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Autoantibody detection in type 2 autoimmune hepatitis using a chimera recombinant protein

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Abstract

Autoantibodies against cytochrome *P*450 2D6 (CYP2D6), known as anti-liver/kidney microsome type 1 (LKM1) and/or anti-human formiminotransferase cyclodeaminase, formally known as anti-liver cytosol type 1 (LC1) define type 2 autoimmune hepatitis (AIH). The aims of this work are to develop a sensitive and specific test to detect anti-LKM1 and/or anti-LC1 autoantibodies and to establish the prevalence of anti-LC1. Sera from children with type 2 AIH ($n=48$) and those from a control group ($n=100$) were evaluated for anti-LKM1 and anti-LC1 by Enzyme-Linked Immunosorbent Assay (ELISA) and Western blotting. Each serum sample was assayed for reactivity against formiminotransferase cyclodeaminase and CYP2D6 alone or as part of a recombinant chimera protein. By ELISA with recombinant chimera protein, 50 serum samples were positive, 48 from patients with type 2 AIH and 2 from patients with chronic hepatitis C. Twenty-five of 48 (52%) patients studied were positive for both CYP2D6 and LC1 autoantibodies. Anti-LC1, either as the only marker or associated with anti-LKM1, was positive in 34/48 (71%). By Western blotting, anti-LC1 was found in 27/48 (56%) patients. This ELISA technique has proven to be antigen-specific and more sensitive than Western blot for the detection of anti-LC1 and anti-LKM1 autoantibodies. The prevalence of anti-LC1 (71%) confirms it as an important immunomarker in type 2 AIH. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Type 2 autoimmune hepatitis; CYP2D6; LKM1; FTCD; LC1; ELISA; Autoantibodies

1. Introduction

Autoimmune hepatitis (AIH) is a chronic, progressive liver disease that responds well in most of the cases to immunosuppressive therapy, but it is associated with high morbidity and mortality if left untreated (Czaja, 1998). Early and accurate diagnosis is therefore of great importance. Several biological and clinical criteria have been defined, and a scoring system has been proposed by the International Autoimmune Hepatitis Group for probable and definitive diagnosis (Johnson and McFarlane, 1993). This system provides a template for the systematic assessment of AIH and its variants. A definitive diagnosis of AIH requires positive laboratory and histological findings, the absence of viral markers and other causes of chronic liver disease, high gamma-globulin levels, a

Abbreviations: aa, amino acid; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; bp, base pair; CYP2D6, *P*450 2D subfamily; FTCD, formiminotransferase cyclodeaminase; HCV, hepatitis C virus; LC1, liver cytosol type 1; LCHC1, liver cytosol human clone 1; LKM1, liver kidney microsome type 1; PCR, polymerase chain reaction.

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favorable response to immunosuppressive treatment, and, particularly, the presence of characteristic autoantibodies.

Although the role of autoantibodies in the immunopathogenesis of AIH remains unclear, they are important diagnostic criteria (Alvarez et al., 1999). Many autoantibodies have been found in sera associated with AIH; some define patients with distinctive clinical and prognostic features (Homberg et al., 1987; Czaja and Manns, 1995; Czaja, 1999). Type 1 AIH is characterized by the presence of smooth muscle antibody (SMA) and/or anti-nuclear antibody (ANA) in patient sera. Type 2 AIH is associated with anti-liver/kidney microsome antibody type 1 (LKM1) directed against a cytochrome *P*450 of the 2D subfamily, CYP2D6, in humans and/or with anti-liver cytosol antibody type 1 (LC1). The molecular target of anti-LC1 was identified recently as formiminotransferase cyclodeaminase (FTCD) (Lapierre et al., 1999). Although anti-LKM1 is the hallmark of type 2 AIH, anti-LC1 is present in 30% of cases and is the only marker in 10% of patients (Martini et al., 1988). In addition, previous work has shown a good correlation between anti-LC1 titers and hepatocyte injury (Muratori et al., 1998). Anti-LKM1 and anti-LC1 antibodies are also detected in some patients suffering from chronic hepatitis C virus (HCV) infection, although there seem to be some differences in epitope specificity (Lenzi et al., 1995; Durazzo et al., 1995; Parez et al., 1996).

In spite of its importance, the clinical use of some autoantibodies in the diagnosis of AIH has been severely limited by the lack of a single and sensitive diagnostic test (Meyer zum Buschenfelde and Lohse, 1995; Alvarez et al., 1999). Consequently, we developed a new Enzyme-Linked Immunosorbent Assay (ELISA) test using a recombinant chimera protein covering CYP2D6 and FTCD antigenic sites. The method was tested for its specificity in a large serum bank. The results were compared with those obtained by Western blotting analyses. Disease activity, estimated by γ-globulin and transaminase levels, was correlated with ELISA titers. This new test for the detection of anti-LKM1 and anti-LC1 autoantibodies could be useful for the diagnosis and follow-up of patients with type 2 AIH.

2. Materials and methods

2.1. Patient sera

Forty-eight anti-LKM1 and/or anti-LC1-positive sera, detected by Western blotting technique from children with type 2 AIH, were used in this study. Tested by Western blotting in microsomal and cytosolic fractions from rat and human livers (Amar-Costesec et al., 1974), 25 of these sera were positive for anti-LKM1, 14 for anti-LC1, and 9 for both autoantibodies.

AIH was diagnosed according to the criteria defined by the International Autoimmune Hepatitis Group (Johnson and McFarlane, 1993) with aggregate scores greater than 15.

As controls, 100 serum samples were chosen randomly from the following groups: 9 patients with type 1 AIH, 29 patients with chronic HCV, 5 patients with primary biliary cirrhosis, 1 patient with chronic hepatitis B associated with Delta virus infection (LKM3+), 1 patient with macrolide-induced cholestasis, 15 liver transplant recipients, 9 patients with idiopathic chronic active hepatitis, 4 patients with autoimmune disorders, 2 patients with Crohn's disease, 5 family members of type 2 AIH patients, and 16 healthy controls. All sera from AIH patients were obtained before immunosuppressive treatment was begun.

2.2. Cloning of recombinant antigens in expression plasmid

2.2.1. Construction of pMAL-cRI/CYP2D6$_{(672-1377)}$

CYP2D6$_{(672-1377)}$ is a cDNA of 705 base pairs (bp) obtained by polymerase chain reaction (PCR) amplification from the full-length cDNA of human cytochrome *P*450 2D6. cDNA encoding human CYP2D6 and cloned in pGEM 3 (Gueguen et al., 1991) was excised with *Eco*RI, fractionated by agarose gel electrophoresis, and the fragment recovered for use as a template in PCR. A pair of primers allowed us to amplify a fragment of 705 bp covering the CYP2D6 cDNA sequence from 672 bp to 1377 bp downstream of the ATG translation initiation codon.

The oligonucleotide CYP2D6-1: 5′-GGG GTA CCG AAT GTC GTC CCC GTC CTC was used as sense primer, and CYP2D6-2: 5′-GGG ATT CGA

GGG AGG TGA AGA AGA GG as antisense primer. Each forward and reverse primer contained artificial Kpn1 and *Eco*RI sites, respectively, for subcloning the fragment amplified into the bacterial expression vector pMAL-cRI (New England Biolaboratories, Beverly, MA). Totally, 0.1 μg of DNA was amplified in 50 μl of PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$) with 0.2 mM dNTP, 20 pmol of each primer, and 1.25 units of Taq Polymerase (Gibco, Gaithetsburg, MD).

Amplification through 27 cycles was as follows: denaturation at 98 °C for 60 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The PCR product was then gel-purified (QIAquick QIAGEN, Mississauga, ON), digested with the endonucleases *Eco*RI and Kpn1, filled and cloned in pMAL-cRI bacterial expression vector, which allowed the expression of $CYP2D6_{(672-1377)}$ as part of a fusion protein with maltose-binding protein.

2.2.2. Construction of pMAL-cRI/$CYP2D6_{(672-1377)}$+ liver cytosol human clone 1 (LCHC1)

pMAL-cRI/LCHC1 consisted of 653-bp cDNA coding 146 amino acids (aa) from C terminal region of human FTCD ligated into an *Eco*RI site at the polylinker region of pMAL-cRI bacterial expression vector. It was shown previously that autoantibodies present in LC1-positive sera are directed against LCHC1 (Lapierre et al., 1999). The pMAL-cRI/$CYP2D6_{(672-1377)}$ + LCHC1 construct was obtained using pMAL-cRI/LCHC1 and pMAL-cRI/$CYP2D6_{(672-1377)}$ (described above) and made as follows. The entire LCHC1 was excised from pMAL-cRI with endonuclease *Eco*RI, purified by gel electrophoresis, and ligated in the *Eco*RI site downstream of $CYP2D6_{(672-1377)}$. The chimera protein was expressed as described above. The molecular mass of the new fusion protein was 84.5 kDa, as established by SDS–PAGE.

2.3. DNA sequencing

The reading frame and identity of major epitopes in the pMAL-cRI/$CYP2D6_{(672-1377)}$ + LCHC1 construct were validated by plasmid sequencing. Sequencing of double-stranded DNA template was achieved with the dideoxy chain termination method using Sequenase version 2.0 T7 DNA polymerase (Amersham Life Science). Specifically designed oligonucleotides primers were employed to sequence our construct.

2.4. Expression and purification of recombinant fusion proteins

2.4.1. Expression of pMAL-CrI/$CYP2D6_{(672-1377)}$ recombinant fusion protein

Briefly, the pMAL-cRI/$CYP2D6_{(672-1377)}$, pMAL-cRI/$CYP2D6_{(672-1377)}$ + LCHC1 and pMAL-cRI/LCHC1 construct were transfected in *Escherichia coli* strain BL21-Gold (Stratagene, La Jolla, CA), and the transformed cells were isolated on selective plates containing Ampicillin. Some colonies were isolated and analyzed for the expression of the fusion protein. After exponential growth in liquid culture medium (LB/Ampicillin), expression was induced by adding isopropyl-1-beta-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 3 h at 37 °C with shaking. After 90 min, the cells were collected by centrifugation and the pellet resuspended in column buffer (20 mM Tris–HCl, pH 7.4, 0.2 mM NaCl and 1 mM EDTA). These solutions were incubated overnight at 4 °C with 1 ml of maltose-resin (New England Biolaboratories) with gentle agitation. The maltose-resin was loaded onto a 0.8 × 4-cm chromatography column (Bio-Rad Laboratories, Richmond, CA). The column was washed with 12 volumes of column buffer and proteins were eluted with column buffer/10 mM maltose. The different fractions obtained were analysed by SDS–PAGE.

2.5. Western blot analysis

For Western blot analysis, 0.2 μg of recombinant proteins was separated by 10% SDS–PAGE and transferred onto nitrocellulose filters, as described previously (Towbin et al., 1979). All sera tested were used at 1:1000 dilution as the first antibody. The second antibody was rabbit anti-human IgG conjugated to peroxidase (Biosource International). Bound peroxidase was detected by chemiluminescense blotting substrate (Roche Mannheim).

2.6. ELISA

Microwell ELISA plates (Corning, New York, NY) were coated, overnight at 4 °C with 0.2 μg of

the purified fusion proteins $CYP2D6_{(672-1377)}$, LCHC1, $CYP2D6_{(672-1377)}$ + LCHC1 and maltose-binding protein in 0.1 M $NaHCO_3$ (pH 8.6). The plates were subsequently washed twice with TBST and incubated with 200 μl of blocking solution (TBST/5% bovine serum albumin) at 37 °C for 1 h. After six washes, the plates were incubated with 100 μl of diluted serum (1:200 to 1:25,600) at 37 °C for 1 h, re-washed six times and incubated with alkaline phosphatase-conjugated anti-human IgG at 1:3000 dilution (Sigma-Aldrich Canada, Oakville, ON), as secondary antibody, for 1 h at 37 °C. Alkaline phosphatase activity retained in the wells was assayed by the addition of 100 μl of *p*-nitrophenyl phosphate (Sigma-Aldrich) and measured at 405 nm by a microtiter plate reader. In each assay, some strong positive and several negative sera were included as controls. Nonspecific background absorbance obtained with diluted sera incubated with maltose-binding protein was subtracted as background. Optical Densities (ODs) exceeding 2.0 times the mean OD of negative controls at each dilution assayed were considered positive. The optimal antigen concentration for ELISA was obtained by measuring ODs versus different concentrations of recombinant fusion proteins. The mean OD of antibody reactivity was linear over a range of 0.1–0.8 μg/well. The highest OD with the least nonspecific background (measured by reading the OD of sera incubated with maltose-binding protein) was obtained at the concentration of 0.2 μg/well.

2.7. Inhibition study

To exclude the possibility of nonspecific reaction with the ELISA method, an inhibition study was conducted by pre-incubating 100 μl of diluted 1:100 sera with 2.5 μg of $CYP2D6_{(672-1377)}$ + LCHC1 recombinant chimera protein and maltose-binding protein (as a control of nonspecific reactivity) overnight at 4 °C. The procedure was performed in triplicate for each serum sample. Sera pre-incubated with recombinant fusion protein, sera pre-incubated with maltose-binding protein and the same sera diluted but not pre-incubated were then tested, at the same time, for reactivity against recombinant proteins by ELISA and Western blotting.

2.8. Statistical analysis

Group data were compared with the Mann–Whitney *U*-test and Fisher's exact probability test. $P < 0.05$ was considered significant. Correlation between two different variables was assessed by the least square method.

3. Results

3.1. Plasmid sequencing

Sequence analysis of the pMAL-cRI/$CYP2D6_{(672-1377)}$ + LCHC1 construct confirmed that the orientations of the two cDNA inserts in pMAL-cRI expression vector were correct and that the reading frame of the inserts extended that of the maltose-binding protein. Plasmid sequencing confirmed the presence of the main epitopes on human CYP2D6 and the C terminal region of human FTCD.

3.2. Anti-$CYP2D6_{(672-1377)}$+LCHC1 autoantibodies in various diseases

Of the 148 serum samples screened by ELISA using $CYP2D6_{(672-1377)}$ + LCHC1 chimera protein, 50 were found to be positive (Table 1). Forty-eight of these ELISA-positive serum samples came from patients fulfilling standard diagnostic criteria for AIH and characterized as type 2 AIH by Western blotting using microsomal and cytosolic fractions from rat and human livers.

Two of the 50 ELISA-positive samples, showing low antibody titers, were detected in the group of patients with chronic HCV infection. No sera from the 16

Table 1
Sensitivity of ELISA binding to CYP2D6, FTCD or the chimera protein CYP2D6 + LCHC1 by sera from different groups of patients

Antigen	Type 2 AIH ($n = 48$)	Control ($n = 100$)
CYP2D6 + LCHC1	48 (100%)	2 (2%)
CYP2D6	39 (81%)	2 (2%)
LCHC1	34 (71%)	0 (0%)

Groups tested: type 2 AIH, type 1 AIH, HCV (chronic hepatitis C virus infection), PBC (primary biliary cirrhosis) healthy controls, others (idiopathic chronic active hepatitis, Crohn's disease, chronic hepatitis B virus infection, other autoimmune disorders).

Table 2
Sensitivity of ELISA and Western blotting to CYP2D6 and/or LCHC1 autoantigens in type 2 AIH patients

	CYP2D6 + LCHC1 (n = 48)	CYP2D6 (n = 48)	LCHC1 (n = 48)
Western blotting (microsomes/cytosol-enriched fractions)	48 (100%)	34 (71%)	23 (47%)
Western blotting (recombinant proteins)	48 (100%)	36 (75%)	27 (56%)
ELISA	48 (100%)	39 (81%)	34 (71%)

healthy controls reacted against $CYP2D6_{(672-1377)}$ + LCHC1 recombinant chimera protein. The results were also negative with sera from 82 patients with type 1 AIH, other autoimmune diseases, idiopathic chronic active hepatitis, chronic active hepatitis B or C or Crohn's disease. The sensitivity of this ELISA to diagnose type 2 AIH using $CYP2D6_{(672-1377)}$ + LCHC1 recombinant chimera protein was 100% (48/48), with specificity ranging from 98% to 100%, including the two patients chronically infected by HCV.

3.3. Titer and nature of autoantibodies detected by ELISA

Patient sera were tested by ELISA for reactivity against recombinant fusion proteins $CYP2D6_{(672-1377)}$, LCHC1 and $CYP2D6_{(672-1377)}$ + LCHC1 at dilutions ranging from 1:200 to 1:25,600. The mean positive titer was 1:9000 (range 1:200–1:25,600) for $CYP2D6_{(672-1377)}$ autoantibody and 1:6000 (range 1:200–1:25,600) for anti-LC1. Sera tested against $CYP2D6_{(672-1377)}$ + LCHC1 had a mean titer of 1:16,000 (range 1:400–1:25,600). By ELISA, 25/48 positive samples showed concurrence of anti-LKM1 and anti-LC1 antibodies, 14/48 were positive for anti-LKM1 and 9/48 for anti-LC1. The ELISA data indicate that when only one autoantibody was found, the titer for this marker was identical to that obtained against $CYP2D6_{(672-1377)}$ + LCHC1 chimera protein with the same serum. According to this result, antigenic site accessibility was similar in $CYP2D6_{(672-1377)}$, LCHC1 and $CYP2D6_{(672-1377)}$ + LCHC1 recombinant proteins as tested by ELISA.

Antibodies measured by ELISA were compared with Western blotting reactivity, using either human liver microsomes/cytosol-enriched fractions or the same recombinant antigen employed in ELISA (Table 2). Tested by ELISA, 25/48 (52%) sera were found to be positive against both CYP2D6 and LCHC1. Anti-LC1, as the only marker or associated with LKM1 antibody, was found in 34/48 (71%) type 2 AIH patient sera. When these 48 positive sera were studied by Western blotting using liver microsomes/cytosol-enriched fractions, only 9/48 (19%) samples exhibited reactivity against both autoantibodies, and the prevalence of anti-LC1 was 23/48 (47%). Using recombinant fusion proteins to test reactivity by Western blotting, 15/48 (31%) positive sera were positive for both autoantigens, and the prevalence of anti-LC1 antibody in sera of type 2 AIH patients was 27/48 (56%) Altogether, these results showed that ELISA was more sensitive, allowing low titers of either autoantibody to be detected. The sensitivity of traditional Western blotting could be improved by using recombinant fusion proteins to test anti-LKM1 and/or anti-LC1 antibodies. Anti-LC1 antibody, as tested by ELISA, was found in 71% of patients in the group with type 2 AIH. The results and predictive value of the ELISA test using the chimera fusion protein are summarised in Table 3.

3.4. Relationship between autoantibodies detected by ELISA and other indicators

ELISA titers using $CYP2D6_{(672-1377)}$ + LCHC1 recombinant chimera protein were compared with IgG concentration and aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels in sera of type 2 AIH patients obtained before immunosuppressive treatment. Patient sera with titers higher than 1:1600 showed a mean IgG concentration of 32.5 g/l, whereas sera with titers lower than 1:1600 had a mean IgG concentration of 23.4 g/l. No correlation

Table 3
Predictive value of ELISA analysis

	Disease (AIH)	Normal control	Total
Positive test (ELISA)	48	2	50
Negative test (ELISA)	0	98	98
Total	48	100	148

Sensitivity: 48/48 = 100%; specificity: 98/100 = 98%.
Positive predictive value: 48/50 = 96%.
Negative predictive value: 98/98 = 100%.

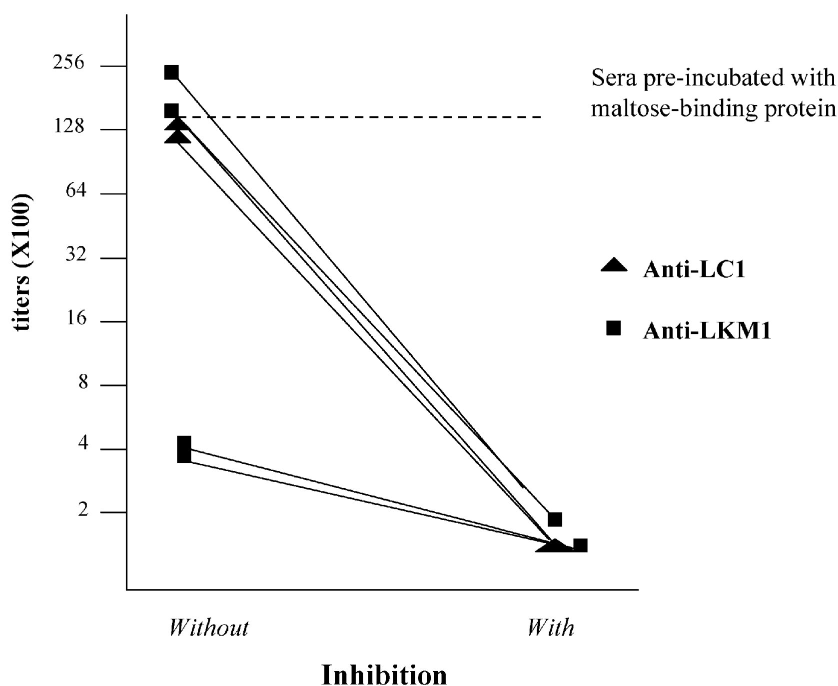

Fig. 1. Inhibition test with ELISA. Antibody binding of tested sera was inhibited by pre-incubation with recombinant chimera protein. In contrast, the titers remained unchanged by prior incubation with maltose-binding protein alone.

was found between autoantibody titers and AST or ALT levels, which were elevated (5–20 times above normal values) in the sera of all patients.

3.5. Inhibition study

An inhibition study was performed to assess the specificity of this ELISA method. More than a 90% drop in autoantibody titers was observed after pre-incubation with $CYP2D6_{(672-1377)}$ + LCHC1 recombinant chimera protein in four serum samples containing high levels of anti-LKM1, anti-LC1 or both antibodies and in two sera with low anti-LKM1 titers belonging to the group of patients with chronic HCV infection (Fig. 1). In contrast, titers of these six sera were unchanged, at the same dilutions after pre-incubation with maltose-binding protein.

4. Discussion

Even though anti-LKM1 (anti-CYP2D6) antibody is the hallmark of type 2 AIH, anti-LC1 is a liver-specific autoantibody detectable either alone or in association with LKM1 in a significant proportion of patients with type 2 AIH. There is ample evidence of anti-LKM1 as an immunomarker, but previous works show that the prevalence of anti-LC1 autoantibody associated with type 2 AIH is variable (Martini et al., 1988; Han et al., 1995; Muratori et al., 1995, 1998). The sensitivity of different detection methods could explain this variability. Anti-LC1 antibody is frequently missed in routine immunofluorescence testing since its specific staining pattern is masked when there is anti-LKM1 autoantibody in the same serum (Abuaf et al., 1992). Consequently, the prevalence of this marker in type 2 AIH could have been underestimated along with its importance as an immunomarker.

The availability of cDNA coding for CYP2D6 and FTCD autoantigens makes it possible to develop new specific diagnostic tests. The $CYP2D6_{(672-1377)}$ sequence encodes aa 224 to 454 of human cytochrome *P*450 2D6. The CYP2D6 region used in our ELISA was analyzed and identified previously as the most immunoreactive portion of human *P*450 2D6 (Gue-

guen et al., 1991; Yamamoto et al., 1993). The four main linear epitopes recognized by anti-LKM1 sera on human CYP2D6 are localized among aa 254–271, 321–371, 373–389, and 410–428. The current "gold standard" technique for the detection of anti-LC1 is the immunoprecipitation (Abuaf et al., 1992; Muratori et al. 1995). Recently, it was described by Lapierre et al. (1999) that all the anti-LC1 antibodies detected by immoprecipitation recognize the LCHC1 cDNA sequence encoding the 146 aa from the C terminal region of human FTCD. This result showed that the 146 aa C terminal region is enough to detect all anti-LC1 positive sera.

This study, using recombinant fusion proteins in ELISA to detected autoantibodies, showed anti-LC1 autoantibody prevalence as high as 70% and confirmed that it is a marker closely associated with anti-LKM1 in type 2 AIH. The ELISA described here is a quantitative procedure applicable to the diagnosis and monitoring of type 2 AIH. It appears to be specific for anti-LKM1 and anti-LC1 autoantibody detection, more sensitive than Western blotting and technically simple. Although the sensitivity of traditional Western blotting technique was improved by testing reactivity with recombinant fusion proteins, ELISA remained the most sensitive technique for detecting anti-LKM1 and anti-LC1 antibodies. The titers of anti-LC1 antibody were lower than those of anti-LKM1 antibody. As a consequence, the improved sensitivity of the ELISA technique using recombinant proteins raised the prevalence of this autoantibody in the group of type 2 AIH patients studied.

Some works have correlated anti-LC1 autoreactivity with hepatocyte injury (Muratori et al., 1998). According to its sensitivity and specificity, our proposed quantitative ELISA appears useful to study the changes in anti-LC1 and anti-LKM1 autoantibody titers to follow disease activity and the response to immunosuppressive treatment.

When ELISA is compared to immunofluorescence, counterimmunoelectrophoresis and Ouchterlony double diffusion to detect anti-LC1, it has a further advantage, which is an easier interpretation of the results, providing a nonsubjective readout. The results are quantitatively expressed and related to specific autoantibody titers.

Two patients with chronic hepatitis C presented low titers (1:400) for $CYP2D6_{(672-1377)}$ and for $CYP2D6_{(672-1377)}$ + LCHC1 recombinant chimera protein by ELISA. Including these two sera in the positive reaction group, specificity of the ELISA method for diagnosis of type 2 AIH could be measured as 98%. However, it is well-known that anti-LKM1 can be detected in sera from patients infected with HCV (Lenzi et al., 1990, 1991; Herzog et al., 1999). Analysis of reactivity reveals that sera from patients with chronic HCV infection can develop antibodies against linear and conformational epitopes of CYP2D6 (Durazzo et al., 1995; Parez et al., 1996). Therefore, a positive reaction in the sera of patients infected with HCV should be considered as a true positive. In addition, the reactivity of these sera was inhibited by pre-incubation with $CYP2D6_{(672-1377)}$ + LCHC1 chimera protein. Accordingly, the specificity of the ELISA for anti-CYP2D6/LC1 detection could be interpreted as 100%.

Taking all these results into consideration, we believe that this new ELISA test combines the specificity of molecular biology techniques through the use of recombinant antigens to the inherent sensitivity of ELISA technology. We strongly feel that the test will improve type 2 AIH diagnosis in clinical laboratories.

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References

Abuaf, N., Johanet, C., Chretien, P., Martini, E., Soulier, E., Laperche, S., Homberg, J.C., 1992. Characterization of the liver cytosol antigen type 1 reacting with autoantibodies in chronic active hepatitis. Hepatology 16, 892.

Alvarez, F., Berg, P.A., Bianchi, F.B., Bianchi, L., Burroughs, A.K., Cancado, E.L., Chapman, R.W., Cooksley, W.G., Czaja, A.J., Desmet, V.J., Donaldson, P.T., Eddleston, A.L., Fainboim, L., Heathcote, J., Homberg, J.C., Hoofnagle, J.H., Kakumu, S., Krawitt, E.L., Mackay, I.R., MacSween, R.N., Maddrey, W.C., Manns, M.P., McFarlane, I.G., Meyer zum Buschenfelde, K.H., Zeniya, M., et al., 1999. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. J. Hepatol. 31, 929.

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., Berthet, J., 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver.

Preparation and composition of microsomal fraction. J. Cell Biol. 61, 201.

Czaja, A.J., 1998. Treatment of autoimmune hepatitis. In: Krawitt, E.L., Wiesner, R.H., Nishioka, M. (Eds.), Autoimmune Liver Diseases, 2nd edn. Elsevier, Amsterdam, p. 499.

Czaja, A.J., 1999. Behaviour and significance of autoantibodies in type 1 autoimmune hepatitis. J. Hepatol. 30, 394.

Czaja, A.J., Manns, M.P., 1995. The validity and importance of subtypes in autoimmune hepatitis: a point of view. Am. J. Gastroenterol. 90, 1206.

Durazzo, M., Philipp, T., Van Pelt, F.N., Luttig, B., Borghesio, E., Michel, G., Schmidt, E., Loges, S., Rizzetto, M., Manns, M.P., 1995. Heterogeneity of liver–kidney microsomal antibody in chronic hepatitis C and D virus infection. Gastroenterology 108, 455.

Gueguen, M., Boniface, O., Bernard, O., Clerc, F., Cartwright, T., Alvarez, F., 1991. Identification of main epitope on human cytochrome P450 IID6 recognized by anti-liver kidney microsome antibody. J. Autoimmun. 4, 607.

Han, S., Tredger, M., Gregorio, G.V., Mieli-Vergani, G., Vergani, D., 1995. Anti-liver cytosolic antigen type 1 (LC1) antibodies in childhood autoimmune liver disease. Hepatology 21, 58.

Herzog, D., Yamamoto, A.M., Jara, P., Maggiore, G., Sarles, J., Alvarez, F., 1999. Sera of children with hepatitis C infection and anti-liver–kidney microsome 1 recognize different CYP2D6 epitopes than adults with LKM+/HCV + . J. Pediatr. Gastroenterol. Nutr. 29, 551.

Homberg, J.C., Abuaf, N., Bernard, O., Islam, S., Alvarez, F., Khalil, S.H., Poupon, R., Darnis, F., Levy, V.G., Grippon, P., et al., 1987. Chronic active hepatitis associated with anti-liver/kidney microsome antibody type 1: a second type of "autoimmune" hepatitis. Hepatololgy 7, 1333.

Johnson, P.J., McFarlane, I.G., 1993. Meeting report: International Autoimmune Hepatitis Group. Hepatology 18, 998.

Lapierre, P., Hajoui, O., Homberg, J.C., Alvarez, F., 1999. Formiminotransferase cyclodeaminase is an organ-specific autoantigen recognized by sera of patients with autoimmune hepatitis. Gastroenterology 116, 643.

Lenzi, M., Ballardini, G., Fusconi, M., Cassani, F., Selleri, L., Volta, U., Zauli, D., Bianchi, F.B., 1990. Type 2 autoimmune hepatitis and hepatitis C virus infection. Lancet 335, 258.

Lenzi, M., Johnson, P.J., McFarlane, I.G., Ballardini, G., Smith, H.M., McFarlane, B.M., Bridger, C., Vergani, D., Bianchi, F.B., Williams, R., 1991. Antibodies to hepatitis C virus in autoimmune liver disease: evidence for geographical heterogeneity. Lancet 338, 277.

Lenzi, M., Manotti, P., Muratori, L., Cataleta, M., Ballardini, G., Cassani, F., Bianchi, F.B., 1995. Liver cytosolic 1 antigen-antibody system in type 2 autoimmune hepatitis and hepatitis C virus infection. Gut 36, 749.

Martini, E., Abuaf, N., Cavalli, F., Durand, V., Johanet, C., Homberg, J.C., 1988. Antibody to liver cytosol (anti-LC1) in patients with autoimmune chronic active hepatitis type 2. Hepatology 8, 1662.

Meyer zum Buschenfelde, K.H., Lohse, A.W., 1995. Autoimmune hepatitis. N. Engl. J. Med. 333, 1004.

Muratori, L., Cataleta, M., Muratori, P., Manotti, P., Lenzi, M., Cassani, F., Bianchi, F.B., 1995. Detection of anti-liver cytosol antibodies type 1 (anti-LC1) by immunodiffusion, counter immunoelectrophoresis and immunoblotting: comparison of different techniques. J. Immunol. Methods 187, 259.

Muratori, L., Cataleta, M., Muratori, P., Lenzi, M., Bianchi, F.B., 1998. Liver kidney microsomal antibody type 1 and liver cytosol antibody type 1 concentrations in type 2 autoimmune hepatitis. Gut 42, 721.

Parez, N., Herzog, D., Jacqz-Aigrain, E., Homberg, J.C., Alvarez, F., 1996. Study of the B cells response to cytochrome P450II6 in sera from chronic hepatitis C patients. Clin. Exp. Immunol. 106, 336.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets. Proc. Natl. Acad. Sci. U. S. A. 76, 4350.

Yamamoto, A.M., Cresteil, D., Boniface, O., Clerc, F.F., Alvarez, F., 1993. Identification and analysis of cytochrome P450IID6 antigen sites recognized by anti-liver–kidney microsome type 1 antibodies (LKM1). Eur. J. Immunol. 23, 1105.