A New Epitope of CYP2D6 Recognized by Liver Kidney Microsomal Autoantibody from Japanese Patients with Autoimmune Hepatitis

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Liver-kidney microsomal antibodies type 1 (LKM-1) are a diagnostic marker for autoimmune hepatitis type II (AIH-II). However, LKM autoantibodies are also detected in a small percentage of patients with chronic hepatitis C. The autoantigen to anti-LKM-1 has been identified to be CYP2D6. To identify the specific antigenic site of CYP2D6 for LKM-1 serum, we established an ELISA with peptides spanning the entire sequence of CYP2D6. Human CYP2D6 containing histidine tag was expressed in *Escherichia coli*. Purified CYP2D6 was digested by lysyl endopeptidase. The linker including the histidine tag has a lysine residue in its C-terminal and can be removed by digestion. Digested peptides were separated by reversed-phase HPLC and coated on ELISA plates chemically with glutaraldehyde. The immunoreactivity of two LKM-1-positive sera (HCV-negative) and five HCV-positive sera from Japanese patients was investigated with the plates. These sera recognized peptides 1–146, 181–214, 246–281, 284–391, and 412–429. The peptide 1–146 was recognized by LKM-1-positive sera but not HCV-positive sera and is a new epitope found in this study. Seven short peptides spanning peptide 1–146 were synthesized and ELISAs were conducted with these peptides. However, two sera recognized none of these peptides, suggesting that two LKM-1-positive sera recognize the conformational immunogenic site of peptide 1–146.

Key words liver kidney microsomal (LKM) antibody; CYP2D6; cytochrome P450; epitope analysis

Autoantibodies to liver-kidney microsomes (LKM) were first characterized by immunofluorescence microscopy using rat liver and kidney sections.¹⁾ These autoantibodies have been classified into three groups (anti-LKM-1, 2, and 3) according to differences in immunofluorescent staining patterns.^{1–3)} Anti-LKM-1 is found in a subgroup of patients with autoimmune hepatitis (AIH) II and the presence of LKM-1 antibodies is one of the most important criteria for the diagnosis of AIH-II. But anti-LKM-1 is not completely specific to AIH-II. LKM-1 antibody is also produced in the course of chronic hepatitis (CH) C and is detected in about 2–10% of CHC patients.^{4,5)} In the case of AIH-II, there is no evidence of HCV infection. The pathological profile of AIH-II is quite different from that of CHC. Patients with AIH-II are young and responsive to immunosuppressive drugs. In contrast, patients with CHC tend to be older and have different pathological conditions. Manns *et al.*⁶⁾ suggested that molecular mimicry between the LKM-1 antigen and CHC viral antigen causes LKM-1 antibody to be produced in CHC patients and the presence of LKM-1 antibody does not affect the clinical and biochemical profiles of CHC. In Japan, patients with AIH-II are very rare while there are many patients with CHC, implying that LKM-1-positive patients with CHC outnumber those with AIH-II.⁷⁾ Therefore, it is very important to detect AIH-II specifically.

The major autoantigen for LKM-1 has been identified as CYP2D6 which is an isoform of cytochrome P450s and has an important role in the metabolism of many drugs in the liver.^{8,9)} The mechanism of production of LKM-1 antibodies remains unclear but ethnic differences among patients with AIH-II may provide a clue.¹⁰⁾ CYP2D6 reveals extensive ge-

netic polymorphism and catalytic activity toward a range of drugs. About 10% of Caucasians have a complete deletion of the CYP2D6 gene but less than 1% of Japanese have such a mutation.^{11,12)} In Japan, patients with AIH-II are very rare.⁷⁾ There may be a relationship between genetic polymorphisms in CYP2D6 and AIH-II in Japanese.¹⁰⁾ LKM-1 antibody recognition sites has been identified in the internal amino acid sequence in CYP2D6. The sequence 257–269 of CYP2D6 was found to be the core of an immunodominant epitope recognized by most anti-LKM-1.^{6,9)} The antibody also recognizes the amino acid sequences 196–218 and 321–351.^{9,13)} The antibody from CHC also recognizes some of these sequences.

In this study, we systemically investigated epitopes of CYP2D6 recognized specifically by LKM-1 antibody but not CHC antibody to develop a method of distinguishing LKM-1 antibody from CHC antibody. A full-length CYP2D6 was expressed in *Escherichia coli* and purified. CYP2D6 was digested by several proteases. The resulting peptides were separated by HPLC and each peptide fragment was reacted with LKM-1 and CHC antibodies. The sequence of each peptide was determined and a specific epitope for LKM-1 antibody was identified.

MATERIALS AND METHODS

Patient Sera Sera were collected from patients who admitted to the Osaka City University Medical School Hospital. The sera were taken after informed consent was obtained from the patients and their families. The entire study protocol was approved by the Institutional Human Investigational

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Table 1. Amino Acid Sequences of Peptides Used for ELISA

Peptide No.	Origin	Sequence No.	Amino acid sequence
0	Linker		MRGSHHHHHHGSACELGTPGRPAAKLKKMG
1	CYP2D6	1—30	MGLEALVPLAVIVAIFLLVLDLMHRRQRWA
2	CYP2D6	21—50	DLMHRRQRWAARYPPGPLPLPGLGNLLHVD
3	CYP2D6	41—70	PGLGNLLHVDVFQNTPYCFDQLRRRFGDVFS
4	CYP2D6	61—90	LRRRFGDVFSQLAWTPVVVLNGLAAVREA
5	CYP2D6	81—110	LNGLAAVREALVTHGEDTADRPVPITQIL
6	CYP2D6	101—130	RPPVPITQILGFGPRSQGVFLARYGPAWRE
7	CYP2D6	121—150	LARYGPAWREQRRFSVSTLRNLGLGKKSLE
8	CYP2D6	254—271	LLTEHRMTWDPAQPPRDL

The linker includes a histidine tag indicated by an underline and the first methionine of CYP2D6 indicated in bold. The linker can be removed from purified CYP2D6 by lysyl endopeptidase treatment as it has a lysine residue before the methionine of CYP2D6.

Committee of Osaka City University. Sera from 388 patients who had HCV-positive antibody or HCV RNA were screened with a LKM-1 antibody detection kit (MBL, Tokyo, Japan) and 17 sera reacted with LKM-1 antigen. Five of 17 sera, which reacted with whole CYP2D6 in an enzyme-linked immunosorbent assay (ELISA), were used for the present study.

Expression and Purification of Human CYP2D6 The isolation of human CYP2D6 cDNA was described previously.¹⁴ Full-length CYP2D6 cDNA in pBluescript was cut out with HindIII and subcloned into pQE81L (Qiagen, Hilden, Germany). CYP2D6 which has six histidines (His tag) in its N-terminal was expressed in DH5 α *E. coli* cells cultured with a large scale chamber, Jar Fermentar (K0812, Able Tokyo, Japan). The expressed CYP2D6 was purified on a Ni NTA-agarose column (Qiagen). The expression and purification of CYP2D6 was done according to the manufacturer's recommendations.

Digestion of CYP2D6 by Protease Purified CYP2D6 (480 μ g) was digested with lysyl endopeptidase (0.12 units, Wako Pure Chemical Co., Tokyo, Japan) in 100 mM Tris-HCl (pH 9.0) at 37°C overnight. Digested peptides were separated on a reversed-phase column (5C18 HG, 4.6 $\times$ 150 mm, Wako). Elution was done at a flow rate of 1.0 ml/min with a linear gradient from an aqueous solution containing 10% acetonitrile and 0.5% TFA to one containing 70% acetonitrile and 0.5% TFA for 50 min. Fractions were collected every minute and lyophilized. The resulting residues were used for analysis of the N-terminal amino acid sequence and for ELISA.

ELISA An ELISA plate (amino-type Sumilon) was purchased from Sumitomo Bakelite Co. (Tokyo, Japan). The peptides listed in Table 1 were synthesized by Operon Biotechnologies (Tokyo, Japan). Peptides separated by HPLC or newly synthesized were dissolved in PBS. The ELISA plate was treated with 2.5% glutaraldehyde (Wako) for 2 h at room temperature. After a wash with H₂O, peptide solution (1 μ g) was added to the wells of an ELISA plate and stood at 4°C overnight. Sera from patients were diluted with PBS containing 0.05% Tween 20 and 0.2% BSA (buffer A) 500 to 1000-fold and added to the wells of the plate. After a wash with buffer A, the secondary antibody conjugated by peroxidase, anti-human IgG (Bio-Rad Laboratories, Hercules, CA, U.S.A.), was added to the well. *o*-Phenylene diamine was used as a substrate for peroxidase. Immunoreactivity was measured by a plate reader at 492 nm. Western blotting was done with a vectastain ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) as described previously.¹⁵ Human

hepatic microsomes were purchased from T-Cubed Co. (Edison, NJ, U.S.A.) and human P450s were purified as described previously.¹⁵ The prestained marker protein for Western blotting was obtained from Daiichi Pure Chemicals Co. (Tokyo, Japan).

RESULTS

Immunoreactivity of LKM-1-Positive Serum with P450s First we investigated the specificity of two LKM-1-positive sera (but HCV-negative) for P450 isoforms (patient serum No. 1 and 2 in Table 2). The purified human P450s, CYP1A1, 1A2, 2A6, 2B6, 2D6, 2E1, 2C8, 2C9, 2C18, and 3A4, were subjected to Western blotting. The two sera gave the same results and the result of serum No. 1 is shown in Fig. 1A. The LKM-1-positive sera from these two patients only reacted with CYP2D6 of the eleven P450 isoforms used in this study. Human and rat hepatic microsomes were separated by SDS-polyacrylamide gel electrophoresis and immunoblotting was done with serum No. 1 (Fig. 1B). In this immunoblotting, the two sera gave similar patterns. Several bands including the CYP2D6 appeared. The molecular weight of proteins on Western blotting was calculated to 48, 67, and 92 kDa. The 48-kDa protein corresponds to CYP2D6. The 67-kDa and 92-kDa proteins are unknown.

Immunoreactivity of LKM-1-Positive Serum with Peptides from CYP2D6 To identify the epitope of LKM-1 antibody, we performed an ELISA using digested peptides of CYP2D6. CYP2D6 which had a linker peptide including a His-tag in its N-terminal region (shown in Table 1) was expressed in *E. coli*. Purified CYP2D6 was digested by lysyl endopeptidase. The linker peptide has a lysine residue in its C-terminal and can be removed from purified CYP2D6 by lysyl endopeptidase. The peptide fragments were separated by reversed-phase HPLC. Each fraction was collected and coated on ELISA plates with glutaraldehyde. The reactivity of LKM-1 serum was evaluated by ELISA. The results are shown in Fig. 2. The theoretical digestion pattern of CYP2D6 is indicated in Fig. 3. CYP2D6 is separated into eleven peptides. Two LKM-1-positive, HCV-negative sera (patient No. 1 and 2) and LKM1-positive, HCV-positive serum (patient No. 5) were used for the ELISA. Two sera (No. 1 and 2) gave the same results. The results for No. 1 and 5 are shown in the inset of Fig. 2. No. 1 revealed three peaks (a, b and c) and No. 5, two peaks (d and e) on ELISA. The corresponding fractions from HPLC were analyzed by a protein sequencer to identify the peptide. The peaks a, b, c, d, and e included

Fig. 1. Immunoblot Analysis of LKM-1-Positive (But HCV-Negative) Sera

(A) Immunoblot with purified P450s. Patient serum (No. 1 in Table 2) was used at 1000-fold dilution. Human hepatic microsomes (6 μ g) and purified human P450s (1 pmol) were used. Lane 1, marker proteins (62 k and 47.5 k); lane 2, CYP1A1; lane 3, CYP1A2; lane 4, CYP2A6; lane 5, CYP2B6; lane 6, CYP2D6; lane 7, CYP2C9; lane 8, CYP2C18; lane 9, CYP2C19; lane 10, CYP2E1; lane 11, CYP3A4; lane 12, human hepatic microsomes. (B) Immunoblot with human and rat hepatic microsomes. Two human hepatic microsomes (6 μ g), rat hepatic microsomes (2 μ g), and purified CYP2D6 (1.5 pmol) were used. Lane a, marker proteins (175 k, 83 k, 62 k, 47.5 k, 32.5 k, 25 k); lane b, rat hepatic microsomes; lanes c and d, human hepatic microsomes; lane e, purified human CYP2D6.

Fig. 2. Chromatographic Profile of Peptides Digested from CYP2D6 and ELISA of LKM-1-Positive Sera with These Peptides

Purified CYP2D6 containing histidine tag was digested by lysyl endopeptidase and the digested peptides were separated by reversed-phase HPLC. The separation was done at a flow rate of 1.0 ml/min with a linear gradient from an aqueous solution containing 10% acetonitrile and 0.5% TFA to one containing 70% acetonitrile and 0.5% TFA for 50 min. The fractions were collected every minute. Each fraction was analyzed with ELISA. The immunoreactivity of patient sera with these fractions is indicated in the inset. The positive fractions are indicated by an arrow in the chromatogram with a fraction number. Peaks a, b, c, d, and e indicate positive immunoreactivity. The upper graph was obtained with LKM-1-positive and HCV-negative serum (patient No. 1) and the lower graph, with LKM-1-positive and HCV-positive serum (patient No. 5).

peptides 246–281, 284–391, 1–146, 412–429, and 181–214, respectively. The peptide 1–146 was a new epitope recognized by LKM-1 serum. We synthesized peptides including 30 amino acid residues spanning the peptide 1–146 (sequences listed in Table 1). Using these peptides, an ELISA was performed under the same conditions as in above experiment. However, two LKM-1 sera did not react with these peptides (Fig. 4) although peptide 254–271, a typical epitope for LKM-1 antibody,^{6,9,16} was recognized by the two

Fig. 3. Theoretical Pattern of Digestion of CYP2D6

Complete digestion of CYP2D6 by lysyl endopeptidase gives 11 peptide fragments. The peptides that reacted with patient sera in this study are indicated by bold characters and a thick line.

Fig. 4. Immunoreactivity of LKM-1-Positive Sera with Synthetic Peptides in the ELISA

The sequences of synthetic peptides are indicated in Table 1. Two sera (patients No. 1 and 2) were used in this study.

Table 2. Immunoreactivity of Patient Sera toward Peptides Digested from CYP2D6

Patients	CYP2D6 peptides					
	HCV	1–146	181–214	246–281	284–391	412–429
1	–	+	+	+	+	–
2	–	+	+	+	+	–
3	+	–	–	–	+	–
4	+	–	–	–	+	–
5	+	–	+	–	+	+
6	+	–	+	–	+	–
7	+	–	+	–	+	–

These sera were selected by ELISA with full length CYP2D6 from Japanese patients suffering from hepatitis.

LKM-1 sera used.

Comparison of Immunoreactivity of LKM-1 Serum with HCV-positive Serum Two LKM-1-positive and HCV-negative sera and five LKM-1-positive and HCV-positive sera were used to react with peptides derived from digestion of CYP2D6 (Table 2). Five LKM-1-positive sera were picked from HCV-positive sera by ELISA with whole CYP2D6 as an antigen. The major epitopes recognized by LKM-1 antibody were reported to span amino acids 257–269 and 321–351 of CYP2D6.^{6,16,17} Peptides 246–281 and 284–391 in this study include these regions. Peptide 284–391 was recognized by both HCV-negative and positive sera but

peptide 246—281 was recognized only by HCV-negative sera. Recently peptide 193—212 was found as a new major epitope of LKM-1 antibody.¹⁸⁾ Our peptide 181—214 was also recognized by two HCV-negative and three HCV-positive sera. Peptide 410—419 is a minor epitope recognized by LKM-1 antibody¹⁹⁾ and was recognized by only one HCV-positive serum in this study. The new epitope found in this study, peptide 1—146, was recognized by two LKM-1-positive and HCV-negative sera but not HCV-positive sera.

DISCUSSION

LKM autoantibodies are markers of AIH-II and are also found in a subset of patients with CHC. Western blotting with LKM-1 sera from two Japanese patients gave a band for a 48-kDa protein corresponding to CYP2D6 in human hepatic microsomes. These sera also gave two bands, 67-kDa and 92-kDa proteins. Durazzo *et al.*²⁰⁾ found a 70-kDa protein recognized by LKM-1 serum other than the antigen, CYP2D6. Our 67-kDa protein may be the same protein. In Japan, LKM-1 autoimmune hepatitis is rare disease.⁷⁾ The molecular mechanism of LKM-1 hepatitis is not clear. As many antipsychotics are metabolized by CYP2D6, a complex of CYP2D6 and the active metabolites of a drug might cause autoimmune hepatitis. In fact, production of LKM-2 antibody is caused by tienilic acid metabolized by CYP2C9.¹⁾ Yamamoto *et al.*⁹⁾ investigated the epitopes recognized by LKM-1 sera systematically. They found the epitope peptides 257—269, 321—351, 373—389, and 410—429, and reported that regions 1—125 and 431—522 were not recognized by the LKM-1 sera used in their study. Later, Kerkar *et al.*¹⁸⁾ synthesized 34 peptides covering all of CYP2D6 and investigated the activity of LKM-1 sera and HCV-positive sera against these peptides. They found the epitopes, 253—272, 373—392, and 418—437, and new epitopes, peptides 192—212, 238—257, 268—287, and 478—497. The epitopes found in this study covered the peptides other than epitope 478—497. Region 257—269 is reported as a major epitope for HCV-positive sera as well as LKM-1 sera^{6,16,17)} but peptide 246—281 containing this region was recognized only by LKM-1 sera in this study. Furthermore, we found a new epitope, 1—146, recognized by LKM-1 sera but not HCV-positive sera. To specify the region, we synthesized the peptides covering epitope 1—146 and the His-tag linker. However, none of these peptides were recognized by LKM-1 sera. Similar results were reported by Sugimura *et al.*¹⁹⁾ They found the epitope 321—494 which was recognized by 92% of LKM-1 sera and 88% of HCV-positive sera but the small peptides containing 30 amino acids were not recognized by these sera. They concluded that these sera recognized the three-dimensional structure of the epitope region. In this study, LKM-1 sera may recognize the conformational immunogenic site of region 1—146.

In conclusion, we established an ELISA using plates which were covalently bound by peptides digested from

CYP2D6. We found a new epitope, 1—146, recognized by sera from Japanese AIH patients but not from HCV-positive patients. As the number of sample was small, it is not clear that this epitope is specific for LKM-1. There may also be ethnic differences. It is necessary to investigate the reactivity with peptide 1—146 using a greater number of serum samples.

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