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Anti-LKM-1 antibodies determined by use of recombinant P450 2D6 in ELISA and Western blot and their association with anti-HCV and HCV-RNA

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SUMMARY

Several subtypes of anti-liver-kidney microsome antibodies (LKM) are known. LKM-1 antibodies associated with autoimmune chronic active hepatitis recognize P450 2D6, a cytochrome P450 monooxygenase. The frequent association of anti-LKM-1 antibodies and hepatitis C virus (HCV) infections and the probable existence of an infectious and autoimmune form of anti-LKM-1-associated hepatitis, requiring different therapeutical strategies, necessitates the exact determination of anti-LKM-1 specificities. Therefore, we compared various antibody tests (immunofluorescence, ELISA with recombinant P450 2D6, and Western blot with recombinant and natural antigens and agargel double diffusion) with sera of 27 anti-LKM-1-positive chronic active hepatitis (CAH) patients, with 61 sera harbouring anti-mitochondrial antibodies, 100 sera each from HCV-RNA-positive and HCV-RNA-negative patients, and 50 sera of healthy persons. Western blot techniques using recombinant MS2-polymerase P450 2D6 fusion protein were found to be the most sensitive and specific method for anti-LKM-1 antibody determination in routine laboratory. The recently recognized association of anti-LKM-1 antibody and HCV infection was confirmed by the results of this study. In anti-HCV and HCV-RNA-positive patients with anti-LKM-1 antibodies there was a preponderance of males with higher mean age and lower antibody titres. The results support the hypothesis of the existence of an autoimmune as well as an infectious (HCV triggered) subgroup of anti-LKM-1-positive hepatitis.

Keywords chronic active hepatitis anti-LKM-1 anti-HCV HCV-RNA cytochrome P450 2D6

INTRODUCTION

Several subsets of autoantibodies to liver and kidney microsomes (anti-LKM) have been described. Anti-LKM-1 reacting with human cytochrome oxydase P450 2D6 (CYP 2D6) [1-3] have been regarded as serological markers of type II autoimmune chronic active hepatitis (CAH) [4]. Anti-LKM-2 directed against cytochrome P450 2C9 [5,6] was associated with ticrynafen-induced hepatitis [7], anti-LKM-3, the specificity of which is unknown, was reported in patients with chronic hepatitis delta virus infection (HDV) [8]. Recently, antibodies specific for cytochromes P450 1A2 and P450 2C3 have also been observed [9-11].

Anti-LKM-1-associated hepatitis has been regarded as an autoimmune disease because of the absence of infectious or toxic causes of liver damage [4]. Also, in patients suffering from non-A, non-B hepatitis anti-LKM-1 antibodies were believed to be rare [12,13]. The demonstration of antibodies to the hepatitis C virus (anti-HCV) in a high percentage of anti-LKM-1-positive patients [14,15] was therefore surprising. Initial doubts regarding the specificity of the anti-HCV assays [16] could be rejected after re-evaluations with second generation anti-HCV ELISA and recombinant immunoblotting assay (RIBA) [17-20] as well as by demonstration of HCV-RNA in the patients' sera [18,21]. In these studies, anti-LKM-1 antibodies were determined by indirect immunofluorescence, agargel diffusion and/or counter current electrophoresis, respectively, methods which do not allow the exact assignment of their specificity. Only one group [22] used a recombinant P450 2D6 β-galactosidase fusion protein in Western blot techniques. Because there are only a few comments on anti-LKM-1 antibody tests by ELISA [23,24] we here evaluated the specificity and sensitivity of anti-LKM-1 tests by ELISA, Western blot and other techniques using natural antigens as well as a recombinant P450 2D6 MS2-polymerase fusion protein, thereby also considering the association of anti-LKM-1 and HCV infection.

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PATIENTS AND METHODS

Sera

From 27 hepatitis B marker negative German patients with the clinical diagnosis of CAH, one to four (Table 1) serum samples

Table 1. Cumulative data of patients

Patient (sera*)	Sex	Age	Anti-LKM-1 IIF titre	Anti-LKM-1 Blot recombinant†	Anti-LKM-1 Blot natural‡ (kD)	Anti-LKM-1 ELISA recombinant, U/ml	HCV-RNA	Anti-HCV Core	Anti-HCV C33	Anti-HCV C100
1 (2)	M	60	640	200 000	50	81	+	−	+	+
2	M	38	1280	200 000	50	78	+	−	+	−
3	M	35	2560	200 000	50	80	+	+	+	+
4 (2)	F	20	10240	800 000	50	128	−	−	−	−
5 (3)	F	27	1280	400 000	50/64	96	−	−	−	−
6	F	19	2560	200 000	50	85	−	−	−	−
7 (4)	F	12	1280	100 000	50	78	−	−	−	−
8 (2)	F	29	2560	400 000	50	83	−	−	−	−
9	F	24	640	200 000	50	81	−	−	−	−
10	F	19	1280	400 000	50/64	77	−	−	−	−
11 (4)	F	9	1280	200 000	50	84	−	−	−	−
12 (2)	F	16	2560	200 000	50	82	−	−	−	−
13 (2)	F	34	1280	400 000	50	118	−	−	−	−
14	F	28	2560	400 000	50	104	−	−	−	−
15 (2)	F	57	640	12 800	50	44	+	+	+	+
16	F	63	320	6400	50	35	+	+	+	−
17	M	46	160	6400	50	40	+	+	+	+
18	M	66	640	1600	50	10	+	+	−	−
19 (2)	M	26	160	800	50	12	+	+	+	+
20 (2)	M	64	160§	800	50	18	+	+	+	+
21	M	38	320	1600	50	6	+	+	+	+
22 (2)	F	6	320	1600	50/64	12	−	−	−	−
23 (2)	M	?	40¶	Neg.	Neg.	9	±	−	+	−

* Number of more than one samples received in parentheses. Results of the first sample tested are given in this Table. Results of anti-HCV and HCV-RNA were identical in all tested samples except for no. 23.

† Immunoblot with recombinant M2-polymerase P450 2D6 fusion protein. Results presented as endpoint titres.

‡ Immunoblot with natural microsomal antigens staining of protein bands indicated.

§ Centrolobular IIF pattern on rat liver.

¶ Typical anti-LKM-1 IIF pattern, AMA negative.

IIF, Indirect immunofluorescence.

showing anti-LKM-like pattern by immunofluorescence were available. At the time of submission of the first sample, none of the patients received corticosteroids or other immunosuppressive drugs. Furthermore, 100 sera each of HCV-RNA-positive and negative patients as well as 61 sera containing antimitochondrial antibodies of anti-M2 specificity were randomly selected. Fifty sera of healthy persons selected from our serum bank served as controls. None of these sera harboured any antibody detectable by methods described herein.

Anti-LKM-1 antibodies

Indirect immunofluorescence tests were performed with 4 μm thick cryostat sections of rat and mouse liver, stomach and kidney. Sera were tested at a dilution of 1:20 and positive results were given in endpoint titres of geometrical dilutions thereof. Mean error of immunofluorescence tests in our laboratory is regarded ± 1 titre step. For anti-LKM-1 screening by ELISA and immunoblotting, recombinant P450 2D6 protein was synthesized. RNA from HepG-2 cells (ATCC HB 8065) was primed with oligo dT and reverse transcribed. The entire P450 2D6 coding sequence [25] was amplified by a nested polymerase chain reaction (PCR) assay. The primers for the second round contained, in addition to recognition sequences for EcoRI and BamHI, codons for six histidine residues (5′-CGCGAATTCGCTAGAAGCACTGGTGCC-3′ and 5′-CGCGGATCCCTAATGGTGATGGTGATGGTGGCGGGGCACAGCACAAAG-3′). The 1·5-kb long cDNA fragment was bacterially expressed [26,27] and the P450 2D6 MS2-polymerase fusion protein produced on a preparative scale according to previously described methods [28]. The insoluble inclusion body fraction was extracted successively with 8 ml 1 M urea and 15 ml solution A (6 M guanidinium-HCl, 10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8·0, 100 mM sodiumphosphate, pH 8·0). The 6 M guanidinium-HCl fraction containing the fusion protein was loaded onto a nickel-NTA-agarose column (DIAGEN), washed with solution A and thereafter with solution B (8 M urea, 10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8·0, 100 mM sodiumphosphate, pH 8·0). Proteins were eluted by an imidazole step gradient (3 mM, 10 mM and 100 mM in solution B). Fractions between 10 mM and 100 mM imidazole contained the P450 2D6 fusion protein in pure form (>95%).

Optimal antigen concentrations for ELISA, appropriate dilutions of conjugates and patients' sera were established by chess board titrations. The P450 2D6 MS2 polymerase fusion protein was applied to Immunolon microtitre plates (Dynatech) at a concentration of 1 μg/ml 0·1 M sodium carbonate buffer pH 9·6 (100 μl/well, 16 h; 4°C) and blocked with 300 μl PBS, 0·5% bovine serum albumin (BSA; 16 h, 4°C). Two hundred micro-

litres serum/well (diluted 1:250 in PBS, 0·05% Tween, 0·05% bovine gamma globulin (BGG), 0·01% BSA) were incubated for 90 min (room temperature). After washing (5 × PBS, 0·05% Tween) plates were incubated with rabbit anti-human-IgG/A/M-POD (Dako; 1:3000 dilution in PBS, 0·05% Tween, 0·01% BGG, 0·05% BSA, 200 μl/well, 90 min, room temperature). Bound antibodies were visualized with OPD. ODs were measured bichromatically at 492 nm/620 nm (Multiscan, Flow). All assays were performed in duplicate on each plate, including serum blanks. ODs exceeding 2·5 × the mean OD of three negative controls were regarded as positive (corresponding to > 25 arbitrary units (aU)).

For immunoblots and agargel double diffusion, microsomes from rat liver were isolated as described [29]. Subcellular particles as well as recombinant P450 2D6 MS2 polymerase fusion protein, respectively, were boiled in Laemmli-buffer and separated on SDS–PAGE (9% acrylamide; 80 μg protein/cm). The proteins were blotted on immobilon membranes (Millipore) overnight (0·3 mA/cm²). Accessory binding sites were blocked with 10% dry milk, 1% BSA, 0·15 M NaCl, 10 mM Tris pH 7·4 and 0·3% Tween 20. In experiments with recombinant fusion protein, sera were geometrically diluted until a band was no longer detectable. Rabbit POD labelled anti-human-IgG or IgM ($F(ab)_2$) (Dianova, Dako) were used in a 1:1000 dilution. Colour was developed with *o*-dianisidin as substrate. Agargel double diffusion was performed with 0·9% (PBS) agarose gels. Protein concentration of microsome preparations was 25 mg/ml.

Other autoantibodies

In addition to the above-mentioned indirect immunofluorescence tests, which also permitted detection of antinuclear, anti-smooth muscle and anti-mitochondrial (AMA) antibodies, all sera were tested for the presence of AMA-M2 specificity (74, 55, 51, 45, 36 kD proteins of the 2 oxoacid dehydrogenase complexes) by immunoblots. Submitochondrial particles were prepared by differential centrifugation of bovine heart homogenates as described [30], separated on SDS–PAGE and blotted as outlined above.

Anti-HCV and HCV-RNA (PCR)

For anti-HCV screening, commercially available assays (ORTHO ELISA II and RIBA II) were used. For anti-HCV ELISA coefficient of intra-assay variation was 9·8, that of interassay variation was 10·8 using the positive control serum of the test kit. For determination of HCV-RNA, 500-μl samples were concentrated by ultracentrifugation through a sucrose cushion (Beckman TL 60; 40 000 rev/min, 60 min, 4°C) followed by RNA extraction. Sediments were dissolved in 100 μl of lysis buffer (2% SDS, 10 mM Tris-HCl, pH 7·5, 1 mM EDTA, 10 μg proteinase K, 0·1 μg t-RNA), incubated for 2 h at 55°C followed by two organic extractions (phenol/chloroform and chloroform only) and ethanol precipitation. The RNA pellets were dissolved in 20 μl of a solution containing 50 units RNase inhibitor (RNasin, Promega) and 1 mM dithiothreitol, divided into two 10-μl aliquots, one of which was stored at −80°C and the other used directly for cDNA synthesis. The RNA solution was heated to 80°C (1 min), chilled on ice (2 min) and synthesis of cDNA was executed for 60 min at 45°C with Moloney murine leukaemia virus reverse transcriptase (10 units) in the presence of 5 mM Tris-HCl, pH 8·5, 50 mM KCl, 2·5 mM $MgCl_2$, 1 mM of dNTPs, 20 pmoles of first-round primers (nt −321 to −292) GACACTCCACCATAGATCACTCCCCTGTGA, (nt −29 to −58) CACTCGCAAGCACCCTATCAGGCAGTACCA, and 50 units of RNasin in a volume of 20 μl. After the addition of water (50 μl) the cDNA was denatured by heat (98°C, 3 min) and amplified by PCR in the first round in a final volume of 100 μl containing 10 mM Tris-HCl, pH 8·4, 50 mM KCl, 2·5 mM $MgCl_2$, 0·2 mM of the four dNTPs, 2 units of Taq DNA polymerase with the temperature-time profile 93°C/1 min, 50°C/2 min; 72°C/2 min for 37 cycles. A volume of 10 μl of the first-round PCR was amplified similarly in a second PCR with an internal pair of primers (nt −298 to −274) CCTGTGAGGAACTACTGTCTTCACG, (nt −52 to −75) AGTACCACAAGGCCTTTCGCGACC.

RESULTS

Anti-LKM-1 ELISA and Western blot

Synthesis of the MS2 polymerase P450 2D6 fusion protein occurred in *Escherichia coli* 537 transformed with the expression vector pEx34b containing cDNA sequences of the entire human P450 2D6 protein. A very high purification (> 95%) of the fusion protein was achieved by Ni^{2+} affinity chromatography (Fig. 1). The doublet of the fusion protein seen in immunoblots is probably caused by partial degradation. This MS2-polymerase fusion protein provides a high antigen specificity, because natural antibodies against MS2-polymerase do not occur in humans. Absorption and elution experiments showed that antibodies reacting with the recombinant antigen also stained the 50-kD band in immunoblots of natural liver microsomes (data not shown).

The results of chess board titrations using different coating concentrations of recombinant P450 2D6 MS2-polymerase fusion protein and anti-LKM-1-positive and negative sera are shown in Fig. 2, indicating an optimal coating concentration of 1 μg/ml. An excellent discrimination of positives and negatives was achieved by 1:250 dilution of sera. In the group of 50 healthy control persons, maximum OD was 0·23 (mean OD 0·14 ± 0·03). Coefficient of intra-assay variation was 6·5, that of interassay variation 9·2 using a positive control serum diluted to 60 arbitrary units.

The sera of 22 of the 27 HBs-Ag-negative patients with clinical diagnosis of CAH showed a characteristic fluorescence pattern of anti-LKM-1 antibodies with homogeneous brilliant fluorescence of rat hepatocytes and prominent staining of P3 zone of kidney (Table 1). Five patients' sera, however, revealed a pronounced staining of centrolobular hepatocytes, in four of which (not mentioned in Table 1) no anti-LKM-1 antibodies could be demonstrated either by ELISA or in Western blots using natural as well as recombinant antigens, nor by agargel double diffusion. Because of indefinite immunofluorescence pattern and the absence of anti-LKM-1 antibodies in all the other anti-LKM tests, these four patients were regarded as antibody negative. The presence of anti-mitochondrial antibodies was excluded in all the patients. From the remaining 23 patients, only 17 (74%) reacted with the recombinant fusion protein in ELISA. In 14 patients antibody concentrations were rather high (nos. 1–14, Table 1) reaching arbitrary units of 77–128. In three patients (nos. 15–17) antibody concentrations of 34–44 arbitrary units were measured, already approaching cut-

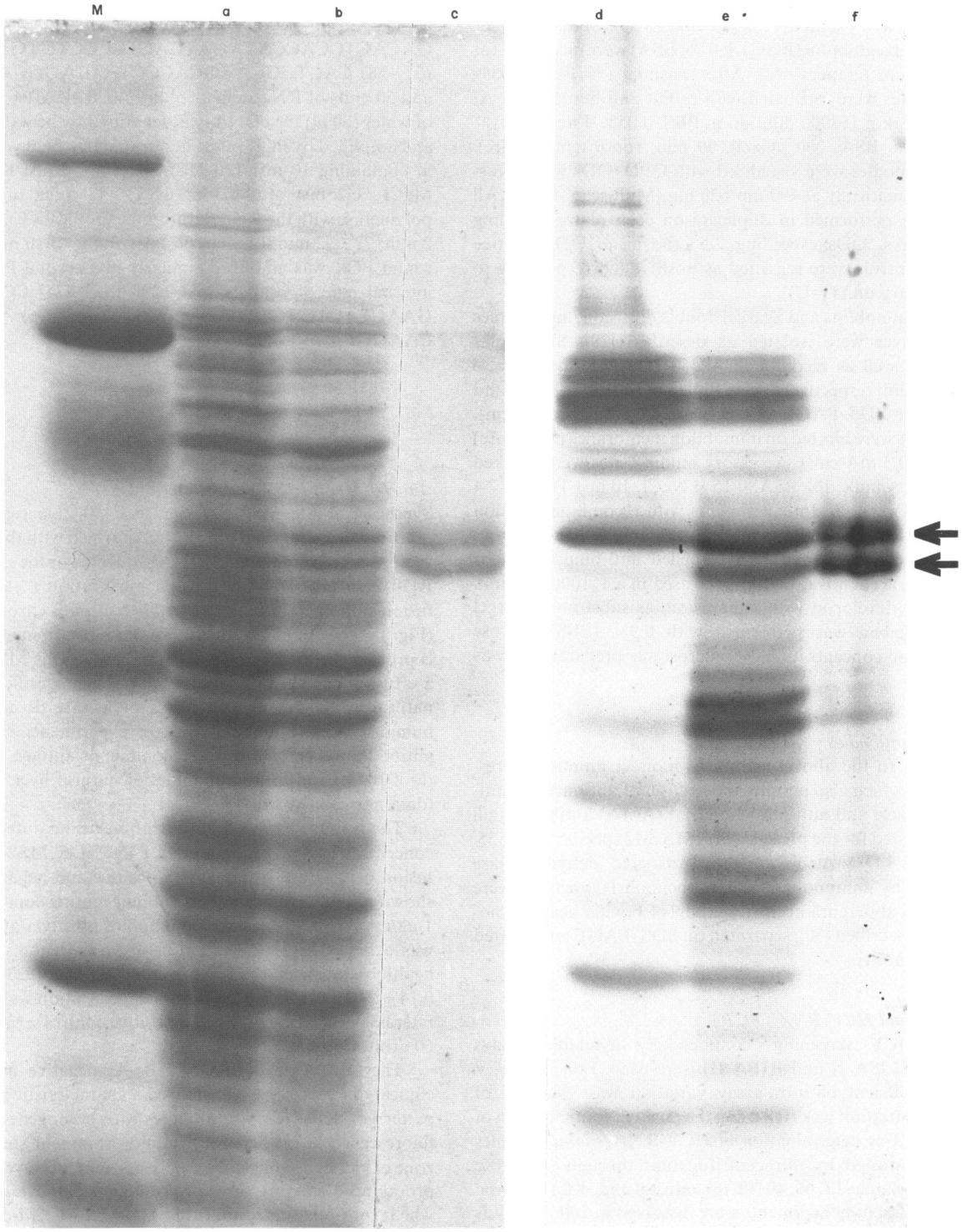

Fig. 1. Coomassie blue stained SDS–PAGE (a, b, c) and Western blot (d,e,f) of recombinant P450 2D6 at various purification steps. Western blot was incubated with anti-LKM-1 serum as outlined in Patients and Methods. Lane M, Molecular weight markers: from bottom to top: 23, 31, 52, 77, 106 and 200 kD; lanes a, d, extract of non-induced bacteria; lanes b, e, extract of induced bacteria; lanes c, f, purified recombinant P450 2D6 at concentrations of 4 μg/lane in c and 1 μg/lane in f.

off values (< 25 aU). Recombinant ELISA was obviously less sensitive than indirect immunofluorescence.

With one exception (no. 23) all the sera showing the anti-LKM-1 immunofluorescence pattern (including the one with centrolobular staining, no. 20) reacted in immunoblots with natural as well as with recombinant antigens. With natural antigens 22 sera reacted with a protein band in the 50/52 kD region, three sera also staining a 64-kD band. One patient (no. 20) with centrolobular immunofluorescence staining which is not regarded as specific for anti-LKM-1, was negative in ELISA

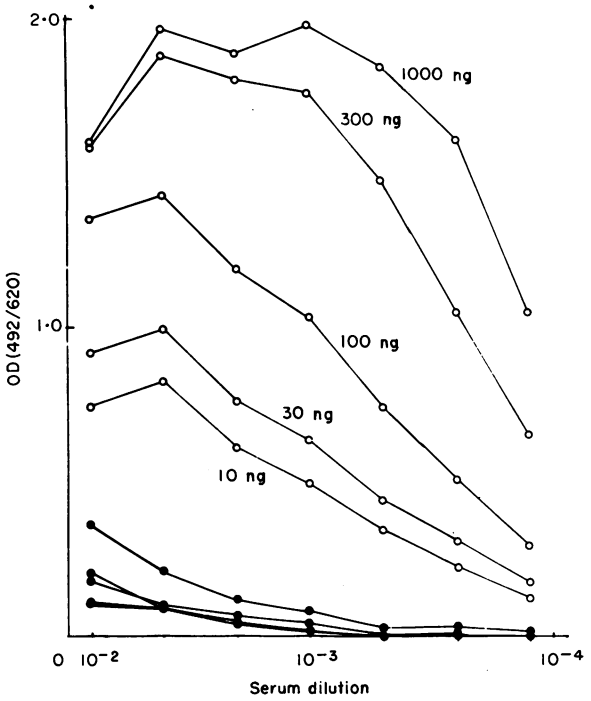

Fig. 2. ELISA: chess board titrations of anti-LKM-1 positive (○) and negative (●) sera with coating concentrations of recombinant CYP P450 2D6 from 10 to 1000 ng/ml. These curves indicate that an antigen concentration of 1 μg/ml is sufficient for coating, which was then used for routine testing.

but actually positive with natural and recombinant antigens in Western blot. Using immunoblots with recombinant antigens, antibody concentrations were measured by endpoint titrations. As can be seen from Table 1, this method is much more sensitive than ELISA and as a rule 10 times more sensitive than immunofluorescence. Inherent to immunofluorescence methods are subjectivity in titre reading and other imponderables concerning antigen presentation, expression and composition, which may be the cause of some titre incompatibilities in both methods. Five ELISA negative patients (nos. 18–22) also revealed precipitating antibodies against liver microsomes in agargel double diffusion, showing a line of identity with ELISA positive sera nos. 1 and 2. No such reaction was observed with the serum of patient no. 23. Whether this patient, negative in both immunoblot and ELISA but weakly positive in immunofluorescence, really harbours anti-LKM-1 cannot be decided.

In some patients up to four serum samples were available (Table 1). In most patients the maximum time interval between testing was 2–3 weeks and comparable results were obtained. From three patients (nos. 5, 7 and 11, Table 1) three (no. 5) and four (nos. 7, 11) samples were tested within time intervals of 1, 3 and 4 years, respectively. Patient no. 11 did receive immunosuppressive therapy for the first time after the fourth antibody test and antibody titres were at an unchanged high level during this period, lasting 4 years. Patients nos. 5 and 7 received corticosteroid and azathioprine followed by an intermittent two to three step decrease of antibody titres.

The results exemplify that antibody determination by Western blot with recombinant antigens is more sensitive than the ELISA in the present design and even more sensitive than indirect immunofluorescence test. Therefore, the use of recombinant P450 2D6 antigen in immunoblots can substitute for immunofluorescence and other tests working with poorly defined natural antigens.

Anti-HCV and HCV-RNA in anti-LKM-1-positive patients

Of the 22 patients harbouring antibodies against recombinant P450 2D6, 10 (45%) were positive for anti-HCV antibodies. Six sera contained antibodies against all the three HCV antigens (Core, C-22, C-100) tested in RIBA (Table 1). Also, all 10 patients were positive for HCV-RNA, indicating an on-going infection. According to the antibody concentration measured in immunoblots or ELISA with the recombinant P450 2D6 antigen, the percentage of HCV infection was much higher in patients with antibody concentrations below 50 aU (ELISA) or a titre of $< 12\,800$ in immunoblot (7 of 8 = 87·5%) than in the group exhibiting higher antibody concentrations (3 of 14 = 21·4%). From 10 anti-LKM-1-positive patients with HCV infection, eight were males but only two females. Thus in our series HCV infection and anti-LKM-1 antibodies are highly correlated in males. In half of the anti-LKM-1-positive patients who exclusively were females, anti-LKM-1 antibodies were not associated with HCV infections. Risk factors of HCV infections, such as drug abuse, homosexuality or polytransfusions, have not been mentioned.

Anti-HCV, HCV-RNA and anti-LKM-1 antibodies in control groups

As shown in Table 2, no anti-LKM-1 antibodies could be detected in sera of 100 patients positive for HCV-RNA. There was only one female showing a very weak immunofluorescence staining and borderline ELISA, but no reaction was observed in Western blot, neither with recombinant nor with natural antigens. Of 100 HCV-RNA-negative patients of the same disease group (mainly dialysis patients and patients with primary liver diseases) none harboured anti-LKM-1 antibodies. The high number of anti-HCV positives (17%) in the latter group may be due to the high percentage of underlying liver diseases. Anti-HCV as well as HCV-RNA was found in one female of 61 patients (57 females, four males) with antimitochondrial antibodies of the anti-M2 specificity. No anti-LKM-1 antibodies were detected in these patients by ELISA and Western blot.

DISCUSSION

Anti-LKM antibodies were first detected in sera of patients with chronic active hepatitis [31]. The anti-LKM-1 subtype of these antibodies reacting with cytochrome oxydase P450 2D6 was associated with a second form of autoimmune hepatitis (CAH type II) which was separated from the ANA or SMA antibody-positive CAH type I [4]. Laboratory tests for anti-LKM-1 antibodies were mainly performed by indirect immunofluorescence, a method which often leads to confusion with antimitochondrial antibodies (results of German autoantibody proficiency testing under supervision of one of the authors, H.P.S.) and which does not allow the assignment of the various LKM-antigen specificities mentioned above. Such problems can certainly be surmounted by use of recombinant P450 2D6 protein as target antigen in ELISA or immunoblot techniques. There are only two reports, however, concerning anti-LKM-1 determinations by recombinant antigen ELISA with 11 sera

Table 2. Anti-LKM-1 antibodies in dialysis patients and patients with primary liver diseases positive or negative for HCV-RNA and healthy control persons (**)

n	HCV-RNA	Per cent anti-HCV positive	Per cent anti-LKM positive			Mean age	
			Blot	ELISA	IFF	Female *n*/y	Male *n*/y
100	Positive	94	0	1*	1*	48/47	52/47
100	Negative	17	0	0	0	45/50	55/49
50**	Negative	0	0	0	0	25/38	25/42

* Borderline results, not significant positive.
n, Number of patients; y, age in years.

[23,24], and their antibody specificity was formerly ascertained by immunoblot techniques using a recombinant antigen. These sera reacted in ELISA with recombinant β-galactosidase P450 2D6 fusion protein, smaller proteins expressed from deletion clones of the encoding gene and synthetic peptides covering main antigenic epitopes, but no comparisons with other methods were reported. Immunoblot analyses using also a β-galactosidase P450 2D6 fusion protein [22,32,33] revealed that 85–90% of the sera showing an anti-LKM-1 pattern reacted with that antigen.

The MS2-polymerase P450 2D6 fusion protein has the distinct advantage that natural antibodies against the fusion part do not occur in humans. The anti-LKM-1 antibody ELISA seems to be highly specific, but of too low sensitivity for routine tests. False positive reactions were seen with neither any of the HCV-RNA-negative disease controls nor with AMA-positive sera, nor with sera of healthy persons. The absence of anti-LKM-1 antibodies in these sera was also confirmed by immunoblots with natural and recombinant antigens as well as by immunofluorescence tests. Therefore, immunoblots using a recombinant P450 2D6 protein in our hands seems to be the most reliable and sensitive method for testing anti-LKM-1 antibodies. Because human anti-LKM-1 might react with several epitopes of the protein, the use of the whole protein should be mandatory despite the existence of major antigenic epitopes [22,24]. Unfortunately, the small number of anti-LKM-1-positive patients available impedes large scale screening with different methods. Therefore the possibility of false negative immunoblots cannot be ruled out with certainty. Patients harbouring antibodies with anti-LKM-1 pattern in immunofluorescence tests but being non-reactive in immunoblots have been found by Manns *et al.* [22] as well as in our series (Table 1, no. 23). As with most of the recombinant or natural peptides and proteins subjected to SDS–PAGE and Western blot, steric hindrance or even destruction of antigenic epitopes has to be considered. On the other hand, antibodies of different antigen specificities can also cause an anti-LKM-1 antibody-like immunofluorescence pattern, and an atypical fluorescence pattern does not rule out anti-LKM-1 antibodies (Table 1, no. 20). Nevertheless, because of clinical demands the exact determination of antibody specificity is necessary and the use of immunoblots with recombinant P450 2D6 should substitute for less specific methods.

When the first generation anti-HCV assays were available a surprisingly high incidence (> 70%) of HCV infections in anti-LKM-1-positive patients was found, mainly in Italian studies [15,17,19,20], which was first thought to be caused by non-specific positive anti-HCV ELISA. Reinvestigations with second generation anti-HCV test and the demonstration of HCV-RNA, however, confirmed these findings. Our results, also, are in agreement with studies of Manns *et al.* [22] and Lunel *et al.* [21], who found a prevalence of 45% and 49%, respectively, of HCV infections in anti-LKM-1-positive patients. In accordance with these authors we found a preponderance of HCV-infected males, but their predominance is far more pronounced in our series (80% *versus* 35% or 45%, respectively). This unexpected high frequency of HCV infections associated with LKM-1 antibodies in males and not in females might be caused by the restriction to a group of patients harbouring a homogeneous antibody population reacting with the recombinant P450 2D6 protein. Further studies with larger collectives of patients of different ethnic background will be necessary to confirm this interesting finding. All anti-HCV-positive patients also harboured circulating HCV-RNA, indicating active hepatitis C virus (HCV) infection. The majority of HCV-infected patients (70%) exhibited lower anti-LKM-1 titres than the non-infected anti-LKM-1-positives. This was demonstrated here for the first time clearly by means of quantitative analyses using a recombinant antigen target. Similar results have been reported by Manns *et al.* [22] working with a competitive radioimmunoassay using natural anti-LKM-1 antibodies and microsome preparations, as well as by Lunel *et al.* [21] recording anti-LKM-1 titres obtained by immunofluorescence. Contrary to Lunel *et al.* [21] who found 3·2% anti-LKM-1-positives within randomly selected anti-HCV-positive patients, we could not detect any unequivocally anti-LKM-positive patients within 100 HCV-RNA positives. Thus, according to our results anti-LKM-1 antibodies do not seem to become a common sequel of HCV infection.

The existence of a distinct group of anti-HCV-negative females of younger age with high titre anti-LKM-1 antibodies supports the hypothesis of classifying anti-LKM-1-positive hepatitis in an infectious (HCV triggered) and an autoimmune form. This differentiation may be important for therapeutic reasons [34]. Improvement of anti-LKM-1 antibody-positive hepatitis after cortisone treatment and relapses after its withdrawal can be regarded as a feature of the autoimmune form, whereas the benefits from antiviral therapy mark the infectious form [19–21,35–37].

Neither the pathomechanism of the autoimmune nor of the

infection-triggered anti-LKM-1 antibodies are known. No major structural defects within the gene coding for P450 2D6 protein have been found in anti-LKM-1-positive patients [38] who also express intact P450 2D6 protein [39]. Viruses have long been suspected of initiating autoimmune diseases either by mimicry of self antigens or by association with self proteins rendering them antigenic. Molecular mimicry may be a cause of anti-LKM-1 antibody development in HCV infections, since there are sequence similarities between the antibody target (P450 2D6) and segments of the HCV encoded polyprotein [22]. On the other hand, an association of self antigens with HCV proteins may trigger antibody formation as was suggested for anti-GOR antibody development in HCV infection [40]. Interestingly, anti-GOR antibodies were also found in 79% of anti-LKM-1-positive HCV-infected patients, but only in 7% with the anti-LKM-1-positive autoimmune form [41]. It is conceivable that such mechanisms also work in anti-LKM-positive patients associated with HDV infections. There may exist a particular interaction between protein synthesis and transporting systems such as endoplasmatic reticulum, Golgi complex and cytoskeleton and autoantibody formation after virus infections. This could be one cause for anti-LKM antibody formation following HCV infections as well as for the development of anti-Golgi antibodies in HIV and rubella infections [42] or anti-cytoskeleton antibodies (part of which are SMA) following HDV or hepatitis A virus (HAV) infections [43].

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