequence and had one base substitution at position 1134 (guanine instead of thymidine) in the 5’ untranslated region. The cDNA encodes an amino-terminal truncated protein of 314 amino acids lacking the first 60 amino acids of the 374–amino acid full-length protein. The amino acid sequence 61–374 of human ADH-γ₂ is 87% homologous to the same region of horse ADH and shares 93%, 96%, 96%, and 95% amino acid sequence identity with human ADH-α, -β₁, -β₂, and -β₃, respectively (Wisconsin Package, version 8; Genetics Computer Group, WI).

Characteristics of Fusion Protein and Horse ADH

Bacterial lysates containing the fusion protein encoded by clone YM1 by immunoblotting. E. coli lysate containing the fusion protein encoded by YM1 was incubated with the following antibodies. Lane 1, guinea pig anti-human LSP antiserum. A band is present at ~146-kilodalton position. Lane 2, guinea pig anti-rabbit LSP antiserum. A band is also detected at ~146-kilodalton position. Lanes 3 and 4, rabbit anti-human ASGPR and guinea pig anti-rabbit ASGPR, respectively. No bands are detected. Lanes 5 and 6, sera from a patient with AIH and one with ASC, respectively. They both recognize the recombinant fusion protein. Lanes 7 and 8, sera from two healthy controls. No staining is observed. Lanes 9 and 10, uninfected E. coli lysate incubated with murine anti-β-galactosidase and with anti-human LSP antiserum, respectively. No relevant bands are detectable with either antiserum. Lane 11, lysate containing fusion protein encoded by clone YM1, incubated with murine anti-β-galactosidase antibody. A band is present at ~146-kilodalton position. An additional band (*) represents a degradation product of β-galactosidase because it is only recognized by the anti-β-galactosidase antiserum.
positivity for patients' sera. Fifteen of 44 with ADH screening were tested for anti-ADH reactivity. A very strong association was found between anti-human ADH-γ1 and anti-horse ADH. Twelve of the 17 anti-human ADH-positive sera (71%) were positive for anti-human ADH, and 20 of 74 anti-human ADH-negative sera (27%) were positive for anti-horse ADH ($\chi^2 = 12; P < 0.001$). Additionally, titers of anti-LSP correlated with both anti-horse ADH ($r = 0.65; P < 0.001$) and anti-human ADH-γ1 antibodies ($r = 0.23; P < 0.05$); titers of anti-horse and anti-human ADH-γ1 antibodies also showed a close correlation ($r = 0.39; P < 0.001$). However, the closest association was found between the prevalence and titers of antibodies against the human β1 subunit and horse ADH, which were identical except that 1 additional patient in each of the AILD and alcoholic liver disease groups reacted with ADH-β1 (Table 2).

Absorption Studies

Results for one of the 2 patients' sera tested in these experiments, as detailed in Materials and Methods, are shown in Figure 3. Unabsorbed sera readily recognized a band at the 80-kilodalton position. After two successive absorptions with horse ADH, the intensity of the band decreased by >90%. Reactivity to horse ADH was also decreased, although to a lesser extent, by preincubating the serum with nitrocellulose strips impregnated with rabbit LSP. When sera were absorbed with rabbit kidney homogenate, no reduction in staining intensity was observed. Very similar results were obtained with the second serum tested (data not shown).

Affinity-Purified Anti-horse ADH Antibody

The affinity-purified anti-horse ADH antibody reacted with both horse ADH (Figure 4) and human recombinant ADH on immunoblotting. The densitometrically assessed intensity of the horse ADH band, obtained with the purified anti-horse ADH antibody (diluted 1:2), was 66% of that obtained with unabsorbed guinea pig anti-LSP antibody (diluted 1:300).

Prevalence and Titer of Anti-ADH Antibodies

The prevalences of anti-human ADH-γ1 and -β1 and horse ADH and their titers in the populations studied are shown in Table 2. In patients with AILD, the prevalence of anti-human ADH-γ1 and anti-horse ADH was 28% and 46%, respectively, with reactivity to both ADHs significantly higher than that in healthy controls.

Figure 2. Immunoblotting with horse ADH (0.75 µg/lane) as the target antigen. Lane 1, guinea pig anti-human LSP antiserum. A band is present at ~80-kilodalton position. Lane 2, guinea pig anti-rabbit LSP antiserum. A less intense band is present at the same position. Lanes 3 and 4, rabbit anti-human ASGPR guinea pig anti-rabbit ASGPR, respectively. No bands are detected. Lane 5, serum of a patient with ANA and SMA-positive AIH. A band is present at ~80-kilodalton position. Lanes 6 and 7, 2 patients with LKM-1-positive AIH. One reacts with ADH (lane 6) but the other is negative (lane 7).

also reacted with this 80-kilodalton band. No reaction was observed with anti-ASGPR antisera (Figure 2).

No positive reactions to purified E. coli β-galactosidase preparation were detected in 10 serum samples from patients with AILD (8 AIH and 2 ASC) chosen at random, 5 of which were positive for anti-human ADH-γ1 (titer, 1/300–1/1000), including 1 serum that was positive for anti-human ADH-γ1 and negative for antibodies to horse ADH and LSP.

The two initial human sera used for cDNA liver library screening were tested for anti-ADH reactivity. One was found to be negative against the recombinant human ADH-γ1 subunit, and the other was positive at a titer of 1/500. When tested against horse ADH, both sera were positive at titers of 1/2000 and 1/3000, respectively.

Association Between Anti-LSP and Anti-ADH and Between Anti-human and Anti-horse ADH Antibodies

A significant association was observed between positivity for anti-LSP and anti-human ADH-γ1 in the patients' sera. Fifteen of 44 anti-LSP-positive sera (34%) were positive for anti-human ADH compared with only 2 of 47 anti-LSP-negative sera (4%) ($\chi^2 = 13; P < 0.0005$). An even stronger association was found between anti-LSP and anti-horse ADH antibodies, which were found in 29 of 44 anti-LSP-positive sera (66%) compared with only 3 of 47 anti-LSP-negative sera (6%) ($\chi^2 = 35; P < 0.00001$). A close association was also found between anti-human ADH-γ1 and anti-horse ADH. Twelve of the 17 anti-human ADH-positive sera (71%) were positive for anti-horse ADH, and 20 of 74 anti-human ADH-negative sera (27%) were positive for anti-horse ADH ($\chi^2 = 12; P < 0.001$). Additionally, titers of anti-LSP correlated with both anti-horse ADH ($r = 0.65; P < 0.001$) and anti-human ADH-γ1 antibodies ($r = 0.23; P < 0.05$); titers of anti-horse and anti-human ADH-γ1 antibodies also showed a close correlation ($r = 0.39; P < 0.001$). However, the closest association was found between the prevalence and titers of antibodies against the human β1 subunit and horse ADH, which were identical except that 1 additional patient in each of the AILD and alcoholic liver disease groups reacted with ADH-β1 (Table 2).
Table 2. Prevalence and Titer of Antibodies to Human and Horse ADH in Different Patient Groups and in Healthy Controls

<table>
<thead>
<tr>
<th>Disease category</th>
<th>No. positive/ total (%)</th>
<th>Titer range (median)</th>
<th>No. positive/ total (%)</th>
<th>Titer range (median)</th>
<th>No. positive/ total (%)</th>
<th>Titer range (median)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Human ADHγ1</td>
<td></td>
<td>Human ADHβ1</td>
<td></td>
<td>Horse ADH</td>
<td></td>
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<tr>
<td>AILD</td>
<td>11/39 (28)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>300–3000 (1000)</td>
<td>19/39 (49)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200–2000 (1000)</td>
<td>18/39 (46)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400–3000 (1000)</td>
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<tr>
<td>Wilson's disease</td>
<td>3/13 (23)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>300–2000 (1000)</td>
<td>ND</td>
<td>ND</td>
<td>5/13 (38)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>500–2000 (1000)</td>
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<td>α&lt;sub&gt;1&lt;/sub&gt;-Antitrypsin deficiency</td>
<td>3/19 (16)</td>
<td>300–1000 (300)</td>
<td>ND</td>
<td>ND</td>
<td>5/19 (26)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200–500 (500)</td>
</tr>
<tr>
<td>HBV infection</td>
<td>0/20</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td>4/20 (20)</td>
<td>500–1000 (500)</td>
</tr>
<tr>
<td>Autoimmune thyroiditis</td>
<td>0/15</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td>0/15</td>
<td>—</td>
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<td>Healthy children</td>
<td>1/37 (3)</td>
<td>1500</td>
<td>1/37 (3)</td>
<td>500</td>
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<td>300, 1000</td>
<td>2/20 (10)</td>
<td>200, 500</td>
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</table>

ND, not done.

<sup>a</sup>P < 0.05 to P < 0.0001, significantly higher than healthy children.

<sup>b</sup>P < 0.01 compared with HBV infections.

<sup>c</sup>P < 0.05 compared with healthy adults.

The anti-horse ADH frequency was significantly increased compared with controls (χ² = 5.4, P < 0.05); both anti-human and anti-horse ADH occurred less frequently (16% and 26%) than in AILD (28% and 46%), but the differences did not reach statistical significance (P = 0.3 and P = 0.15, respectively). Four of the 20 patients with chronic HBV infection (20%) were positive for anti-horse ADH antibodies, whereas none reacted with human ADH-γ1 and horse ADH, respectively). In patients with Wilson’s disease, the prevalences of anti-human (23%) and anti-horse ADH (38%) were also higher than in healthy controls (χ² = 5.4, P < 0.05; χ² = 8.7, P < 0.005) and were similar to those in the patients with AILD. In patients with α<sub>1</sub>-antitrypsin deficiency, only the anti-horse ADH frequency was significantly increased compared with controls (χ² = 5.4, P < 0.05); both anti-human and anti-horse ADH occurred less frequently (16% and 26%) than in AILD (28% and 46%), but the differences did not reach statistical significance (P = 0.3 and P = 0.15, respectively). Four of the 20 patients with chronic HBV infection (20%) were positive for anti-horse ADH antibodies, whereas none reacted with human ADH-γ1 and horse ADH, respectively).

Figure 3. Absorption studies using serum from a patient with AIH. Reactivity to horse ADH was investigated by immunoblotting. Lane 1, unabsorbed serum reacts with horse ADH, and a band is present at ~80-kilodalton position. After absorption with horse ADH once (lane 2) and twice (lane 3), the intensity of the band is decreased more than 90%. Serum was absorbed by rabbit LSP once (lane 4) and twice (lane 5), with the intensity of the reaction with horse ADH finally decreasing by ~20%. After absorption with rabbit kidney homogenate once (lane 6) and twice (lane 7), no reduction of reactivity to horse ADH is observed.

Figure 4. Characterization by immunoblotting of affinity-purified anti-ADH antibody from anti-LSP antiserum. (A) Horse ADH incubated with ADH antibody (lane 1) affinity purified from anti-human LSP antiserum, used at a dilution of 1:2; and with the whole anti-human LSP antiserum (lane 2) used at a dilution of 1:300. A band is present at a 80-kilodalton position in both lanes (arrow); its densitometrically assessed intensity in lane 1 is 66% of that in lane 2. (B) E. coli lysate containing recombinant fusion protein encoded by clone YM1 incubated as in A. Both antibody reagents recognize the recombinant ADH-γ1-containing fusion protein (arrow).
ADH-γ₁, with prevalences for both antibodies significantly lower than in AILD (P < 0.05 and P < 0.01 for anti-horse and anti-human ADH-γ₁, respectively) and similar to those found in the control subjects. In patients with alcoholic liver disease, the prevalence of anti-human ADH-γ₁ (15%) and anti-horse (45%) ADH was higher than in normal adults (P = 0.08 and P < 0.05 for anti-human and anti-horse, respectively). No reactivity to either human ADH-γ₁ or horse ADH was found in patients with autoimmune thyroiditis.

Only 2 of the 37 healthy children were positive for anti-horse ADH (at titers of 1/500 and 1/1000); 1 of the 2 patients was also positive for anti-human ADH-β₁ (at a titer of 1/500). One of the healthy children was positive for human ADH-β₁, antibodies (at a titer of 1/1500). Two of the sera from 20 healthy adults reacted with horse ADH at titers of 1/200 and 1/500 and also were positive for anti-human ADH-β₁ (at titers of 1/300 and 1/1000). None was positive for anti-human ADH-β₁.

The titers of anti-horse ADH antibodies in patients with AILD (range, 1/400–1/3000; median 1/1000) were similar to those in patients with alcoholic liver disease (range, 1/200–1/2000; median, 1/2000) and were significantly higher than in patients with non-AILDs (range, 1/200–1/2000; median, 1/500; P = 0.05).

**Association With Disease Activity**

Overall, reactivity to horse ADH (but not to human ADH-β₁) was significantly more common in patients with serum aspartate aminotransferase (AST) activities of >100 IU/L (i.e., more than twice the upper normal limit of 50 IU/L) than in patients with AST levels of <100 IU/L (19 of 33 [55%] vs. 21 of 69 [30%]; P = 0.019). However, analysis of the individual patient subgroups showed that only in the patients with AILD was there a significant correlation between the presence of antibodies to horse ADH and AST activities of >100 IU/L (9 of 12 [75%] vs. 9 of 27 [33%]; P = 0.016). The titers of anti-horse ADH in the AILD group were also significantly higher in patients with AST levels of >100 IU/L (range, 1/500–1/3000; median, 1/1500) than in those with AST levels of <100 IU/L (range, 1/400–1/3000; median, 1/500; P = 0.04). Among the 20 patients with AILD who underwent liver biopsy at the time of testing, those with histologically moderate or severe liver damage had a significantly higher frequency of anti-horse ADH antibody than patients with mild or minimal liver damage (8 of 11 [73%] vs. 2 of 9 [22%]; P < 0.05). The frequency of anti-horse ADH antibodies was significantly higher in the eight sera collected at diagnosis before immunosuppressive treatment than in treated patients (7 of 8 [88%] vs. 11 of 31 [35%]; P < 0.01). Similarly, titers of anti-horse ADH antibodies were higher in untreated (range, 1/1000–1/3000; median, 1/2000) than in treated patients (range, 1/500–1/3000; median, 1/500; P < 0.005). In patients with alcoholic liver disease, reactivity to horse ADH also tended to be associated with histological evidence of active disease: 8 of the 12 patients (67%) with histologically active disease (including 4 of the 6 with alcoholic hepatitis) were positive for anti-horse ADH compared with only 1 of the 5 patients (20%) with no inflammatory activity (P = 0.08).

**Discussion**

In the present study, we report the identification of human liver ADH-β₁ subunit as a component of LSP by the screening of a human liver cDNA library with a guinea pig antiserum against human LSP. The use of this xenogeneic antiserum was the key to the successful isolation of ADH. A similar approach, but using patients' sera, has been used for the identification of targets of other autoantibodies in liver disease. Thus, Van de Water et al., using patients' sera containing high-titer antimitochondrial antibody, were able to isolate dihydrolipoamide acetyltransferase of the pyruvate dehydrogenase complex as target antigen of antimitochondrial antibody from a human liver cDNA library. Similarly, using high-titer LKM-1 sera from patients with AIH, Manns et al. were able to extract CYP2D6 from a human liver cDNA library, and we succeeded in isolating three clones encoding CYP2D6 from the same human liver cDNA library used in the present study. We were able to obtain evidence indicating that the epitopes recognized by LKM-1 in AIH differ from those of LKM-1 found in chronic hepatitis C virus infection.

Our unsuccessful initial attempt to identify targets of anti-LSP antibodies using patient sera is probably a result of the lower titer of anti-LSP antibodies in these sera compared with antiserum raised in guinea pigs. The use of high-titer guinea pig antiserum, with the possible additional advantages of higher avidity for the target antigens, enabled us to isolate an immunopositive clone. The sequence of its 1.6-kb insert was found to be virtually identical to that of the human ADH-β₁ subunit, with only one substitution, a guanine instead of a thymine, in the untranslated region, which does not affect the predicted 314-amino acid sequence.

Human ADH is a homodimeric or heterodimeric enzyme that is found in multiple molecular forms. Ethanol is metabolized mainly by liver ADH isoenzymes containing α, β, γ, and π subunits. The α, β, and γ cDNAs...
share about 94% identity with respect to their amino acid sequences, whereas \( \pi \) is more distantly related to them (about 60% identity).\(^{13}\) Liver is by far the richest source of the human ADH-\( \alpha \), -\( \beta \), and -\( \gamma \) isozymes, with low levels of activity being detectable in lung, kidney, and the gastrointestinal tract.\(^{44}\) In view of its preferential location in the liver, ADH fulfills one of the criteria for a target autoantigen of liver-specific autoreactions.

Using a high-titer xenogeneic antiserum raised against the antigenically heterogeneous LSP preparation, we have been able to identify a new autoantigenic target of autoantibodies in human liver disease. Thus, autoantibodies against ADH were demonstrable in the patients with liver disease but rarely in normal subjects or patients with a liver-unrelated organ-specific autoimmune disease (thyroiditis). The highest prevalence and titers of anti-ADH antibodies were found in patients with liver diseases believed to have an autoimmune basis (AILD and ASC) and, overall, correlated well with the presence of anti-LSP antibodies. Interestingly, such a correlation was not found in patients with HBV infection, suggesting that anti-LSP antibodies in chronic hepatitis B recognize different antigens to those in the autoimmune liver disorders.

It is also interesting to note that reactivity to horse ADH was found more frequently than to the human ADH-\( \beta_1 \) subunit. This is almost certainly caused by the fact that the full-length, dimeric form of horse ADH is a richer source of antigenic determinants than the truncated, monomeric human counterpart derived from the cDNA clone, which is also devoid of posttranslational modifications. In keeping with this suggestion is the observation that, of the two human sera initially used to screen the liver cDNA library, one was negative and the other was positive at low titer against the prokaryotically expressed \( \gamma \), subunit, whereas they were both positive at high titer against horse ADH. This is also supported by the finding that the prevalence and titers of antibodies to the human \( \beta_1 \) subunit were almost identical to those for anti-horse ADH in the patients with AILD or alcoholic liver disease. These results suggest that the 60–amino acid residues missing from the cloned human ADH-\( \beta_1 \) may be important in determining the B-cell epitopes of ADH, either because that segment contains an additional epitope or because its absence affects the tertiary structure (possible through lack of posttranslational modification) of the cloned \( \gamma \), subunit to an extent that one or more conformational epitopes are lost.

Because the present findings indicate that ADH is a component of the LSP preparation, it is perhaps not surprising to find that patients with alcoholic liver disease have anti-ADH autoantibodies and that these tend to be associated with active liver necroinflammation. Autoimmune reactions to the liver cell have been repeatedly reported in patients with alcoholic liver disease,\(^{25,46}\) and it has been shown that a high proportion of such patients have anti-LSP antibodies and that these correlate with active disease.\(^{47}\) Whether alcohol intake induces alterations of the liver cell with ensuing autoimmunity or alterations of the immune system itself capable of producing liver damage requires further study. However, it seems that the presence of anti-ADH may in some way be related to pathogenesis of liver damage in both AILD and alcoholic liver disease. ADH is known to be a predominately cytosolic protein.\(^{48}\) It is not normally expressed on the cell surface, which is a criterion required for a putative target of tissue-damaging autoantibodies; it therefore seems unlikely that anti-ADH could cause hepatocellular damage. On the other hand, the presence of anti-ADH in these two disorders does not appear to be simply a consequence of liver injury, with release of cryptic antigens that might stimulate an immune response, because the same prevalence and association with active necroinflammation was not found in the patients with chronic HBV infection who (as a group) had comparable disease activity. It seems, therefore, that the development of anti-ADH antibodies may be part of the overall heightened immune responsiveness in AILD and alcoholic liver disease.

In conclusion, our data indicate that patients with AILD and alcoholic liver disease have circulating autoantibodies that react with ADH and are associated with active necroinflammation. By using a high-titer xenogeneic antiserum against a partially purified antigenically heterogeneous liver autoantigen preparation such as LSP, it is possible to effectively immunoprobe a cDNA library and identify individual targets of autoantibody responses in liver disease.

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