Enzyme Immunoassay for Autoantibodies to Human Liver-Type Arginase and Its Clinical Application

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Background: Arginase is an enzyme of the urea cycle, and one of the two isoenzymes is the liver-type enzyme. We examined serum autoantibodies to this liver-type enzyme in patients with hepatitis.

Methods: Antibodies to recombinant human liver-type arginase were measured by ELISA in 95 patients and 55 healthy controls.

Results: The mean absorbance values in the ELISA assays of patients with definite autoimmune hepatitis (n = 11; P < 0.0001), probable autoimmune hepatitis (n = 31; P < 0.0001), and hepatitis C (HCV; n = 20; P < 0.01) were significantly different from those of healthy controls, but the values in patients with hepatitis B (HBV; n = 23) and other autoimmune diseases (n = 10) were not significantly different from those of healthy controls. When the cutoff was fixed at the upper 95th percentile of the absorbance value in healthy controls, positive reactions were found in 18.2%, 32.3%, 20.0%, 13.0%, and 10.0% of patients with definite autoimmune hepatitis, probable autoimmune hepatitis, HCV hepatitis, HBV hepatitis, and other autoimmune diseases, respectively. All of these positive reactions were abolished by inhibition of serum with recombinant antigen. The specificity and sensitivity of this ELISA were 96% and 29%, respectively. The intraassay and interassay coefficients of variation were 2.3–7.5% and 9.8 –11%, respectively. There was no relationship between these antibodies and anti-nuclear, anti-smooth muscle, or anti-cytochrome P450IID6 antibodies.

Conclusions: The ELISA for anti-liver-type arginase autoantibody improved the detectability of autoimmune hepatitis when compared with established assays for liver-specific autoantibodies.

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Autoimmune hepatitis (AIH)4 has been recognized for >30 years, but its diagnostic criteria have only recently been codified (1). To support the diagnosis of AIH, several laboratory tests for autoantibodies, such as anti-nuclear antibodies, anti-smooth muscle antibodies, liver-kidney microsome antibodies, anti-neutrophil cytoplasmic antibodies, and antibodies to soluble liver antigen, have been used (2–4). AIH has been subdivided into three types, based on the different patterns of these antibodies (1, 5). However, most of these antibodies are not organ specific (2) and are unsatisfactory for routine use. Therefore, we searched for a liver-specific antigen and tried to establish an ELISA for liver-specific autoantibodies.

Arginase (EC 3.5.3.1; l-arginine amidinohydrolase) catalyzes hydrolysis of arginine to urea and ornithine in the liver of ureotelic animals. Two forms of arginase have been defined: liver-type arginase (arginase 1) and extrahepatic type arginase (arginase 2) (6, 7). Liver-type arginase differs from extrahepatic type arginase in immunological properties and enzyme kinetics, and it is quite abundant in the liver cytosol. cDNAs encoding human liver-type arginase (8, 9) and extrahepatic arginase (10) have been cloned from human liver and kidney, respectively. Liver-type arginase mRNA is expressed strongly in the liver, whereas extrahepatic arginase mRNA is ex-

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Nonstandard abbreviations: AIH, autoimmune hepatitis; HCV, hepatitis C virus; HBV, hepatitis B virus; and CYP2D6, cytochrome P450IID6.
pressed strongly in the adult kidney and is present in lesser amounts in other extrahepatic tissues (11, 12). In 1985, Mafune et al. (13) reported that rabbit antisera against rat liver arginase showed direct cytotoxic effects and antibody-dependent cell-mediated cytotoxicity against primary cultured hepatocytes of rat. They suggested that autoantibodies to liver-type arginase may have an important role in the pathogenesis of AIH. In this study, we developed an ELISA that uses recombinant human liver-type arginase as an antigen for the detection of anti-arginase autoantibodies in patients with AIH.

Materials and Methods

Patients

Serum samples were obtained from 11 patients with definite AIH, 31 patients with probable AIH, 20 patients with hepatitis C (HCV), 23 patients with hepatitis B (HBV), 10 patients with other autoimmune diseases, and 55 healthy controls. We obtained informed consent from all patients. The mean ages and sex distribution are summarized in Table 1. The mean ages of the various patient groups were not significantly different from those of the controls. Diagnosis of AIH was made by the scoring system of the International Autoimmune Hepatitis Group (1). Definite AIH patients had scores ≥16, and probable AIH patients had scores of 10–15 before treatment. Two patients with definite AIH and two other patients with probable AIH had been receiving steroid therapy at the time of serum sampling, but the rest of the patients had received no immunosuppressive therapy before venesection. Two of the 31 patients with probable AIH had HCV infections. HBV infection was diagnosed by positive reaction in an enzyme immunoassay (Abbott AxSYM HBsAg; Abbott Diagnostics) for hepatitis B surface antigen. HCV hepatitis was diagnosed by positive reaction in an enzyme immunoassay (Abbott AxSYM III; Ortho Clinical Diagnostics) for hepatitis C Ab. HCV hepatitis 20 57.2 10.8 13/7 0.515 0.444 0.0053

Hepatitis B virus (HBV) infection was diagnosed by positive reaction in an enzyme immunoassay (Abbott Diagnostics) for hepatitis B surface antigen. Two of the 31 patients with probable AIH had been receiving steroid therapy at the time of serum sampling, but the rest of the patients had received no immunosuppressive therapy before venesection. Two of the 31 patients with probable AIH had HCV infections. HBV infection was diagnosed by positive reaction in an enzyme immunoassay (Abbott AxSYM HBsAg; Abbott Diagnostics) for hepatitis B surface antigen. HCV hepatitis was diagnosed by positive reaction in an enzyme immunoassay (Abbott AxSYM III; Ortho Clinical Diagnostics) for hepatitis C Ab. HCV hepatitis 20 57.2 10.8 13/7 0.515 0.444 0.0053

Hepatitis C virus (HCV) infection was diagnosed by positive reaction in an enzyme immunoassay (Ortho HCV Ab ELISA Test III; Ortho Clinical Diagnostics) for hepatitis C Ab. HCV hepatitis 20 57.2 10.8 13/7 0.515 0.444 0.0053

Elisa procedure

One of 20 patients with HCV hepatitis also had a HBV infection. Two of the 31 patients with probable AIH had HCV infections. HBV infection was diagnosed by positive reaction in an enzyme immunoassay (Abbott AxSYM HBsAg; Abbott Diagnostics) for hepatitis B surface antigen. HCV hepatitis was diagnosed by positive reaction in an enzyme immunoassay (Abbott AxSYM III; Ortho Clinical Diagnostics) for hepatitis C Ab. HCV hepatitis 20 57.2 10.8 13/7 0.515 0.444 0.0053

Table 1. Age, sex, and anti-arginase antibodies in patients with hepatitis and autoimmune diseases, and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age, years*</th>
<th>Male/female</th>
<th>Anti-arginase antibody absorbance in ELISA, mean ± SD</th>
<th>P for difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Definite</td>
<td>11</td>
<td>46.6 ± 12.5</td>
<td>1/10</td>
<td>0.503 ± 0.225</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Probable</td>
<td>31</td>
<td>53.9 ± 15.0</td>
<td>9/22</td>
<td>0.625 ± 0.458</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HCV hepatitis</td>
<td>20</td>
<td>57.2 ± 10.8</td>
<td>13/7</td>
<td>0.515 ± 0.444</td>
<td>0.0053</td>
</tr>
<tr>
<td>HBV hepatitis</td>
<td>23</td>
<td>47.3 ± 9.2</td>
<td>18/5</td>
<td>0.395 ± 0.468</td>
<td>0.58</td>
</tr>
<tr>
<td>Other autoimmune disease</td>
<td>10</td>
<td>50.9 ± 14.6</td>
<td>1/9</td>
<td>0.388 ± 0.297</td>
<td>0.1408</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>55</td>
<td>50.5 ± 12.2</td>
<td>21/34</td>
<td>0.277 ± 0.201</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD.
absorbance obtained with pooled serum from healthy subjects was subtracted from each value.

INHIBITION STUDY
Inhibition studies were performed according to the method of Ma et al. (15) with slight modifications. In brief, strips of polyvinylidene difluoride membrane (1.0 × 1.0 cm, Hybond-P; Amersham Pharmacia Biotech) were impregnated with 50 μg of recombinant human arginase. Residual nonspecific reactivity of the strips was then blocked by incubation in blocking solution for 1 h at room temperature. The strips were incubated in patient sera diluted 1:100 in reaction solution for 2 h at room temperature; the procedure was performed in triplicate. As a control, nonimpregnated strips were incubated with diluted serum. Sera preincubated with impregnated or nonimpregnated strips were tested for reactivity against recombinant human arginase by ELISA.

DETECTION OF ANTI-CYTOCHROME P450IID6 ANTIBODY
Anti-cytochrome P450IID6 (CYP2D6) antibodies were detected by a radioligand-binding assay, according to the method reported previously (16, 17). In brief, [35S]methionine-labeled recombinant human CYP2D6 was produced by in vitro transcription and translation using a TNT-coupled reticulocyte lysate system (Promega). Patient sera (diluted 1:50) and labeled human CYP2D6 were incubated overnight at 4 °C. The [35S]methionine-labeled recombinant human CYP2D6-antibody complexes were precipitated with protein A-Sepharose 4FF (Amersham Pharmacia Biotech) for 45 min at room temperature. The quantity of precipitated, labeled CYP2D6 was determined by measuring the radioactivity with a 1450 Micro Beta TRILUX apparatus (Amersham Pharmacia Biotech). The results were expressed as a CYP2D6 index calculated as below:

\[
\frac{\text{cpm of unknown sample} - \text{cpm of pooled serum from controls}}{\text{cpm of positive serum calibrator} - \text{cpm of pooled serum controls}} \times 100
\]

OTHER ASSAYS
Anti-nuclear and anti-smooth muscle antibodies were measured by indirect immunofluorescence assays using commercial kits: the Fluorohepana Test (Medical and Biological Laboratories) for anti-nuclear antibodies and the Fluoro AID-1 Test (Medical and Biological Laboratories) for anti-smooth muscle antibodies.

STATISTICAL ANALYSIS
The Mann–Whitney U-test was used to compare absorbance readings of the patient groups and the control group. \( P < 0.05 \) was considered significant. Pearson correlation coefficient analysis was used to examine the correlation between the titers of anti-arginase antibodies and other indicators.

DILUTION CURVE
Sera obtained from three patients with AIH with positive anti-arginase antibodies were diluted in reaction solution. Eight dilutions were obtained by serial 1:1 dilution of a serum from 1:50. Values are given as means of duplicate determinations. The dilution curve was linear in the dilution range between 50- and 800-fold (Fig. 1). Therefore, we decided to use a serum dilution of 1:100 in subsequent experiments.

PRECISION
In the ELISA for anti-liver-specific arginase antibodies, the within-run coefficient of variation (CV) was 2.3% (n = 7) for sera with high antibody concentrations (mean absorbance, 1.82) and 7.5% (n = 7) for sera with medium antibody concentrations (mean absorbance, 1.11). The between-run CV was 11% (n = 7) for sera with high antibody concentrations and 9.8% (n = 7) for sera with medium antibody concentrations.

INHIBITION STUDY
To exclude the possibility of nonspecific reactions in the ELISA method, an inhibition study was performed. The absorbance values at 450 nm of the serum samples containing positive antibodies were markedly decreased (>45%) after inhibition with recombinant human liver-type arginase (Fig. 2). In contrast, absorbance values were unchanged after inhibition with control membranes that were not impregnated with arginase. Positive antibody reactions in the non-AIH patients were also completely abolished by inhibition with liver-type arginase antigen.

Fig. 1. Serum dilution curve in AIH patients with positive anti-liver-type arginase antibodies (●) and in healthy controls (○).
ANTI-LIVER-TYPE ARGINASE ANTIBODIES IN VARIOUS DISEASES

Using this ELISA, we examined serum samples obtained from patients and compared the results with those of healthy controls. As shown in Table 1, the mean absorbance at 450 nm was significantly higher in patients with AIH, either definite or probable, than in healthy controls; the absorbance at 450 nm was also higher in HCV hepatitis patients, but not patients with HBV hepatitis or other autoimmune diseases, than in healthy controls.

Individual absorbance values are shown in Fig. 3. The upper 95th percentile limit of the absorbance value in healthy controls was calculated by a parametric method after square-root-power transformation of the absorbance value. Values greater than this limit (0.640 at 450 nm) were considered positive. As described above, these positive values became negative after inhibition with liver-type arginase in all samples. Accordingly, 18.2%, 32.3%, 20.0%, 13.0%, and 10.0% of patients were positive for definite AIH, probable AIH, HCV hepatitis, HBV hepatitis, and other autoimmune diseases, respectively. Three of 55 healthy controls and 12 of 42 total patients with AIH had positive anti-arginase antibodies. Thus, the specificity and sensitivity of this ELISA were 96% and 29%, respectively.

RELATIONSHIP BETWEEN ANTI-ARGINASE ANTIBODIES AND OTHER INDICATORS

Anti-arginase antibody titers were compared with serum IgG concentrations (Fig. 4A), anti-nuclear antibody titers (Fig. 4B), and anti-CYP2D6 antibody activity (Fig. 4C) in patients with AIH. None of these indicators was related to the anti-arginase antibody titer. Definitive positive antibody reaction against CYP2D6 was observed in only 2 of 42 (4.8%) patients with AIH. The anti-arginase antibody activity was not related to the presence of anti-smooth muscle antibodies or increased serum concentrations of aspartate aminotransferase or alanine aminotransferase. No relationship was observed between positive anti-arginase antibodies and a family history of AIH or other autoimmune diseases. There were no special clinical characteristics in the patients positive for anti-arginase antibodies.

Discussion

Excellent laboratory tests for autoantibodies, such as anti-thyroid microsomal antibody, anti-thyroid-stimulating hormone receptor antibody, and anti-glutamic acid decarboxylase antibody, enable us to screen for subclinical autoimmune diseases and to predict the clinical onset of disease (18, 19). Thus, we tried to establish a new autoantibody test for specific detection of hepatic autoimmune abnormalities.

Among several autoantigens in the liver, human liver-
Arginase (arginase 1) is specifically expressed in liver cells. Our search of the medical literature revealed no report on autoantibodies to human liver-type arginase. Therefore, we tried to develop an ELISA for anti-human liver-type arginase antibodies, and we succeeded in establishing a methodologically satisfactory ELISA system. Unexpectedly, however, the prevalence of positive reactions was not high enough to detect definite AIH. However, one-third of patients with probable AIH showed positive reactions. This result was not induced by immunosuppressive therapy because only 4 of 42 patients with AIH (2 definitive, 2 probable) were receiving immunosuppressive therapy at the time of serum sampling. In some autoimmune diseases, autoantibodies gradually disappear according to the progress of disease. Our data in this study suggest that anti-arginase antibodies might be detectable in mild forms of AIH, although the difference in antibody occurrence was not statistically significant between the two groups.

Many studies have reported on non-organ-specific autoantibodies; however, little is known about the frequency of autoantibodies to liver-specific antigen(s) within patients with AIH. Only 4.8% of patients with AIH in this study had definitive positive antibodies to CYP2D6. Anti-CYP2D6 antibodies usually are found in patients with type 2 AIH, who constitute 5% of all AIH patients. In previous studies, the prevalence of CYP2D6 antibodies in patients with AIH was reported as <5% (22, 23). Antibodies to soluble liver antigen were found in only 11% of patients with AIH (22). Autoantibodies to asialoglycoprotein receptor, which is expressed on the surface of hepatocytes, were found in 50–80% of patients with AIH. However, positive antibodies were also found in 6.5–73.0% of patients with active HBV hepatitis (24, 25). Thus, the specificity of anti-asialoglycoprotein-receptor antibodies toward AIH apparently is low (25). Among all AIH patients, therefore, it is clear that this ELISA for anti-arginase antibodies is far better than the established assay for anti-CYP2D6 antibody or soluble liver antigen from the point of view of screening for autoimmune hepatic abnormality.

It has been speculated that AIH may be triggered by infection with hepatotropic viruses, and the relationship between HCV infection and AIH is of interest. Several patients with HCV or HBV infection had positive anti-arginase antibodies in this study. This may not have been attributable to nonspecific reactions in the ELISA because the positive reactions became negative after specific inhibition with human liver-type arginase antigen. According to the scoring system of the International Autoimmune Hepatitis Group, the presence of viral infection subtracts two or three points from the total score; thus, patients who had both AIH and viral infections might easily be dropped from the group with AIH. Actually, in this study, no patients with definite AIH had hepatitis B surface antigen or HCV antibody.
study is needed to clarify the clinical significance of positive antibodies in patients with viral hepatitis.

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References