Heterogeneity of liver/kidney microsomal antibody type 1 in autoimmune hepatitis and hepatitis C virus related liver disease

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Abstract

Liver/kidney microsomal antibody type 1 (LKM-1), the serological marker of a subset of autoimmune hepatitis, is also present in a proportion of patients with hepatitis C virus (HCV) related chronic liver disease. To characterise further this autoreactivity and to evaluate whether an autoantibody giving an identical immunofluorescence staining, and detected in two different clinical conditions, involves the same antigenic target(s), sera from autoimmune and HCV infected patients were tested with native, recombinant, and synthetic antigens. Sixty five sera were selected on the basis of the typical immunofluorescence pattern: 50 patients had serological markers of HCV infection, the remaining 15 suffered from autoimmune hepatitis. The reactivity of each serum with rat and human microsomal fractions, full length human recombinant CYP2D6, and two synthetic peptides spanning the amino acid regions 257-269 and 373-398 of CYP2D6 was systematically investigated by immunoblotting. Fourteen (93%) sera from autoimmune hepatitis patients and 39 (78%) from HCV infected patients reacted with rat and/or human microsomal polypeptides of 39 kD, 50 kD, 58 kD, and 66 kD in different associations, the 50 kD band being the most frequently observed. Reactivity to CYP2D6 and its amino acid sequence 257-269 was significantly more common in autoimmune hepatitis than in HCV infected patients ($p < 0.001$ and $p < 0.0003$, respectively). LKM-1 reactivity is directed against heterogeneous and not entirely defined autoantigens. The main target in autoimmune sera is CYP2D6 and its 257-269 amino acid region, while sera from patients with HCV infection are more likely to recognise other microsomal targets, the molecular identity of which is currently unknown.

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Keywords: liver/kidney microsomal antibody type 1, hepatitis C virus, autoimmune hepatitis, CYP2D6, immunoblotting.

Liver/kidney microsomal antibody type 1 (LKM-1) is the serological marker of a subset of autoimmune hepatitis (AIH) which affects children and young adults, mainly of the female sex.¹ In adults this autoantibody is

usually found in association with hepatitis C virus (HCV) infection.²⁻⁵ In studies preceding the discovery of HCV, LKM-1 positive sera were shown to react with a 48-50 kD, protein present in rat and human liver microsomal fractions.^{6,7} The finding that cytochrome P450IID6 (CYP2D6) corresponds to the 48-50 kD microsomal protein⁸⁻¹⁰ generated the notion that the LKM-1 target, the 48-50 kD microsomal polypeptide, and CYP2D6 are one and the same. Puzzling results, however, were reported when CYP2D6, extracted and purified or in recombinant form, was used as antigen: anti-CYP2D6 antibodies were detected not only in the presence⁸⁻¹⁰ but also in the absence¹⁰ of the 50 kD band. Moreover, the typical immunofluorescence pattern of LKM-1 was present in occasional sera which did not react with the 48-50 kD protein but only with a distinct 64-66 kD polypeptide.^{10,11} The question as to whether reactivity to 48-50 kD protein, CYP2D6, and the target of LKM-1 is the same, however, remains unsettled.

Another unresolved issue relates to the nature of LKM-1 target antigen(s) in HCV infection. While some studies report that the key target antigen of LKM-1 is CYP2D6, both in patients with LKM-1 positive AIH and patients with LKM-1 positive HCV related chronic liver disease,^{12,13} others suggest different LKM-1 targets¹⁴ or different CYP2D6 antigenic sites¹⁵ in these two conditions. In an attempt to define the relationship between microsomal antigens, CYP2D6, and HCV infection, we have collected 65 sera positive for the typical LKM-1 immunofluorescence pattern obtained from 50 patients with and 15 without HCV infection and we have investigated their reactivity against the different antigenic preparations used in previous reports: rat and human liver microsomal fractions, human recombinant CYP2D6, and two synthetic peptides covering the 257-269 and the 373-389 amino acid sequences of CYP2D6, which have been reported to contain the target epitopes of LKM-1 positive sera obtained from patients with AIH.¹⁶

Methods

PATIENTS AND SERA

Sixty five patients with liver disease were selected on the basis of a typical LKM-1 pattern¹⁷ detected by indirect immunofluorescence. Patients were recruited from an Italian (Cattedra di Medicina Interna, Policlinico S Orsola, Bologna, Italy) and from an English

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TABLE I Demographic, immunological, and histological features of 65 liver/kidney microsomal antibody type 1 (LKM-1) positive patients with chronic hepatitis

	Autoimmune hepatitis	Hepatitis C virus related hepatitis	p Value
No	15	50	
Sex (M/F)	4/11	17/33	NS
Median age (range) (y)	10 (1–38)	47 (16–76)	<0.001
Liver biopsy findings	11 (76%)	38 (73%)	
Cirrhosis	2 (18%)	6 (15%)	NS
Chronic hepatitis:			
Severe	5 (45%)	5 (13%)	<0.04
Mild	0	27 (71%)	<0.00003
Multilobular collapse	4 (36%)	0	<0.002
Median LKM-1 titre (range)	640 (40–10 240)	640 (40–10 240)	NS

NS: not significant.

department (Paediatric Liver Service, King's College School of Medicine and Dentistry, London, UK). Fifty were anti-HCV positive: of these, 24 were tested for serum HCV RNA and were all found to be positive. The 15 anti-HCV negative patients fulfilled internationally agreed criteria¹⁸ for the diagnosis of LKM-1 positive AIH. Five of these patients were tested for serum HCV RNA and were negative. Liver biopsy tissue was available in 49 patients. All the patients with markers of HCV infection were asymptomatic and had been referred because of abnormal liver function tests observed during routine check ups, whereas all patients with AIH were symptomatic, presenting with prolonged acute hepatitis, complications of portal hypertension, or with fulminant hepatic failure. Demographic, immunological, and histological features of the two groups of patients are summarised in Table I.

As controls, we tested sera from 28 healthy blood donors (16 female, median age 37 years, range 20–65) and from 44 LKM-1 negative patients with chronic liver disease of viral and non-viral aetiology. Twenty four patients had autoantibody negative chronic hepatitis C (13 female, median age 45 years, range 18–70), 10 had HBsAg positive (EIA kit, Abbott Laboratories, North Chicago, Illinois, USA) chronic hepatitis B (3 female, median age 48, range 25–68), and 10 had anti-nuclear and/or anti-actin positive AIH (8 female, median age 54 years, range 7–82).

INDIRECT IMMUNOFLUORESCENCE

Sera were tested by indirect immunofluorescence using cryostat sections of rat liver, kidney, and stomach (screening dilution 1:10) as substrate.¹⁷ Each serum was titrated to extinction.

WESTERN IMMUNOBLOTTING

Rat liver microsomes were obtained from male Sprague-Dawley rats; human liver microsomes were prepared from the normal liver tissue of a group 0, HBsAg negative, anti-HCV negative adult male who underwent partial hepatectomy for giant hepatic haemangioma. Preparation of rat and human microsomal fractions was carried out as previously described.¹⁹ Western immunoblotting experiments were performed according to standard techniques.^{20,21} Briefly, 400 µg of rat or human liver microsomal proteins were

separated in 10% SDS-polyacrylamide gel electrophoresis (Mini-PROTEAN II System, Bio-Rad Laboratories, Richmond, CA, USA). After separation, proteins were electrophoretically transferred onto nitrocellulose filters. The filters were then blocked and cut into strips which were subsequently incubated with patients' and controls' sera at 1:100 dilution. The second antibody (peroxidase-conjugated rabbit anti-human IgG, IgA, IgM, kappa and lambda, DAKO, Copenhagen, Denmark) was used at 1:600 dilution. The presence of CYP2D6 in the human microsomal fraction was confirmed using a monoclonal antibody⁸ (monoclonal antibody BI-114/2, a gift of Professor U A Meyer, Basel, Switzerland) which detects the 'extensive metaboliser' phenotype.²² For the isolation of human recombinant CYP2D6, a human liver cDNA library in λgt11 (Clontech, Palo Alto, CA, USA) was screened and CYP2D6 fusion protein extracted using a high titre LKM-1 positive serum obtained from a patient with AIH.²³ Antibodies against CYP2D6 were detected by Western immunoblotting after separation of the recombinant fusion protein in 7.5% SDS-polyacrylamide gel electrophoresis.

PEPTIDE TESTING

Two peptides previously reported to contain epitopes reacting with LKM-1 in AIH were obtained: peptide 257–269 (amino acid sequence: E H R M T W D P A Q P P R) which is the main antigenic epitope of CYP2D6 and includes seven amino acid residues of the octapeptide described by Manns *et al.*,¹² and peptide 373–389 (amino acid sequence: G M T H M T S R D I E V Q G F R I).¹⁶ The two peptides were synthesised by PACEMAKER (AFFINITI Research Products Limited, Nottingham, England, UK), purified by high performance liquid chromatography, and conjugated to keyhole haemocyanin via a glutaraldehyde coupling procedure that gives a peptide:carrier ratio of approximately 200:1. Conjugated peptides were absorbed at three different concentrations (50, 25, and 12.5 µg/ml) to nitrocellulose filters. The filters were then blocked, cut into strips and probed with patients' and controls' sera as in the Western immunoblotting experiments.

ANTI-HCV TESTING

Presence of antibodies against structural and non structural HCV antigens was assessed using a second generation assay (ELISA 2, Ortho Diagnostics, Raritan, NJ, USA), according to manufacturer's instructions. All positive results were validated by second generation RIBA test (Ortho).

HCV RNA TESTING

Twenty nine sterile sera were available for the detection of HCV RNA. Briefly, total RNA was extracted from 100 µl of serum and reverse transcribed. Five µl of cDNA were amplified using a 'nested' polymerase chain

TABLE II Frequency of reactivity of 65 liver/kidney microsomal antibody type 1 positive sera against rat and human microsomes, human recombinant CYP2D6, and synthetic peptide 257–269

	All patients	Autoimmune hepatitis	HCV related hepatitis
Rat microsomes	52/65 (80%) ^a	14/15 (93%)*	38/50 (76%)*
50 kD	33	8	25
50, 58 kD	6	2	4
39, 50 kD	3	1	2
50, 66 kD	4	1	3
39, 50, 58 kD	2	1	1
39, 50, 66 kD	2	0	2
50, 58, 66 kD	1	1	0
58 kD	1	0	1
Human microsomes	40/65 (62%) ^b	11/15 (73%)†	29/50 (58%)‡
50 kD	20	2	18
50, 58 kD	3	1	2
39, 50 kD	3	1	2
50, 66 kD	4	2	2
39, 50, 58 kD	2	1	1
39, 50, 66 kD	2	1	1
50, 58, 66 kD	2	1	1
66 kD	1	1	0
58, 66 kD	2	1	1
58 kD	1	0	1
CYP2D6	32/65 (49%) ^c	13/15 (86%)‡	19/50 (38%)‡
Peptide 257–269	12/63 (19%)	8/14 (57%)§	4/49 (8%)§

HCV: hepatitis C virus; CYP2D6: human recombinant cytochrome P450IID6.
* and † p not significant; ‡p<0.001; §p<0.0003.
a v b p<0.02; a v c p<0.0003; b v c p not significant.

reaction (PCR) with primers derived from the highly conserved 5' non-coding region²⁴ of the HCV genome, as previously described.²⁵ In all the experiments, positive and negative controls were present. Positive sera gave a second round PCR product of the expected size, assessed under ultraviolet light on a 2% agarose gel stained with ethidium bromide.

STATISTICS
Fisher's exact test and Wilcoxon rank sum test were used where appropriate.

Results
There was no difference in LKM-1 titre between the two patient groups (Table I).

Figure 1: Western immunoblotting reactivities of liver/kidney microsomal antibody type 1 positive sera using rat microsomes as antigenic substrate. In lanes A to D are sera from patients no 12, 15, 6, and 13, respectively, all with autoimmune hepatitis; in lanes E to I are sera from patients no 28, 43, 59, 54, and 20, respectively, all with hepatitis C virus infection. A 50 kD band is the most frequent reactivity both in autoimmune hepatitis and hepatitis C virus infected patients. Additional reactivities at 39 kD, 58 kD, and 66 kD were observed in some patients. In lane I (patient no 20) an isolated reactivity with a 58 kD polypeptide was detected.

Patients with AIH were significantly younger and had significantly more histologically active disease than patients with HCV infection, while there was no difference in frequency of histologically diagnosed cirrhosis and in sex ratio between the two groups, females being prevalent in both.

The overall rates of reactivity for rat and human microsomal fractions and recombinant CYP2D6 were 80%, 62%, and 49%, respectively, rat microsomal polypeptides being the most frequently recognised targets ($p<0.02$ v human microsomes and $p<0.0003$ v recombinant CYP2D6) (Table II). Twelve (19%) out of the 63 sera tested recognised the 257–269 peptide, whereas none reacted with the 373–389 peptide. Examples of reactivities to rat and human microsomes, as assessed by Western immunoblotting, and to the 257–269 synthetic peptide of CYP2D6 are shown in Figures 1–3.

The frequencies of reactivity to the antigens tested in patients with AIH and HCV related liver disease are summarised in Table II. Only one of the 15 patients with AIH, whose LKM-1 titre was 1:80, did not react with rat and human microsomal fractions nor with CYP2D6. Among the remaining 14 patients the following frequency of reactivity was observed (Table III): 14 reacted with rat 50 kD microsomal protein, 13 (93%) with recombinant CYP2D6, nine (64%) with human 50 kD microsomal protein, and eight of the 14 tested (57%) with the 257–269 synthetic peptide of CYP2D6. Of the five sera giving a 50 kD band with rat but not with human microsomal fraction, three did not react with human microsomal fractions at all, while two reacted with a human 66 kD polypeptide, alone in one case and in association with a 58 kD polypeptide in the other. Reactivity to a 66 kD polypeptide was more common against human (6 patients) than rat microsomal preparation (2 patients), while reactivity to a 58 kD and a 39 kD polypeptides was similarly frequent against rat (4 and 2 patients, respectively) or human (4 and 3 patients, respectively) microsomes. The reactivity against the non-50 kD microsomal polypeptides obtained from the two species overlapped only partially (Table III).

Of the 50 patients with LKM-1 positive chronic hepatitis with markers of HCV infection (table IV), 11 did not react with rat and human microsomal fractions nor with recombinant CYP2D6, nor with synthetic peptide 257–269. The remaining 39 patients (78%) had antibodies against one or more of the antigens tested. The following frequency of reactivity was observed: 37 (95%) reacted with a rat 50 kD microsomal protein, 27 (69%) with a human 50 kD microsomal protein, 19 (49%) with recombinant CYP2D6, and four of the 38 tested (10%) with the 257–269 synthetic peptide of CYP2D6. Of the 10 sera giving a 50 kD band with rat but not with human microsomal fractions, nine did not react with human microsomal fractions at all, while one reacted with a human 58 kD polypeptide. The frequencies of reactivity to 66 kD, 58 kD, and 39 kD polypeptides were similar against human (5, 6, and 4

Figure 2: Western immunoblotting reactivities of liver/kidney microsomal antibody type 1 positive sera using human microsomes as antigenic substrate. In lanes A to F are sera from patients no 8, 15, 12, 2, 11, and 6, respectively, all with autoimmune hepatitis; in lanes G to L are sera from patients 24, 58, 54, 62, 16, and 59, respectively, all with hepatitis C virus infection. A 50 kD band is the most frequently recognised target both in autoimmune hepatitis and hepatitis C virus infected patients. Additional reactivities at 39 kD, 58 kD, and 66 kD were observed in some patients. In lane K (patient no 16) the 50 kD band is absent.

patients, respectively) and rat microsomal preparation (5, 6, and 5 patients, respectively). Akin to AIH, the reactivity against the non-50 kD microsomal proteins obtained from the two species overlapped only partially (Table IV).

Patients with either AIH or HCV infection, seropositive for antibody to CYP2D6, invariably reacted with the rat 50 kD polypeptide. All sera showing reactivity to the 257–269 synthetic peptide also recognised human recombinant CYP2D6. Reactivity against CYP2D6 and peptide 257–269 was significantly more often observed in autoimmune cases ($p < 0.001$ and $p < 0.0003$, respectively) (Table II). Overall, positivity for anti-CYP2D6 was significantly associated with higher LKM-1 titres ($p < 0.0002$), while patients who did not react with any of the antigens tested had significantly lower LKM-1 titres than patients showing positive reactions ($p < 0.007$).

None of the healthy controls and patients with LKM-1 negative liver disease showed reactivity to any of the antigens tested.

TABLE III Individual demographic, histological, immunological, and viral data and immunoblotting reactivities with rat and human microsomes, human recombinant CYP2D6, and synthetic peptide 257–269 of CYP2D6 in the 15 patients with liver/kidney microsomal antibody type 1 (LKM-1) positive autoimmune hepatitis studied

Patient	Sex	Age (y)	Liver histology	LKM-1 titre	HCV RNA	Rat microsomes (kD)	Human microsomes (kD)	CYP2D6	Peptide 257–269
1	F	7	s CAH	40	ND	50	66	+	–
2	F	17	s CAH	40	ND	50	39 50 58	+	+
3	F	8	ND	80	–	Neg	Neg	–	ND
4	M	6	s CAH	160	ND	50 58	50 58 66	–	–
5	F	21	ND	160	ND	50	Neg	+	+
6	F	10	Cirrhosis	160	ND	50 66	50 66	+	+
7	F	38	ND	320	ND	50	58 66	+	–
8	F	10	ND	640	ND	50	50	+	–
9	M	20	s CAH	640	ND	50	Neg	+	+
10	M	1	MC	2560	–	50	50	+	+
11	F	12	Cirrhosis	5120	ND	39 50 58	50 58	+	+
12	F	2	MC	5120	–	50	50 66	+	+
13	M	14	MC	5120	–	50 58	Neg	+	–
14	F	7	s CAH	10240	ND	50 58 66	39 50 66	+	–
15	F	15	MC	10240	–	39 50	39 50	+	+

HCV: hepatitis C virus; CYP2D6: human recombinant cytochrome P450IID6.

LKM-1 titre given as reciprocal of serum dilution; s CAH: severe chronic active hepatitis; MC: multilobular collapse; ND: not done.

Figure 3: Immunoblotting reactivity against different concentrations of CYP2D6 (cytochrome P450IID6) peptide 257–269 in autoimmune hepatitis (lanes 1, 3, and 5) and hepatitis C virus infected cases (lanes 2, 4, and 6). Lane 7: negative control. Peptide concentration was 50 µg/ml in A, 25 µg/ml in B and 12.5 µg/ml in C. D represents an internal negative control.

Discussion

In recent years, LKM-1 reactivity has generated a great deal of excitement. From being a simple marker of a subset of autoimmune hepatitis it has now taken on the role of a potential effector of damage²⁶ and a link, through molecular mimicry, between viral infection and autoimmune disease.²⁷ The nature of its target antigen has been extensively investigated. Thus, LKM-1 has been described to react with a 48–50 kD polypeptide present in both rat and human microsomal preparations^{6, 7}; the polypeptide has been identified as CYP2D6,^{8–10} and a short amino acid sequence of this enzyme is reported to account for most of the LKM-1 reactivity.¹⁶ This attractive scenario has been pieced together with information provided by studies using patient populations selected with different criteria and using different techniques. Reservations on the possibility to apply this scenario universally have been put forward by various reports. The typical immunofluorescence LKM-1 pattern has been found, for example, in the presence of a 66 kD but in the absence of the characteristic 50 kD microsomal reactivity¹²; different epitopes on CYP2D6 have been suggested to be the target of LKM-1 in patients with and without HCV infection¹⁵; the correspondence between the target of LKM-1 and CYP2D6, particularly in patients with evidence of HCV infection, has been challenged.¹⁴ To clarify how the described target antigens of LKM-1 relate to one another, we have used for the first time a large number of sera giving the typical

immunofluorescence LKM-1 pattern from patients with or without HCV infection, to test simultaneously their reactivity against rat and human liver microsomal fractions, recombinant CYP2D6, and its synthetic peptides spanning the amino acid sequence 257–269 and 373–389.

Analysing the patient groups undivided, the following hierarchy of reactivity was noted: four fifths of the sera recognised a rat 50 kD polypeptide, fewer than two thirds the human 50 kD analogue, less than half the recombinant fusion CYP2D6 protein, only a fifth the 257–269 peptide, and none the 373–389 peptide. Twenty per cent of our LKM-1 positive sera reacted with none of the antigens on test. Thus, the antibody responsible for the LKM-1 immunofluorescence does not react univocally with any of the suggested target antigens, and can even be present without any of the previously described reactivities. Why the 50 kD rat microsomal polypeptide is more frequently recognised than its human counterpart is unclear, since both rodent and human hepatic tissue were freshly obtained and processed under identical conditions. The fact that rodent livers express higher amounts of

cytochrome P450 proteins than humans is a possible explanation. The observation that the LKM-1 positive sera recognised CYP2D6 significantly less frequently than both rat and human microsomal preparations may be due to the lack of conformational epitopes and post-translational modifications in the recombinant CYP2D6 protein. Lastly, the fact that LKM-1 may be present without any of the previously described reactivities suggests that it may recognise additional targets.

The comparison between LKM-1 positive patients with or without HCV infection indicates clear differences in the two groups. Akin to previous reports, HCV negative patients with classic autoimmune hepatitis are younger, have a more severe histological picture, and more frequently present with fulminant hepatitis. In contrast to previous reports,^{4 12} we find similar LKM-1 titres and prevalence of female sex in the two patient groups.

Within the AIH group, autoantibody reactivity tended to be more homogeneous. Not only were these sera found to react almost invariably with rat microsomal preparations and CYP2D6, but also, in two thirds of those tested, with the putative immunodominant

TABLE IV Individual demographic, histological, immunological, and viral data and immunoblotting reactivities with rat and human microsomes, human recombinant CYP2D6, and synthetic peptide 257–269 of CYP2D6 in the 50 patients with liver/kidney microsomal antibody type 1 (LKM-1) positive/anti-HCV positive chronic liver disease studied

Patient	Sex	Age (y)	Liver histology	LKM-1 titre	HCV RNA	Rat microsomes (kD)	Human microsomes (kD)	CYP2D6	Peptide 257–269
16	F	20	CPH	40	+	50 58	58 66	–	–
17	F	52	m CAH	40	+	50	Neg	–	–
18	M	63	CPH	40	+	Neg	Neg	–	–
19	M	22	ND	80	ND	Neg	Neg	–	–
20	M	26	CPH	80	+	58	Neg	–	–
21	M	58	ND	80	ND	Neg	Neg	–	–
22	M	40	m CAH	80	+	50 58	Neg	–	–
23	M	64	Cirrhosis	80	+	50	50	–	–
24	F	57	CPH	80	+	50	50	+	+
25	F	51	CPH	80	+	50	Neg	+	–
26	M	45	CPH	80	+	Neg	Neg	–	–
27	F	28	m CAH	160	ND	Neg	Neg	–	–
28	F	38	CPH	160	+	50	50	–	–
29	F	37	m CAH	160	ND	50	Neg	–	–
30	F	23	ND	160	ND	Neg	Neg	–	–
31	F	52	m CAH	320	+	50	50	–	–
32	M	76	ND	320	+	39 50	66	–	–
33	F	42	CPH	320	ND	Neg	Neg	–	–
34	F	45	ND	320	ND	Neg	50	–	–
35	M	49	s CAH	320	+	Neg	Neg	–	–
36	F	62	m CAH	320	ND	50	50 58	+	–
37	M	50	ND	640	ND	50	50 58	–	–
38	F	59	CPH	640	ND	50	50	–	–
39	M	49	m CAH	640	ND	50	50	–	–
40	F	29	CPH	640	+	50	50	–	–
41	F	20	m CAH	640	ND	50	50	–	–
42	F	56	Cirrhosis	640	ND	50	50	–	–
43	F	48	ND	640	ND	39 50	Neg	–	–
44	F	26	ND	640	+	Neg	Neg	–	–
45	M	60	ND	640	ND	Neg	Neg	–	–
46	M	45	CPH	640	+	50	50	+	–
47	F	56	s CAH	640	ND	50	Neg	+	–
48	F	61	CPH	640	ND	Neg	Neg	–	–
49	F	71	Cirrhosis	640	ND	50	50	+	–
50	M	43	ND	640	ND	50	50	–	–
51	F	53	m CAH	640	+	50	50	+	–
52	F	54	ND	640	ND	50	50	+	ND
53	F	39	s CAH	640	ND	50	50	–	–
54	F	32	ND	640	ND	50 58	50 66	–	–
55	F	47	m CAH	640	ND	50	50	+	+
56	M	19	s CAH	640	+	50 58	58	+	–
57	F	32	s CAH	1280	+	39 50	66	50 66	–
58	M	16	s CAH	1280	+	50	39 50	+	–
59	F	57	m CAH	2560	+	50	66	50	–
60	F	67	Cirrhosis	2560	+	50	Neg	+	–
61	M	48	m CAH	2560	ND	50	Neg	+	–
62	F	49	m CAH	2560	ND	50	66	39 50 66	+
63	F	28	m CAH	2560	+	39 50	39 50 58	+	–
64	F	16	m CAH	5120	ND	50	66	50 58 66	+
65	F	45	Cirrhosis	10240	+	39 50 58	39 50	+	–

HCV: hepatitis C virus; CYP2D6: cytochrome P450IID6; LKM-1 titre given as reciprocal of serum dilution; CPH: chronic persistent hepatitis; m CAH: mild-moderate chronic active hepatitis; s CAH: severe chronic active hepatitis; ND: not done.

CYP2D6 epitope contained within the 257–263 peptide. These results support the notion that a major target of LKM-1 antibody in AIH is a linear epitope of CYP2D6. Our observations, though, are partially at variance with previous reports. Yamamoto *et al*¹⁶ found that 14 of 15 LKM-1 positive sera from their patients with AIH reacted with the 257–269 peptide, while a third of our AIH cases did not. This discrepancy may be accounted for by the fact that Yamamoto's sera were highly selected, being positive not only for LKM-1 in conventional immunofluorescence – the sole recruiting criterion in our study – but also for LKM-1 in ELISA, for rat 50 kD/human 48 kD microsomal polypeptides, and for recombinant CYP2D6. Another possible explanation is the use by Yamamoto *et al*¹⁶ of bovine serum albumin as peptide carrier, in view of the described occurrence of antibodies to this protein in humans.²⁸ Conversely, our data showing no reactivity against a second potential epitope on CYP2D6 present in the 373–389 amino acid sequence agree with those of Yamamoto *et al*¹⁶ who found this reaction in only one of their sera. The high frequency of 257–269 peptide reactivity in AIH has prompted these authors to propose the establishment of a test based in the reactivity to this peptide as a diagnostic tool for LKM-1 positive AIH.¹⁶ The observation that only two thirds of our autoimmune cases and four patients with HCV infection reacted with this synthetic peptide questions, however, the disease specificity of such a test.

Autoantibody reactivity in patients with HCV infection was much more heterogeneous than that in patients with AIH. Since the LKM-1 titre was similar in the two groups, the differences probably derive from dissimilarities in the target antigens in the two conditions. Interestingly, 22% of LKM-1 positive/HCV positive patients did not react with any of the antigens tested. This could be due to their lower LKM-1 titre, but other patients with similar low titres did show positive reactions with one or more antigenic preparations. Alternatively, LKM-1 antibodies may be directed against a target different from CYP2D6, as previously proposed.¹⁴ The lack of reactivity to microsomal polypeptides may also be related to the timing of testing, since we have shown a temporal dissociation between the appearance of LKM-1 immunofluorescent antibody and reactivity to the 50 kD liver microsomal polypeptide.²⁹ In the HCV positive sera that reacted with the various antigenic preparations, the hierarchy of reactivity was different from that of AIH, 95% recognising the rat 50 kD polypeptide, 69% the human 50 kD analogue, 49% the recombinant fusion CYP2D6 protein, and only 10% the 257–269 peptide. These results indicate, firstly, that the 257–269 peptide does not contain the immunodominant epitope for LKM-1 positive sera from patients with HCV infection, and, secondly, that these sera react preferentially with conformational epitopes, as suggested by the discrepancy between the results obtained with microsomal and recombinant targets.

Sera from several patients in both groups reacted with a number of non-50 kD rat or human microsomal polypeptides, usually in association with the main 50 kD band, but occasionally in its absence. Among the HCV positive patients this multiple reactivity tended to be associated with the highest LKM-1 titres (Table IV). The discrepancy between reactivities to the microsomal preparations obtained from the two species was even more obvious than that observed for the main 50 kD target antigen. These results further emphasise the diversity of LKM-1 reactivity.

In conclusion, this study provides evidence that LKM-1 reactivity is directed against heterogeneous and not entirely defined microsomal antigens. CYP2D6, the best characterised and studied antigenic target of LKM-1, and its main linear epitope peptide 257–269, account for most of the reactivity of AIH sera, while HCV related LKM-1 positive sera more often recognise other targets, the nature of which is still unknown. LKM-1 positive AIH and LKM-1 positive liver disease with markers of HCV infection can therefore be considered as two different entities not only with distinct clinical and histological features, but also in terms of LKM-1 antigenic specificity.

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