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Occurrence and significance of IgG liver membrane autoantibodies (LMA) in chronic liver diseases of different aetiology

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SUMMARY

The prevalence of liver cell membrane antibodies (LMA) was evaluated in the sera of 124 untreated patients with various chronic liver diseases, in 17 acute hepatitis patients and in 40 normal controls by indirect immunofluorescence on rabbit hepatocytes, isolated by non-enzymatic method. The presence of LMA was compared with the presence of HBs Ag, anti-HBc and non-organ specific autoantibodies (anti-nuclear antibody, ANA; smooth muscle antibody, SMA; anti-mitochondrial antibody, AMA; liver–kidney microsomal antibody, LKM). LMA was found in 83% of autoimmune chronic active liver disease (CALD), in 47% of cryptogenic CALD and in 42% of primary biliary cirrhosis (PBC). LMA prevalence both in HBsAg positive and HBsAg negative/anti-HBc positive CALD was 11%, significantly lower than in the other three groups. In the cryptogenic group the prevalence of non-organ specific autoantibodies was significantly lower than LMA prevalence. The 35 LMA positive sera were titred to end point dilution. Autoimmune cases presented titres higher than those of all the other groups. Adsorption experiments showed that in autoimmune cases LMA fluorescence is not blocked by pre-incubation with liver antigens LSP and LP2, while a mild blocking effect was observed in some HBsAg positive cases or PBC sera. No cross-reaction with mitochondrial antigens was observed in PBC sera. LMA can still be considered a marker of autoimmune CALD only when present at high titre and without cross-reactivity with other liver antigens.

INTRODUCTION

Antibodies against two different hepatocyte membrane antigens may be detected in sera of patients with chronic active liver disease (CALD) (Tage-Jensen *et al.*, 1977; Jensen *et al.*, 1978a).

Antibodies directed against a liver specific lipoprotein complex (LSP) are studied by radioimmunoprecipitation and have been found in patients with HBsAg negative and positive chronic active hepatitis (CAH) and in the acute phase of viral hepatitis (AVH) (Jensen *et al.*, 1978a, 1978b; Meliconi *et al.*, 1981a, 1982).

Liver membrane autoantibody (LMA), detected by indirect immunofluorescence (IF) on isolated rabbit hepatocytes, has been found mainly in HBsAg negative CAH and cirrhosis (Tage-Jensen *et al.*, 1977).

This antibody was first described in association with the presence of membrane bound IgG on

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the hepatocytes isolated from patients' biopsies; IgG was present with a linear pattern (Hopf *et al.*, 1976). It has been suggested that LMA and membrane bound linear IgG are the same antibody which reacts *in vivo* and *in vitro* with liver cell membrane (Hopf *et al.*, 1976) and it has been considered to represent an exclusive marker of HBsAg negative autoimmune chronic liver disease (Kaiser *et al.*, 1981).

In the present investigation the prevalence of IgG class LMA in chronic liver disease was evaluated in relation to the presence of hepatitis B virus (HBV) markers (HBsAg, anti-HBc) and non-organ specific autoantibodies and studies were carried out on the distribution of LMA titres in order to evaluate whether LMA positive patients present homogeneous features of autoimmunity.

Finally we looked for a cross-reactivity between the target antigen of membrane autoantibodies and the liver specific protein (LSP) and a liver specific cytoplasmic antigen (LP2) (Behrens & Paronetto, 1979) or antigens present on the mitochondrial membrane.

MATERIALS AND METHODS

Patients. Sera were obtained from 141 patients admitted to our department with various forms of liver disease, during a 31 month period and from 40 normal controls (medical students and laboratory staff without clinical or laboratory signs of liver disease; HBsAg and anti-HBc negative by radioimmunoassay, RIA).

Ninety-three patients had chronic active hepatitis or active cirrhosis and were classified as CALD. In this group 55 cases were HBV related (37 HBsAg positive and 18 HBsAg negative/anti-HBc positive) 32 patients were HBsAg negative/anti-HBc negative with absent or low titre autoantibodies and therefore classified as cryptogenic; six patients were HBV markers negative, high titre autoantibodies positive and were considered autoimmune. Non-organ specific autoantibodies distribution is shown in Table 1.

No significant difference was found in the γ-globulin levels (g/dl, mean $\pm$ s.d.) among the various CALD groups (autoimmune $1{\cdot}82 \pm 0{\cdot}60$, HBsAg positive $1{\cdot}77 \pm 0{\cdot}45$, HBsAg negative/anti-HBc positive $1{\cdot}81 \pm 0{\cdot}38$, cryptogenic $1{\cdot}89 \pm 0{\cdot}58$).

Seven patients presented chronic persistent hepatitis (CPH). Four had prolonged acute viral hepatitis (PVH) and 12 had PBC. Eight patients presented Wilson's disease (in four cases liver biopsy was performed and revealed the features of chronic active hepatitis).

Seventeen patients were tested during the acute phase of viral hepatitis (AVH) type B and non-B.

All patients tested were off immunosuppressive treatment for at least 6 months.

The diagnosis in all the chronic patients was obtained on the basis of clinical, biochemical and histological findings according to the diagnostic criteria of an international committee (Leevy, Popper & Sherlock, 1976). Drugs and alcohol were excluded as possible aetiological agents on the basis of careful history taking and histological features.

Immunofluorescence test on isolated rabbit hepatocytes. Isolation of hepatocytes and LMA testing by immunofluorescence were performed according to the technique described by Hopf *et al.* (1976) with minor modifications.

Briefly, in the anaesthetized rabbit, the liver was perfused with solution 1 (stock solution 90 g of NaCl, 2·4 g KCl, 21 g $NaHCO_3$ with water to 1,000 ml, and 10 ml of stock solution with 2·5 g of bovine serum albumin (BSA), 20 mg of ethylene glycol-bis-aminoethyl-ether-*N*-*N*-tetra-acetate and water to 100 ml) and solution II (80 g of NaCl, 4 g KCl, 2 g of $MgSO_4.7\ H_20$, 0·6 g of $Na_2HPO_4.2H_20$, 0·6 g of NaH_2PO_4 and water to 1,000 ml) for 8 min.

After removal the liver was placed in a new medium: solution III (15 ml of stock solution II, 315 mg of Na_2HCO_3, 3·75 g BSA, 19·5 mg of $CaCl_2.H_2O$ and distilled water to 150 ml) and cut into small pieces which were forced through a strainer and poured into a flask. This was slowly shaken for 15 min at 37°C. The liver cells were filtered through nylon wool and washed twice in RPMI 1640 (GIBCO, Grand Island, New York, USA).

Fifty microlitres of the isolated hepatocyte pellet (mean concentration 2×10^6 cells/ml) were incubated with 100 μl of patients' serum diluted 1:4 for 30 min at 37°C. After two washings, the pellets were incubated with 100 μl of fluorescein isothiocyanate labelled anti-human IgG. After two washings a drop of the sediment was mounted and quickly read under a Zeiss microscope (Fig.1).

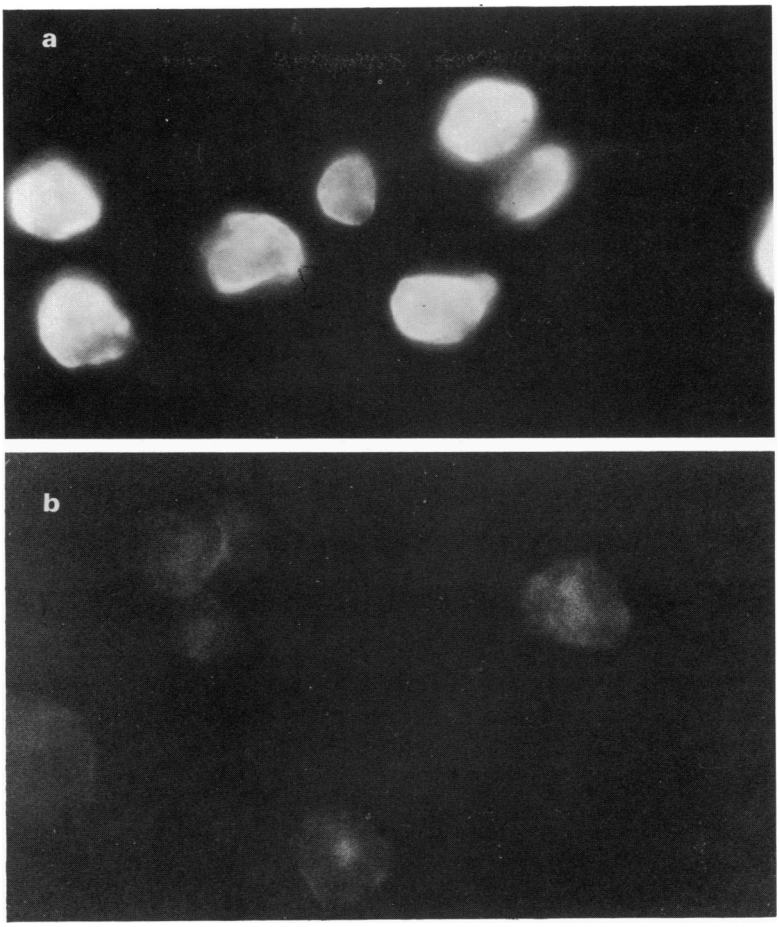

Fig. 1. (a) Liver membrane antibody detected in the serum of a patient with autoimmune chronic active liver disease. (b) Isolated hepatocytes after incubation with serum from a patient with HBsAg positive chronic active liver disease and incubation with fluorescein conjugated anti-human IgG antiserum: negative membrane fluorescence.

Non-specific IgG binding was excluded by negative findings after incubation of isolated liver cells with RPMI 1640 and fluorescein conjugated (FITC) antiserum alone.

LMA positive sera were titred to end point dilution.

Blocking experiments. LSP and LP2 were isolated from normal human liver according to the technique described by Meyer zum Büschenfelde, Kossling & Miescher (1972) and by McFarlane *et al.* (1977).

Suspension of rabbit liver mitochondria was kindly provided by Dr Giovanna Lenaz (Istituto di Biochimica, Università di Bologna).

Two hundred microlitres of sera from 11 high titre LMA positive patients (five autoimmune CALD, three PBC and three HBsAg positive CALD) were pre-incubated with 200, 400, 800 μg of LSP and LP2 for 1 hr at 37°C and for 15 hr at 4°C with occasional gentle agitation. After 30,000 g centrifugation for 15 min, the pellet was discharged and the supernatant was utilized in the LMA test.

The same procedure was performed for the pre-incubation of the three PBC sera with isolated mitochondria.

Detection of non-organ specific autoantibodies was performed by indirect immunofluorescence on cryostat sections of human thyroid and stomach and rat kidney and liver.

Table 1. Autoantibodies prevalence in liver patients and normal controls

Diagnosis	*n*	LMA *n* (%)	ANA *n* (%) (titre range)	SMA *n* (%) (titre range)	AMA *n* (%) (titre range)	LKM *n* (%) (titre range)
Autoimmune CALD	6	5(83)	2(33) (1:160–1:320)	0	0	4(66) (1:160–1:1,280)
PBC	12	5(42)	1(9) (1:20)	2(18) (1:20)	12(100) (1:320–1:1,280)	0
Cryptogenic CALD	32	15 (47)	5(17) (1:20–1:80)	4(12) (1:20–1:40)	0	0
HBV related CALD						
HBsAg+	37	4(11)	5(14) (1:20–1:40)	4(11) (1:20–1:40)	0	0
HBsAg−/anti-HBc+	18	2(11)	3(17) (1:20–1:40)	3(17) (1:20–1:40)	1(5) (1:160)	0
CPH	7	1(14)	0	0	0	0
PVH	4	1(25)	1(25) (1:20)	1(25) (1:20)	0	0
Wilson's disease	8	0	0	0	0	0
AVH	17	1(11)	n.t.	n.t.	n.t.	n.t.
Normal controls	40	1(2)	1(2) (1:20)	0	0	0

n.t. = not tested.

HBsAg, anti-HBc, anti-HBs were tested by solid phase radioimmunoassay (AUSRIA II, CORAB, AUSAB, Abbott, Chicago, USA).

LMA prevalence in the groups of chronic liver patients was compared using the χ square test with Yates' correction for small numbers. LMA titres were compared utilizing the non-parametric Wilcoxon rank sum test.

RESULTS

The results are summarized in Table 1. LMA were found mainly in autoimmune CALD (83%), in cryptogenic CALD (47%) and in PBC (42%).

The prevalence of LMA in the two groups of HBV related CALD was identical (11%) and significantly lower than in autoimmune cases ($P < 0{\cdot}0005$, $P < 0{\cdot}005$), and in cryptogenic cases $P < 0{\cdot}005$, $P < 0{\cdot}025$).

One of the seven CPH patients and two of the 17 AVH patients were positive for LMA.

One patient with prolonged viral hepatitis, with histological signs of chronicization was LMA positive, but negative for HBsAg and anti-HBc. Of the three other patients with PVH, one was HBsAg and anti-HBc negative, and two were HBsAg negative but anti-HBc positive.

None of Wilson's patients was positive for LMA and only two of the acute viral hepatitis cases presented circulating LMA. Among the normal controls only one case was LMA positive.

The immunofluorescence pattern was similar in all the positive cases. However two HBsAg positive patients and three PBC cases presented a slightly more granular fluorescence.

The titres of the liver membrane antibody in the 35 positive patients are shown in Fig. 2. Autoimmune cases presented LMA titres significantly higher than the HBsAg positive patients ($P < 0{\cdot}02$); the HBsAg negative/anti-HBc positive cases ($P < 0{\cdot}05$), the cryptogenic cases ($P < 0{\cdot}001$), the PBC cases ($P < 0{\cdot}05$) and the CPH, PVH and AVH patients ($P < 0{\cdot}025$). The results of the blocking experiments with human LSP, human LP2 and rabbit liver mitochondria are presented in Table 2.

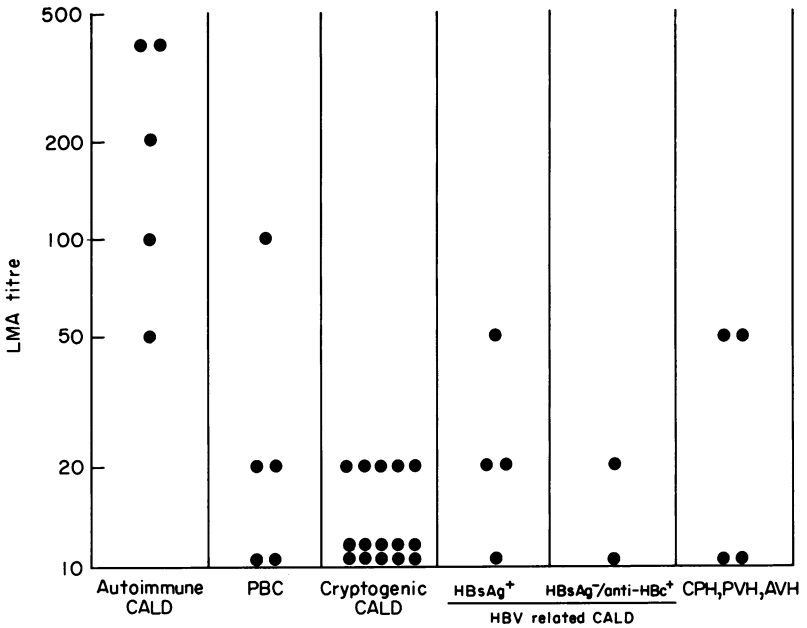

Fig. 2. Autoantibody titres of the 35 LMA positive cases. LMA titre is expressed as the reciprocal of serum dilution.

The hepatocyte membrane fluorescence obtained with autoimmune sera was not affected by pre-incubation with LSP or LP2. The membrane fluorescence presented by one HBsAg positive CALD was completely adsorbed either by LSP or LP2, while in another HBsAg positive CALD LMA was adsorbed only by the highest LSP and LP2 concentrations. In one PBC serum the fluorescence was abolished only by the highest concentration of LSP or LP2 added. No significant modification of the membrane fluorescence was observed after the pre-incubation with rabbit

Table 2. Blocking experiments

Serum No.	Diagnosis	LMA titre	Membrane fluorescence after adsorption with: Human LSP 200 μg	Human LSP 400 μg	Human LSP 800 μg	Human LP2 200 μg	Human LP2 400 μg	Human LP2 800 μg	Rabbit mitochondria 200 μg	Rabbit mitochondria 400 μg	Rabbit mitochondria 800 μg
1	Autoimmune CALD	1:400	+++	+++	+++	+++	+++	+++	n.d.	n.d.	n.d.
2	Autoimmune CALD	1:400	+++	+++	+++	+++	+++	+++	n.d.	n.d.	n.d.
3	Autoimmune CALD	1:200	+++	+++	+++	+++	+++	+++	n.d.	n.d.	n.d.
4	Autoimmune CALD	1:100	+++	+++	+++	+++	+++	+++	n.d.	n.d.	n.d.
5	Autoimmune CALD	1:50	++	++	++	++	++	++	n.d.	n.d.	n.d.
6	PBC	1:100	++	+	+	++	++	++	++	++	++
7	PBC	1:20	++	+	−	++	+	−	++	++	+
8	PBC	1:20	+	+	+	+	+	+	+	+	+
9	HBsAg+ CALD	1:50	+	+	+	+	+	+	n.d.	n.d.	n.d.
10	HBsAg+ CALD	1:20	−	−	−	−	−	−	n.d.	n.d.	n.d.
11	HBsAg+ CALD	1:20	+	−	−	+	+	−	n.d.	n.d.	n.d.

+ = weak positive; ++ = moderate positive; +++ = strong positive; n.d. = not done.

mitochondria in the three PBC sera. The prevalence of ANA and SMA in HBV related and in cryptogenic CALD was not significantly different.

The patients with CPH were found to be negative for non-organ specific autoantibodies. Two patients with PVH were autoantibody positive, one was SMA positive and one (positive for LMA) was ANA positive.

All the PBC patients were AMA positive, one was also ANA positive and two SMA positive.

In the cryptogenic CALD group, 10 patients positive for LMA were negative for ANA, SMA, LKM. In this subpopulation of CALD, LMA prevalence was higher than the prevalence of non-organ specific autoantibodies (47–19%, $P < 0.05$).

DISCUSSION

Earlier studies showed that LMA is strictly associated with HBsAg negative chronic liver disease, particularly in patients with autoimmune markers. These findings have recently been confirmed by Manns *et al.* (1980) using a solid phase radioimmunoassay for LMA. In this study the group of HBsAg positive patients was small (seven cases) and all the HBsAg negative CAH patients also presented anti-nuclear antibodies and therefore represented a homogeneous autoimmune group.

In the present study four groups of CALD patients, classified according to the presence of viral markers (HBsAg, anti HBc) and autoantibodies are studied.

It was demonstrated that LMA may also be detected even at a low percentage (11%) in HBsAg positive patients. In HBsAg negative patients, we identified a sub-group positive for anti-HBc which presented LMA prevalence identical to that observed in HBsAg positive patients. The highest LMA prevalence was found in autoimmune and cryptogenic CALD and in PBC. No significant difference was found in the prevalence of non-organ specific autoantibodies in the cryptogenic and HBV related CALD. LMA is significantly more frequent than non-organ specific autoantibodies in cryptogenic CALD, and therefore seems to represent a more sensitive marker of autoimmunity.

The high LMA prevalence in PBC cases confirms the findings of previous reports on autoimmune aggression against membrane antigens in this disease (Geubel *et al.*, 1976; Perperas *et al.*, 1980; Meliconi *et al.*, 1981b), particularly in cases with severe piecemeal necrosis (Perperas *et al.*, 1980).

Some conclusions may be drawn from these findings and from the study of the distribution of LMA titres in the 35 patients positive for this antibody.

LMA positive patients do not represent a homogeneous population from the point of view of autoimmune features or virological markers.

Definite LMA titre can be found in autoimmune and cryptogenic CAH, HBV related CAH, PBC. However the highest prevalence and above all the highest titres are found in autoimmune CALD.

Finally, low LMA titres are frequently detected in HBsAg negative/anti-HBc negative CALD patients who rarely present markers of autoimmunity (anti-nuclear antibodies). In these patients it is possible to suggest that the autoimmune reaction against liver cell membrane antigens may be triggered by a viral infection other than hepatitis B virus infection, probably due to the non-A, non-B hepatitis viruses.

The recent findings of Tage-Jensen *et al.* (1980), who found nine non-A, non-B CAH patients out of ten positive for LMA, are in keeping with this hypothesis.

Preliminary data from our laboratory on a large series of chronic non-A, non-B hepatitis again show high prevalence of LMA in this form of chronic viral infection (Meliconi *et al.*, 1981c).

It is noteworthy that patients with a metabolic disorder such as Wilson's disease do not present an autoimmune reaction against hepatocyte membrane.

The results of our blocking experiments show that in autoimmune cases the membrane fluorescence is not related to LSP and LP2. This finding is in keeping with the data reported by Meyer zum Büschenfelde *et al.* (1979). On the other hand, in PBC and HBsAg positive CALD a reaction against LSP could be involved in determining the membrane fluorescence. As far as LP2 is concerned, the blocking efficiency of this cytoplasmic antigen is observed only in sera with low

LMA titre and only at high antigen concentration: therefore it could be due to non-antigen specific interaction. Finally no cross-reaction was observed between LMA and anti-mitochondrial antibody.

Although sera from various liver diseases can produce a hepatocyte membrane fluorescence, LMA can still be considered a marker of autoimmune liver disease when present at high titre and without cross-reactivity with other liver antigens.

In viral and cryptogenic forms of CALD the presence of LMA is an expression, as anti-LSP antibody (Meliconi *et al.*, 1981a) and lymphocytotoxic reaction to liver cells (Facchini *et al.*, 1978) of an autoimmune component whose role in the pathogenesis of liver damage is still under investigation.

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