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Antibodies against human liver-specific protein (LSP) in acute and chronic viral hepatitis types A, B and non-A, non-B

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SUMMARY

Sera from 42 patients with acute viral hepatitis (AVH), 97 patients with chronic active liver disease (CALD) and 89 controls were tested by radioimmunoprecipitation for the presence of antibodies against human liver-specific protein (LSP). Anti-LSP were found in all but one patient with AVH type A (93%) and in a smaller percentage of AVH type B (55%). In non-A, non-B cases, anti-LSP were found in low percentages: 27% in acute cases, 10% in chronic cases. Furthermore, in CALD, a significant difference was found between HBsAg-positive CAH and 'autoimmune' CAH, both in anti-LSP prevalence (21%, 67%; $P<0{\cdot}005$) and in anti-LSP titre ($1:154\pm170$, $1:316\pm186$; $P<0{\cdot}005$). In HBsAg-negative/anti-HBc-positive CAH, three of 15 patients were anti-LSP positive. Anti-LSP were found only in three of 57 patients with various non-hepatic diseases with autoimmune features. None of the 12 healthy HBsAg carriers was positive. Hence there is evidence for a considerable heterogeneity in anti-LSP response in acute and in chronic inflammatory HBsAg-negative liver diseases. These data suggest that anti-LSP antibodies do not play a prominent role in the process of transition to chronicity of acute viral hepatitis particularly in non-A, non-B cases, whereas these antibodies may be important in the mechanism of ongoing liver cell injury in patients with 'autoimmune' CAH, and can represent a useful diagnostic marker of this type of hepatitis.

INTRODUCTION

In recent years the identification and partial characterization of two distinct hepatocyte membrane antigens, namely the liver-specific protein (LSP) and the liver membrane antigen (LM-Ag), have raised several questions and controversies on the role of the immune response to these proteins in the pathogenesis of different types of acute and chronic liver cell damage (Dienstag & Isselbacher, 1978; Meyer zum Büschenfelde *et al.*, 1979).

Sensitization against LSP was demonstrated by means of different *in vitro* techniques in patients with chronic liver diseases of various aetiology, and transiently in patients with viral hepatitis during the acute phase of the illness (Thomson *et al.*, 1974; Cochrane *et al.*, 1976a; Facchini *et al.*, 1978; Miller *et al.*, 1972; Ortona *et al.*, 1980; Stefanini *et al.*, 1978, 1981). The development of radioimmunoassay (RIA) techniques has allowed the detection of circulating anti-LSP antibodies in patients with chronic active hepatitis (CAH) and acute viral hepatitis (AVH) (Jensen *et al.*, 1978a,

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1978b; Kakumu *et al.*, 1979; Manns, Meyer zum Büschenfelde & Hess 1980a). So far, high titres of anti-LSP have been found with similar prevalence in both HBsAg-negative and -positive acute and chronic hepatitis (Jensen *et al.*, 1978b; Manns *et al.*, 1980a).

Recently, new agents of viral hepatitis have been discovered, the so-called non-A, non-B viruses which appear responsible for an increasing number of post-transfusion HBsAg-negative acute hepatitis cases with frequent progression to chronicity (Alter *et al.*, 1978; Berman *et al.*, 1979; Mosley *et al.*, 1977; Rakela & Redeker, 1979; Shirachi *et al.*, 1978).

These discoveries have indicated a vast heterogeneity among acute and chronic hepatitis cases, which were previously defined merely as HBsAg negative.

In this study we have evaluated the anti-LSP antibody response in acute and chronic hepatitis in relation to aetiology in order to assess further the pathogenetic and diagnostic relevance of this autoimmune response in viral and non-viral forms of liver injury.

MATERIALS AND METHODS

Patients

Acute viral hepatitis. Forty-two patients who presented clinical and biochemical manifestations of acute hepatitis were included in this study. A total of 130 sera were available from the 42 patients. Thirty-five cases were studied within 15 days after clinical onset.

Subsequent samples were available at different intervals during the acute phase of illness and afterwards up to 9 months from the onset.

Sixteen cases had a positive IgM-anti-HAV test and were classified as acute type A hepatitis, 11 HBsAg-positive cases were considered type B hepatitis, while 15 patients negative for HBsAg, anti-HBc, and IgM-anti-HAV were considered acute non-A, non-B cases (five post-transfusion cases).

Chronic liver disease. Ninety patients with CAH, classified according to the diagnostic criteria of an international committee (Leevy, Popper & Sherlock, 1976), were studied. Among these patients, 56 were HBsAg positive (48 were untreated and nine received immunosuppressive treatment—steroids and/or azathioprine). Fifteen patients were HBsAg negative but positive for anti-HBc (eight were also anti-HBs positive); 12 were untreated, three were on immunosuppressive treatment. Ten patients were investigated after progression to chronicity of post-transfusion non-A, non-B hepatitis; all these patients were untreated at the time of the study.

Nine patients were negative for HBV markers (HBsAg and anti-HBc) but positive for anti-nuclear antibodies (ANA) (titre $\geqslant 1:40$) and smooth muscle antibodies (SMA) or liver–kidney microsomal antibodies (LKM); they were therefore classified as ‘autoimmune’ CAH (three patients received immunosuppressive treatment).

Seven patients presented histological, biochemical and immunological features of primary biliary cirrhosis. None of these patients received immunosuppressive therapy.

Control groups. As controls we screened the sera from 89 subjects: 20 healthy subjects (blood donors, medical students) negative for HBsAg and anti-HBc; 12 healthy HBsAg chronic carriers (five with normal liver histology and seven with focal parenchymal necrosis); 10 patients with glomerulonephritis (all HBsAg negative and on immunosuppressive treatment); 10 patients with rheumatoid arthritis (all positive for ANA and rheumatoid factor); 10 patients with systemic lupus erythematosus; 10 patients with autoimmune thyroiditis; 10 with inflammatory bowel disease without liver involvement; seven patients with coeliac disease (positive for reticulin antibodies; R).

Methods

Isolation of LSP from human liver. All procedures were performed according to the method of McFarlane *et al.* (1977). A piece of normal human liver of about 500 g, obtained from a kidney donor at the time of transplant, was cut into small cubes and washed 10 times in cold 0·25 M sucrose (pH 8·0) for 30 min. A Waring blender and a Potter homogenizer were then used at 4°C to obtain a 50% w/v homogenate. After a 1-hr centrifugation at 105,000 *g* at 4°C, penicillin and gentamicin were added to the supernatant and stored at −20°C. Twenty millilitres of supernatant thawed at

4°C were centrifuged at 20,000 *g* for 15 min and then applied to a series of gel filtration elutions (Sephadex G-100, Sepharose 6B and Sepharose 4B columns).

Human LSP was then tested in immunodiffusion with anti-LSP antisera and always produced a single precipitin line with the antisera used.

Double immunodiffusion for LSP was performed in 0·6% agarose in borate/EDTA buffer. Five per cent Na-deoxycholate was added both to LSP and antiserum wells; the results were read after a 24-hr incubation at 37°C.

Guinea-pig antiserum, sheep antiserum and reference anti-LSP-positive human sera were kindly provided by Dr A. L. W. F. Eddleston, Dr I. G. McFarlane (London) and Professor K. H. Meyer zum Büschenfelde (Berlin).

^{125}I-radiolabelling of LSP. Fifteen microlitres of LSP solution (1 mg/ml) were radiolabelled with Bolton–Hunter reagent (Radiochemical Centre, Amersham, UK) in a fume cupboard at room temperature (Jensen *et al.*, 1978a). After iodination, 2 ml of borate/EDTA buffer containing 0·2 M glycine were added to ^{125}I-LSP. This stock solution was then dialysed in $7 \times 2{,}000$ ml borate/EDTA buffer at 4°C. The mean specific radioactivity obtained after dyalisis was 54×10^4 bequerels/μg. Mean percentage ^{125}I-LSP bindings obtained testing an antiserum, two positive reference sera and a pool of sera from healthy normal subjects (PNHS) are shown in Fig. 1.

Radioimmunoprecipitation for anti-LSP. The technique used was that described by Jensen *et al.* (1978a) with minor modifications. Each serum was screened in duplicate at 1 : 20 dilution; when found positive, it was tested again in three serial dilutions. Borate/EDTA buffer with 1% bovine serum albumin, 0·1% sodium azide, 10 μg/ml gentamicin and 600 units/ml penicillin was used in the assay.

Twenty-five microlitres of ^{125}I-LSP (100 ng/ml, 25,000 c.p.m./test-tube) were added to 25 μl of heat-inactivated serum and incubated overnight (15 hr) at 4°C.

One hundred microlitres of buffer containing 1 mg staphylococcal dried cells (Sigma Chemical, London) were then added. After a 1-hr incubation, 850 μl of buffer were added and the tubes were centrifuged at 30,000 *g* for 10 min in a microcentrifuge at 4°C.

Five hundred microlitres of the supernatant were transferred into another tube and both supernatant and infranatant with the pellet were counted in a gamma-counter (PRIAS PGD Autogamma, Packard) for 1 min.

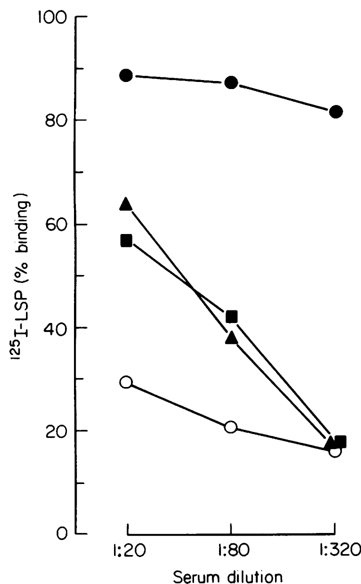

Fig. 1. ^{125}I-LSP binding in three serial dilutions of anti-LSP antiserum (●), two positive sera from CAH patients (▲, ■), and a pool of sera from healthy subjects (○).

The percentage binding of ^{125}I-LSP was calculated according to the formula:

$$\frac{\text{c.p.m. infranatant} - \text{c.p.m. supernatant}}{\text{c.p.m. infranatant} + \text{c.p.m. supernatant}} \times 100.$$

The titre of positive anti-LSP sera was calculated according to the method of Jensen *et al.* (1978b).

Screening for HBsAg, anti-HBs, anti-HBc and IgM-anti-HAV was performed by solid-phase radioimmunoassay (AUSRIA II, AUSAB, CORAB, HAVAB-M; Abbott, Chicago, USA).

Non-organ-specific autoantibodies (ANA, SMA, AMA, LKM, R) were detected by indirect immunofluorescent technique using cryostat sections of human thyroid and stomach and rat kidney and liver.

Statistical methods. The prevalence of anti-LSP in the different disease groups was compared using the Chi-square test with Yates' correction for small numbers, and Fisher's test.

Titres of anti-LSP were compared using the non-parametric Wilcoxon rank sum test.

RESULTS

Acute viral hepatitis

The results obtained in acute hepatitis cases types A, B and non-A, non-B are shown in Figs 2, 3 & 4 and in Table 1. During the first 2 weeks of illness, 14 of 15 patients in group AVH type A were positive for anti-LSP (93%; titre range 1:20 to 1:1,500).

In all patients, transaminase values declined regularly after the first sample. Only the patient who presented a late anti-LSP peak (7th week) showed two AST peaks, the second one appearing 7 days after the highest anti-LSP value (1:150).

Four of five patients tested after the 3rd month were negative. Four patients presented a second anti-LSP peak during the 4th week of illness, without any modification of transaminase behaviour.

In hepatitis B, five of nine patients tested during the first 2 weeks of illness were anti-LSP positive (55%, titre range 1:80 to 1:1,000). Three patients became negative during the 4th week, the other two were negative when tested after the 3rd month. None of these patients developed chronic liver disease.

In non-A, non-B hepatitis only three of 11 patients were positive during the first 2 weeks of illness (27%; titre range 1:70 to 1:400). A fourth patient was found positive at the first available sample obtained 22 days after onset. Among the four anti-LSP-positive patients, three presented sporadic forms, and one was a post-transfusion case. Patients still positive after the 3rd month eventually underwent a complete clinical and biochemical recovery, whereas two of the three

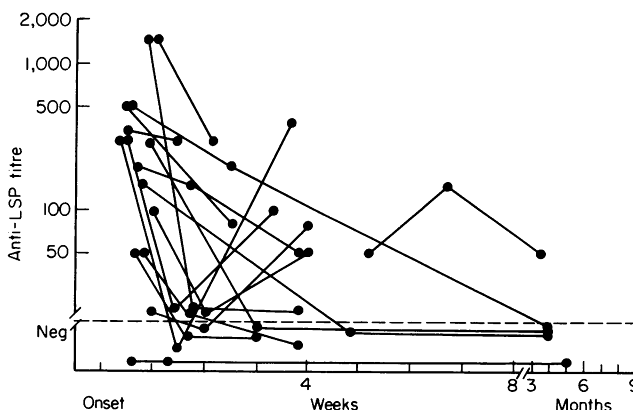

Fig. 2. Serial titres of antibody to LSP in patients with acute viral hepatitis type A. Anti-LSP titre is expressed as the reciprocal of serum dilution.

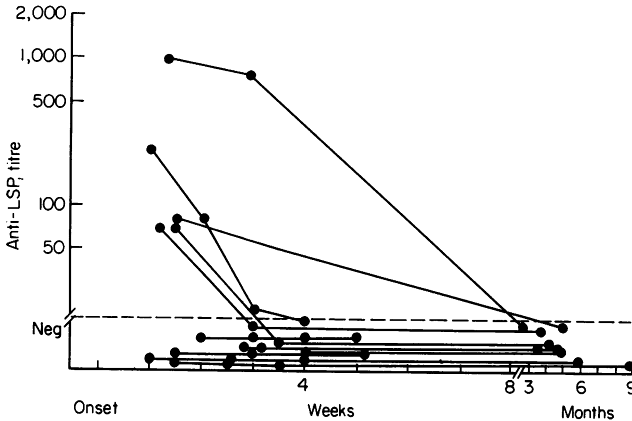

Fig. 3. Serial titres of antibody to LSP in patients with acute viral hepatitis type B. Anti-LSP titre as in Fig. 2.

patients who progressed to chronic liver disease were negative in all samples tested, and one became positive at low titre at the 5th month, but became negative again at the 9th month.

Comparing the prevalence of anti-LSP in the three groups of patients during the first 2 weeks of illness, we found a significant difference between AVH type A and non-A, non-B patients ($P < 0{\cdot}005$), while anti-LSP prevalence in patients with AVH type B did not reach statistical significance when compared with cases of A and non-A, non-B hepatitis.

No significant difference was found comparing the peak transaminase values in the three groups of patients.

Among the 24 AVH patients positive for anti-LSP, 13 were males and 11 females.

Chronic liver disease

The results obtained in chronic liver disease patients are summarized in Fig. 5 and in Table 1.

In HBsAg-positive CAH, only 12 patients (21%) were anti-LSP positive (titre range 1:20 to 1:500). A similar prevalence is found if we consider the untreated cases alone (10 of 48; 20%). The three patients with the highest anti-LSP values were untreated.

In HBsAg-negative, anti-HBc-positive CAH, three of 15 patients were found positive (20%); all three were anti-HBs negative. Only three patients of this group were receiving immunosuppressive treatment and were anti-LSP negative.

In chronic non-A, non-B hepatitis, only one patient (10%) was positive at a low titre (1:80). It is

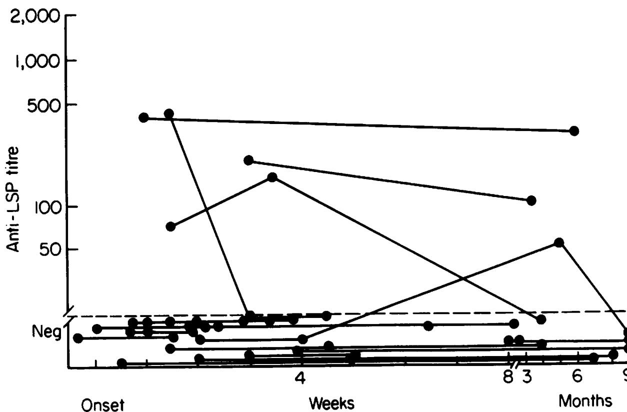

Fig. 4. Serial titres of antibody to LSP in patients with acute viral hepatitis type non-A, non-B. Anti-LSP titre as in Fig. 2.

Table 1. Anti-LSP-positive patients in acute and chronic liver diseases and in control groups.

Liver diseases	Anti-LSP	Control groups	Anti-LSP
Acute viral hepatitis type A*	14/15	Normal controls	0/20
Acute viral hepatitis type B*	5/9	Healthy HBsAg carriers	0/12
Acute viral hepatitis type non-A, non-B*	3/11	Glomerulonephritis	1/10
$HBsAg^+$ chronic active hepatitis	12/56	Systemic lupus erythematosus	2/10
$HBsAg^-$, $anti\text{-}HBc^+$ chronic active hepatitis	3/15	Rheumatoid arthritis	0/10
Non-A, non-B chronic active hepatitis	1/10	Autoimmune thryoiditis	0/10
'Autoimmune' chronic active hepatitis	6/9	Inflammatory bowel disease	0/10
Primary biliary cirrhosis	2/7	Coeliac disease	0/7
Total	46/132		3/89
(%)	(34·8)		(3·4)

* Anti-LSP-positive cases during the first 2 weeks of illness.

noteworthy that all these patients were untreated and among anti-LSP-negative cases, three presented with very severe piecemeal necrosis and portal inflammation in the liver biopsy.

In 'autoimmune' CAH, six of nine patients (67%) were anti-LSP positive (titre range 1:50 to 1:600); four of these were untreated and two were receiving immunosuppressive treatment at the time of the study.

The prevalence of anti-LSP in 'autoimmune' CAH was significantly higher than in HBsAg-positive CAH ($P<0{\cdot}005$) and in non-A, non-B CAH ($P<0{\cdot}025$). Furthermore, the mean ± s.d. titre of anti-LSP-positive cases in 'autoimmune' CAH was significantly higher than that in HBsAg-positive CAH (1:316 ± 186, 1:154 ± 170; $P<0{\cdot}005$).

In PBC patients, anti-LSP were present in two of seven cases (29%; titres 1:50, 1:400).

In patients with chronic liver diseases positive for anti-LSP, 13 were males and 11 females, with male prevalence (12:4) in virus-related chronic liver diseases and female prevalence (7:1) in the 'autoimmune' type.

In acute and chronic viral hepatitis we did not find any significant correlation between non-organ-specific autoantibodies and anti-LSP antibodies.

Control groups

All but three patients were anti-LSP negative (Table 1). A female patient with chronic glomerulonephritis had anti-LSP at a titre of 1:100. This patient was HBsAg negative and, when

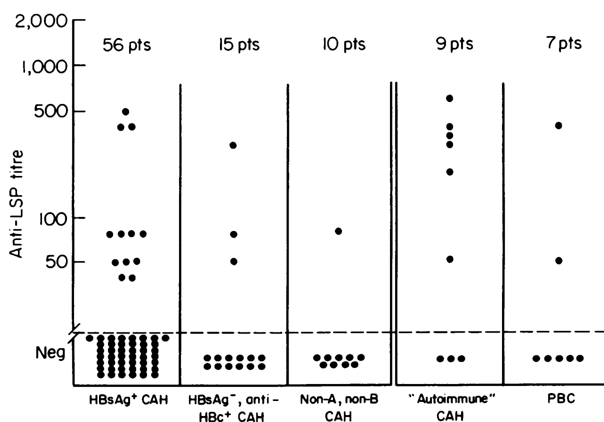

Fig. 5. Antibody titres in 90 patients with CAH and in seven with PBC. Anti-LSP titre as in Fig. 2.

checked after anti-LSP testing, was found to have had a slight increase in transaminase values 6 days earlier, which returned to normal 5 days later.

Two patients with systemic lupus erythematosus without biochemical signs of liver disease were positive at titres of 1:20 and 1:80.

The overall prevalence of anti-LSP in chronic liver disease (24 of 97; 25%) was significantly higher than in the control groups (three of 69; 4%; $P<0{\cdot}001$).

DISCUSSION

Antibodies to a liver membrane lipoprotein have been suggested to mediate the lymphocytotoxicity demonstrable *in vitro* in patients with HBsAg-negative and -positive CAH (Cochrane *et al.*, 1976b; Stefanini *et al.*, 1978. Jensen *et al.* (1978b) found a correlation between anti-LSP antibody titres and the extent of piecemeal necrosis in CAH patients. They therefore postulated that humoral reaction to LSP may play an important role in the pathogenesis of hepatocyte injury in chronic active liver disease. This hypothesis has been confirmed by Tsantoulas *et al.* (1980) in patients with primary biliary cirrhosis.

In the present study we investigated a large number of acute and chronic liver disease patients with definitive virological characterization. This enabled us to find significant variations in anti-LSP prevalence among the three main types of AVH and between viral (B and non-A, non-B) and 'autoimmune' types of CAH.

In AVH we found a considerable heterogeneity in anti-LSP response in patients with non-B (HBsAg negative) acute viral hepatitis. In fact, all but one of the patients with AVH type A were positive at the first sample, while in non-A, non-B patients only a minority (27%) were positive.

In HBsAg-positive acute cases, anti-LSP prevalence was lower than in type A cases (55%). However, this difference is not statistically significant and can be related to the different timing of the first sample in HBsAg-positive patients. In fact, these patients were studied between the 7th and the 14th day after clinical onset, while the first sample in type A AVH patients was obtained between onset and the 7th day. Therefore, we can postulate a similar transient sensitization against LSP during the acute phase of both types A and B AVH cases probably linked to early liver cell necrosis or to loss of tolerance for membrane components due to viral replication (Meliconi *et al.*, 1981a).

On the other hand, the investigation of a highly selected group of patients with acute and chronic non-A, non-B hepatitis has shown that anti-LSP response is only seldom triggered in this acute viral infection. Furthermore, it is also absent in chronic patients, even when severe piecemeal necrosis and portal inflammation is present in the liver biopsy. These data are confirmed by the lack of relationship we found between the process of transition to chronicity in acute hepatitis and the persistence of anti-LSP in patients with acute non-A, non-B hepatitis.

Hence, a substantial lack of sensitization to LSP is present in this viral infection and different membrane antigens could be taken into account as targets of possible immune reactions, especially in chronic cases (Meliconi *et al.*, 1981b). Alternatively, since circulating immune complexes have been described in sera of a high percentage of acute and chronic non-A, non-B patients (Dienstag *et al.*, 1979), negative results in anti-LSP tests can be explained by the presence of antibodies complexed to LSP, which are not detectable by our technique. On the other hand, immune complexes have also been detected in sera of patients with type A or B AVH or with CAH (Thomas *et al.*, 1978; Abrass, Border & Hepner, 1980).

The striking difference in anti-LSP response between non-A, non-B CAH and 'autoimmune' CAH emphasizes the possible role of this antibody in the immunopathogenesis of hepatocyte necrosis in the latter form of chronic hepatitis. Furthermore, we suggest that the finding of high-titre anti-LSP in patients with HBsAg-negative, anti-HBc-negative chronic active liver disease could be more relevant than non-organ-specific autoantibodies in differentiating the 'autoimmune' type of CAH from the form due to the process of progression to chronicity of non-A, non-B virus infection.

In our series of HBsAg-positive CAH, anti-LSP prevalence was not particularly high. A possible explanation could be linked to the clinical and immunological features of HBsAg-positive CAH in Italy. In our country this disease is relatively common, with a prevalence of mild forms with no signs

of abnormal immunological reactivity (Bianchi *et al.*, 1972). Evidence for lack of altered immunological regulation in HBsAg-positive CAH and non-A, non-B CAH has been produced by the study of Tremolada *et al.* (1980) who found normal T-suppressor function in these types of CAH, while a significant deficit was present in autoimmune, cryptogenetic and anti-HBs- and anti-HBc-positive forms of CAH.

Finally, the still incomplete purification and characterization of LSP should be taken into consideration. Recent studies by Behrens & Paronetto (1979) demonstrated that LSP preparation contains non-organ-specific components. Manns *et al.* (1980b) initially presented evidence for species-specific and non-species-specific determinants on LSP. The same group (Manns & Meyer zum Büschenfelde, 1980) then isolated a species-specific determinant by CsCl density-gradient centrifugation, which proved to be organ-specific and reacted only with sera from autoimmune HBsAg-negative CAH.

Thus the use of purified smaller components from LSP is now necessary for better characterization of the immune response to liver-specific membrane antigens, and for analysing their pathogenetic significance in acute and chronic liver disease.

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