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The Role of Liver Membrane Antigens as Targets in Autoimmune Type Liver Disease

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1. Introduction

Autoimmune type chronic active hepatitis is clinically characterized by the presence of non-organ-specific autoantibodies, high gammaglobulin levels, circulating liver membrane autoantibodies, and the absence of hepatitis B virus markers. Host immune reactions against liver membrane antigens are regarded to be of significant importance for hepatic injury. Organ-specific determinants are of special interest whereas non-organ-specific determinants are believed to account for extrahepatic symptoms in chronic inflammatory liver diseases. In the present paper a review is given of the published data concerning the identification of liver membrane antigens. Furthermore the present knowledge of immune reactions against these target antigens will be reported and their clinical significance will be discussed.

2. Characterization of Liver Membrane Antigens

2.1. The Liver Specific Protein (LSP)

The liver specific protein (LSP) is part of a macrolipoprotein complex and can be detected in the first peak of Sephadex G 200 chromatography of 100,000 *g* supernatants of fresh human liver homogenates [37]. A further purification and an improved stability of the labile components of this lipoprotein fraction has been achieved by chromatography on Sepharose 6B in a Tris buffer system containing 1 mM EDTA [32]. This high molecular weight liver specific protein complex contains a membrane antigen localized on the cell surface of liver cell membranes as shown by immunofluorescence studies using isolated hepatocytes and heterologous anti-LSP sera [15].

Small amounts of human LSP were purified by affinity chromatography on insolubilized anti-LSP serum prepared in a sheep [6]. After elution from the affinity chromatography columns using 3 M sodium iodide, human LSP showed only one precipitin band with the sheep antiserum whereas native LSP complex reveals both

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species-specific and non-species-specific determinants. The non-species-specific determinant is altered by the sodium iodide treatment and seems to be less stable [31].

Molecular weight determinations on calibrated Sepharose 4B and Sepharose 6B columns suggest that the molecular weight of the LSP fraction is between 4×10^6 and 20×10^6 daltons. The lipid and apolipoprotein moieties of the liver specific protein were partially characterized after separation on LH-20 column chromatography. Thin layer chromatography of the different fractions showed that LSP contains large amounts of phosphatides and triglycerides [17]. A further analysis of the phosphatides revealed cephalin, sphingomyelin, lecithin, and lysolecithin. The antigenicity of LSP depends on its lipid content. Delipidated LSP does not react with antisera against native LSP when tested by double immunodiffusion [17]. Polyacrylamide gel electrophoresis of LSP isolated from different species demonstrated a similar mobility of the macrolipoprotein. When the same experiment is performed in the presence of sodium dodecylsulphate (SDS) five major and several minor components can be distinguished demonstrating the existance of different polypeptides [17, 32]. In recent studies immunoelectrophoretic methods were used for the identification of species-specific and non-species-specific determinants of the LSP complex. Human LSP shows in crossed immunoelectrophoresis two different precipitin lines with anti-human LSP serum prepared in a sheep whereas rabbit, rat, mouse, and swine LSP exhibited one precipitin line with similar mobility. No immunoprecipitate was found with sheep and bovine LSP [31]. In addition to the non-species-specific determinant present in several mammals human LSP complex contains a species-specific determinant. Analogous results were obtained by fused rocket immunoelectrophoresis (Fig. 1).

When LSP of these seven different species was tested against the sheep anti-human LSP with fused rocket immunoelectrophoresis a pattern of identity is seen of one human precipitin line with rabbit, rat, swine, and mouse LSP. These species share a common non-species-specific determinant. A second precipitate is only seen with human LSP (Fig. 1).

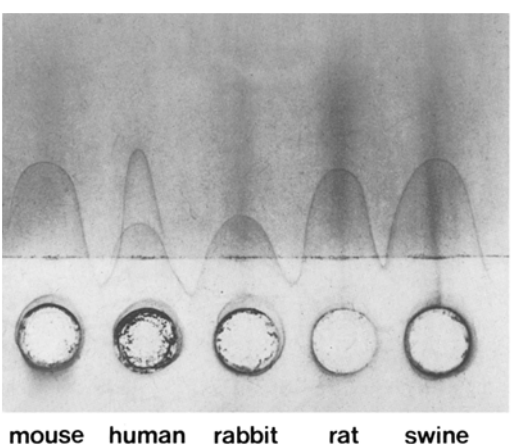

Fig. 1. Fused rocket immunoelectrophoresis: LSP of different species tested against sheep anti-human LSP serum

Autoantibodies of rabbits with experimentally induced chronic active hepatitis (CAH) react against human LSP with additional precipitin peaks in crossed immunoelectrophoresis. Using rabbit LSP only one precipitate is found. This autoantibody specificity is directed against the non-species-specific determinant of LSP as confirmed by absorption experiments with LSP from different species [31].

The hypothesis of different determinants requires that the LSP complex displays microheterogeneity. Microheterogeneity can readily be assessed by isoelectric focusing (IEF). Human and raddit LSP have been isoelectrically focused in a 4.3% macroporous polyacrylamide slab gel through a pH 3 to 10 gradient (Möller, B., Meyer zum Büschenfelde, K.-H., unpublished data). A series of six to seven bands with identical isoelectric points in human and rabbit LSP can be identified (Fig. 2). The protein stained bands indicate a similar microheterogeneity of the LSP in both tested species. The protein bearing pH-regions of the slab gel obtained after IEF were separated and used for short time immunization of rats.

The antisera raised against different protein bands of isoelectrically focused human and rabbit LSP were compared in fused rocket immunoelectrophoresis. Before absorption of rat anti-LSP sera with plasma, kidney, and blood cells, the immunoelectrophoretic pattern of different antigenic proteins in the elution profile of Sepharose 6B by fractionated soluble liver proteins is shown in Fig. 3. The unabsorbed antiserum contains antibodies reacting with different proteins of the first and second peak of Sepharose 6B chromatography. The antisera of all four isoelectrically focused protein zones show a similar immunoelectrophoretic pattern

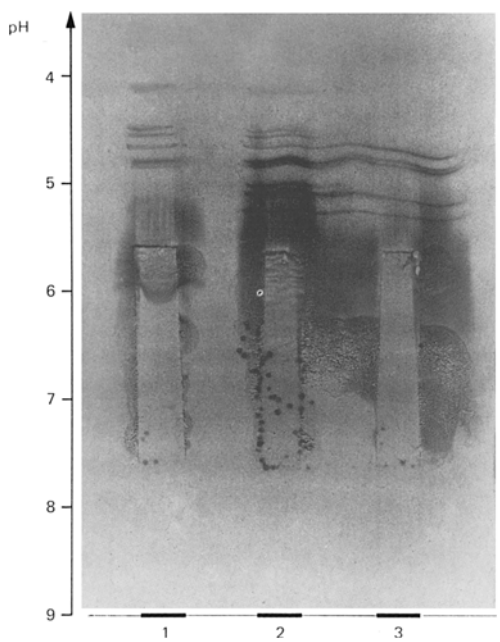

Fig. 2. Isoelectric focusing (pH 3–10) of purified human-LSP [1] and rabbit-LSP [2, 3] prepared by gel chromatography on Sepharose 6B

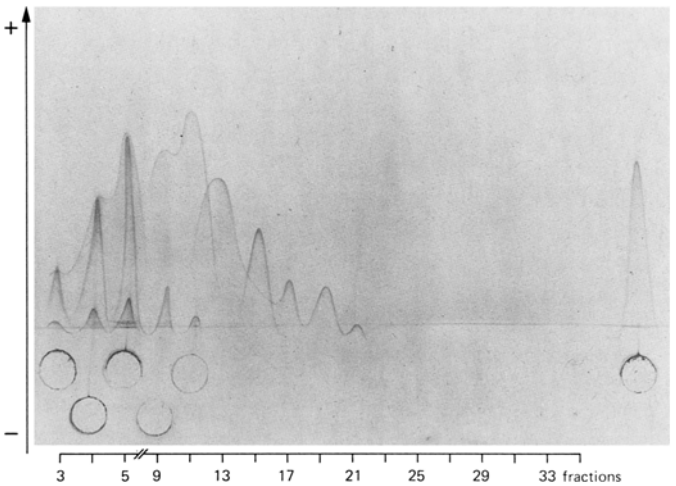

Fig. 3. Fused rocket immunoelectrophoresis: Fractions of rabbit liver proteins obtained after Sepharose 6B chromatography tested against non-absorbed rat anti-rabbit-LSP (immunizing antigen: isoelectric focused LSP, pH gradient 3–10)

in fused rocket immunoelectrophoresis suggesting a similar distribution of several antigenic determinants on each isoelcetrically focused constituent of the LSP complex.

The absorbed antiserum forms only one precipitate in the region of the first peak of Sepharose 6B chromatography (Fig. 4). It is identical with the species-specific moiety of LSP and is also detectable in all isoelectric-focused LSP-fractions.

Recently new radioimmunoassay systems for the detection of autoantibodies against LSP were developed [20, 22, 30]. Such highly sensitive test systems may allow further characterization of the liver specific lipoprotein complex LSP and its antigenic determinants.

Behrens and Paronetto [3] recently reported additional immunochemical studies on LSP. Comperative investigations on LSP, using Sepharose 6B first peak, and the kidney equivalent, designated KSP, revealed no major immunochemical differences of both antigen preparations. These authors were not able to produce organ-specific antibodies in rabbits, which may be due to different immunization procedures when compared to previous reports. Nevertheless these authors derived from cytotoxicity studies [4] the conclusion that the LSP preparation contains organ-specific determinants.

2.2 The Liver Membrane Antigen (LM-Ag)

Absorption studies revealed that liver membrane autoantibodies (LMA) which were detected by indirect immunofluorescence [13] could not be absorbed by purified LSP in many sera. This observation was the first evidence that LSP may not be the only target antigen in liver diseases. By affinity chromatography on insolubilized serum from patients with HBsAG-negative CAH a protein was isolated which reacted in crossed immunoelectrophoresis with HBsAg-negative LMA-positive

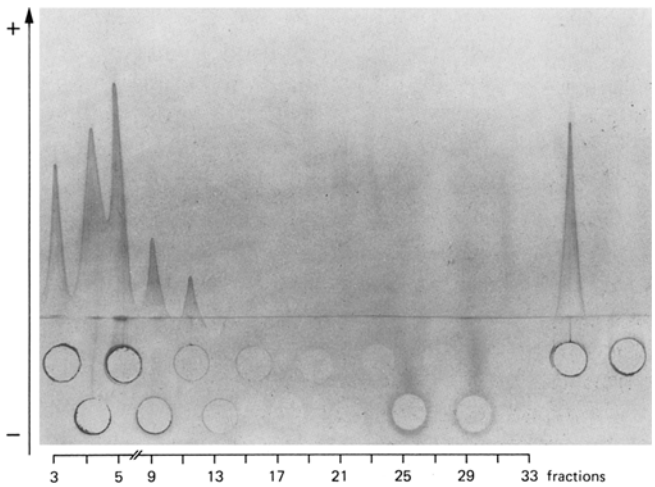

Fig. 4. Fused rocket immunoelectrophoresis: Fractions of rabbit liver proteins obtained after Sepharose 6B chromatography tested against rat anti-rabbit-LSP (immunizing antigen: isoelectric focused LSP, pH gradient 3–10) after absorption with rabbit plasma and rabbit kidney homogenate

CAH serum [36]. This protein was purified from human and rabbit soluble liver proteins. Immunological identity was confirmed by tandem crossed immunoelectrophoresis. The antibody activity to this new antigen, called liver membrane antigen (LM-Ag), could be absorbed by isolated rabbit hepatocytes. LM-Ag could not be purified by affinity chromatography on LMA-negative HBsAg-positive CAH-serum, normal human serum, normal sheep serum, and anti-LSP-serum prepared in a sheep.

Schuurman, H. J., Vogten, A. J. M., Schalm, S. W. (personal communication) were able to prepare liver membrane antigen (LM-Ag) from normal human liver by homogenization, sucrose density gradient centrifugation, treatment of the 1.18–1.20 g/cm^3 inter-face with Triton X 100, and subsequent Sepharose 6B chromatography. Using a reference serum from a patient with HBsAg-negative CAH which contained high titre liver membrane autoantibodies as detected by the LMA-test [13], LM-Ag was located in the second peak of Sepharose 6B. This reference serum was anti-LSP negative. At present it is unknown whether both LM-Ag preparations represent identical antigens.

3. Humoral Immunity Against Liver Membrane Antigens

3.1. Autoantibodies Against Liver Specific Lipoprotein (Anti-LSP)

Jensen et al. [20] first reported a sensitive radioimmuno-precipitation test for the detection of circulating anti-LSP. LSP in this test system was radio-labelled with I^{125} by the Bolton-Hunter technique [5] and LSP-anti-LSP complexes were precipitated by Cowan I staphylococcal cells, which contain protein A in their cell walls that avidly binds the Fc region of IgG. Ninety seven percent (29/30) of patients

with untreated CAH had anti-LSP detectable in their serum. The mean titre was higher in HBsAg negative cases, although the difference was not significant. Ninety five percent (20/21) of patients with acute viral hepatitis had anti-LSP within two weeks of the onset of jaundice. In uncomplicated acute viral hepatitis sera became anti-LSP negative within 12 weeks. Anti-LSP was found in 60% (10/17) of patients with chronic persistent hepatitin (CPH.) A highly significant correlation was observed between antibody titre and histological and biochemical parameters of activity of disease in CAH. No correlation existed with the presence of non-organ-specific autoantibodies. Smooth muscle antibodies (SMA), antinuclear antibodies (ANA) and antimitochondrial antibodies (AMA).

A similar technique was used by Gerber et al. [9] who detected anti-LSP in 63% (38/60) of patients with chronic active hepatitis (CAH) irrespective of the presence of non-organ-specific autoantibodies (ANA, SMA, AMA) or HBsAg. The incidence of anti-LSP was significantly higher in untreated patients. Anti-LSP were further found in patients with primary biliary cirrhosis (PBC), chronic persistent hepatitis. (CPH) and acute viral hepatitis (AVH) not in patients with alcohol induced liver diseases, but anti-LSP were detected in 18% (3/17) of patients with glomerulonephritis (Table 1).

Kakumu et al. [22] used a different radioimmunoprecipitation technique to detect circulating anti-LSP in patient sera. In their studies LSP was labelled using the Chloramine T method [19] and rabbit anti-human IgG serum was used as second antibody. These authors found anti-LSP in 57% of patients with chronic

Table 1. Frequency (% positive) of anti-LSP in patient sera

	Jensen et al. [20]		Kakumu et al. [22]	
	%	No.	%	No
Chronic active hepatitis	97	29/30	57	25/44
Chronic persistent hepatitis	60	10/17	22	5/23
Acute viral hepatitis	95	20/21	40	12/32
Alcohol induced liver disease	–	–	0	0/8
Miscellaneous liver diseases	0	0/14	0	0/22
Cirrhosis of the liver	–	–	38	8/21
Primary biliary cirrhosis	–	–	33	2/6
Drug induced hepatitis	–	–	0	0/11
Primary non-hepatic auto-immune diseases	–	–	0	0/60
Healthy blood donors	–	–	0	0/50

active hepatitis irrespective of HBsAg status. Twenty two percent of patients with CPH, 38% of patients with liver cirrhosis, and 40% of patients with acute viral hepatitis had anti-LSP. In uncomplicated cases anti-LSP disappeared within two months after the onset of disease. In cases which progressed to CAH anti-LSP remained positive and indicated development of CAH before the diagnosis was made histologically. The frequency of anti-LSP was significantly correlated with the presence of non-organ-specific autoantibodies. No correlation was observed with other biochemical data.

Manns et al. [30] developed a radioimmunoprecipitation test for anti-LSP similar to the one described by Kakumu et al. [19]. Anti-LSP was detected in 44% of patients with (Chronic active liver disease), 56% with CPH, 42% with AVH, and 20% with inactive cirrhosis irrespective of HBsAg status. Anti-LSP was also found in 29% of patients with alcohol-induced liver disease, 14% with miscellaneous liver diseases, and 10% of patients with primary non-hepatic autoimmune diseases (Table 1); anti-LSP was not detectable in 31 healthy blood donors. In this study within the group of CALD no correlation was observed transminase between anti-LSP and sex, age, HBsAg, gamma-globulin level, serum glutamic-oxaloacetic (SGOT), or non-organ-specific autoantibodies (ANA, AMA, SMA).

Human LSP was used as test antigen in the assays described by Jensen et al., Gerber et al., and Kakumu et al. Kakumu et al. could absorb the antihuman LSP activity using human and rat LSP, indicating that non-species-specific determinants

Gerber et al. [9]		Manns et al. [30]		Uibo et al. [47]	
%	No	%	No	%	No
63	38/60	44	27/62	39	9/23
56	5/9	56	9/16	–	–
40	4/10	42	14/33	8	2/25
0	0/13	29	4/14	0	0/10
0	0/14	14	1/7	0	0/24
–	–	20	2/10	–	–
43	9/21	–	–	100	45/45
–	–	–	–	–	–
18	3/17	10	6/58	–	–
–	–	0	0/31	–	–

of LSP were targets for anti-LSP autoantibodies in the sera tested by these authors. No information is given in this paper as to whether all sera tested were absorbed by rat LSP. As species-specific and non-species-specific determinants of LSP could be demonstrated by heterologous antisera [31] we tested all groups of patients for anti-human and anti-rabbit LSP. The incidence for anti-rabbit LSP was similar although below the percentage obtained for anti-human LSP. A striking difference was observed in AVH. Only 9% of patients had anti-rabbit LSP whereas 42% had antibodies to human LSP in this group of patients. As antibodies against LSP in AVH were found to be transient in uncomplicated AVH [20, 22] and as in rabbits, antibodies against non-species-specific determinants of LSP were only found in animals with histological signs of CAH [31], it may be speculated whether only antibodies against non-species-specific determinants reflect a real self-perpetuating state of autoimmunity.

Originally LSP was defined by heterologous antisera obtained after absorption with kidney proteins, human plasma, and blood cells [37]. Furthermore the present LSP preparation contains non-organ-specific contaminants [3]. Therefore one may question whether these non-organ-specific contaminants are targets for circulating autoantibodies. A kidney protein fraction prepared in the same way as LSP served as labelled antigen in the anti-LSP radioimmuno precipitation test [30]. Antibodies against the kidney equivalent of LSP were found in 10% (6/62) of patients with CALD, 14% (1/7) of patients with alcohol-induced liver disease, and in 3% (2/58) of patients with primary non-hepatic autoimmune diseases, indicating that naturally occurring anti-LSP are predominantly directed against organ-specific determinants of the LSP preparation.

Very recently Uibo et al. (47) reported the detection of circulating anti-LSP by enzyme linked solid phase immunosorbent assay (ELISA) (Table 1). These authors found circulating anti-LSP with human LSP, rabbit LSP, and bovine LSP as antigen.

The described radioimmunoprecipitation tests for anti-LSP indicate that autoantibodies against the antigen fraction LSP are of diagnostic and prognostic value in inflammatory liver diseases. As all test systems use an antigen of partial purification and the monospecificity of the autoantibodies detected by these test systems has still to be proved, a further purification of these molecule LSP and its antigenic determinants seems necessary. From the reported data on anti-LSP it cannot be determined whether detected autoantibodies are reacting with identical determinations of the LSP antigen complex. Furthermore one has to question whether the organ-specific and non-organ-specific determinants are located on identical or different molecules.

3.2. Liver Membrane Autoantibodies Detected by Indirect Immunofluorescence on Isolated Hepatocytes (LMA)

Liver membrane autoantibodies can further be detected by indirect immunofluorescence on isolated rabbit hepatocytes [13]. When isolated rabbit hepatocytes are incubated with patient serum the existence of circulating liver membrane autoantibodies is indicated by a characteristic linear membrane staining (LMA-test). In the original study [13] LMA were found in 7/10 patients with HBsAg negative CAH

but not in patients with AVH, CPH, HBsAg positive CAH, healthy HBsAG carriers, alcoholic liver disease, and healthy controls. These original observations were followed by a larger clinical study [43]. In this study LMA were predominantly found in HBsAg negative chronic inflammatory liver diseases: 38% of HBsAg negative CAH, 61% of HBsAg negative cirrhosis, and rarely in other liver diseases. Schuurman et al. [42] tested 100 sera for the presence of LMA. While the above reported data resemble LMA of IgG type, Schuurman et al. tested for IgM antibodies as well. They found LMA positive sera (including IgG and IgM antibodies) in 71% of HBsAg negative CAH, 14% of HBsAg positive CAH, 35% of PBC, 57% in drug induced liver diseases, and in 67% of AVH. No LMA were found in alcohol induced liver disease, a group of miscellaneous liver diseases, and in inflammatory bowel diseases. Thus the sensitivity of the LMA test was confirmed for HBsAg negative CAH; LMA in PBC, AVH, and drug induced liver disease were mainly referable to IgM antibodies. The LMA titre in this study was decreased during immunosuppressive therapy.

Similar results were obtained by Junge et al. [21]. These authors could absorb the immunofluorescence by liver homogenate but not by equivalent kidney tissue fractions.

Kawanishi and McDermott [25] detected liver membrane autoantibodies by indirect immunofluorescence. Human and rabbit hepatocytes were isolated after liver perfusion in the presence of 0.1% collagenase. Sera from 10 patients with CALD, preabsorbed with human kidney tissues possessed antibodies against the surface membrane of human and rabbit hepatocytes. The detected autoantibodies were only of IgG class. The five HBsAg negative CALD sera exhibited a linear membrane staining whereas the remaining five sera from patients with HBsAg positive CALD showed a mixed granular and diffuse fluorescence of lower intensity.

3.3 Heterogeneity of Liver Membrane Autoantibodies

The LMA-test seems to be a valuable marker for autoimmune type liver disease. Nevertheless no information is available concerning the target antigen(s) to which these LMA are directed. First it was suggested that LMA resemble antibodies against LSP [12, 20]. Absorption studies revealed that many of the LMA positive sera could not be absorbed by LSP [36]. Recently, testing 231 patient sera for LMA and anti-LSP [30], it could be demonstrated that the LMA-test detects liver membrane autoantibodies against further membrane antigens besides LSP. As the linear membrane immunofluorescence staining of the LMA-test could be absorbed by 100,000 *g* supernatants of liver homogenates the corresponding target antigens had to be looked for in this protein fraction. A solid phase radioimmunoassay (RIA) [29] was developed to further characterize liver membrane autoantibodies. The IgG fraction of 38 patient sera was prepared by ammonium sulphate precipitation and labelled with I^{125} using the Chloramine T method [19]. The IgG fraction was used to coat the assay tubes and as labelled antibody, 100,000 *g* supernatants served as test antigen. Positive results were only obtained with 10/14 sera from patients with HBsAg negative CAH, not with sera from three patients with HBsAg positive CAH, two with CPH, six patients with AVH, six with miscellaneous liver diseases, seven with primary nonhepatic autoimmune diseases, and two healthy blood

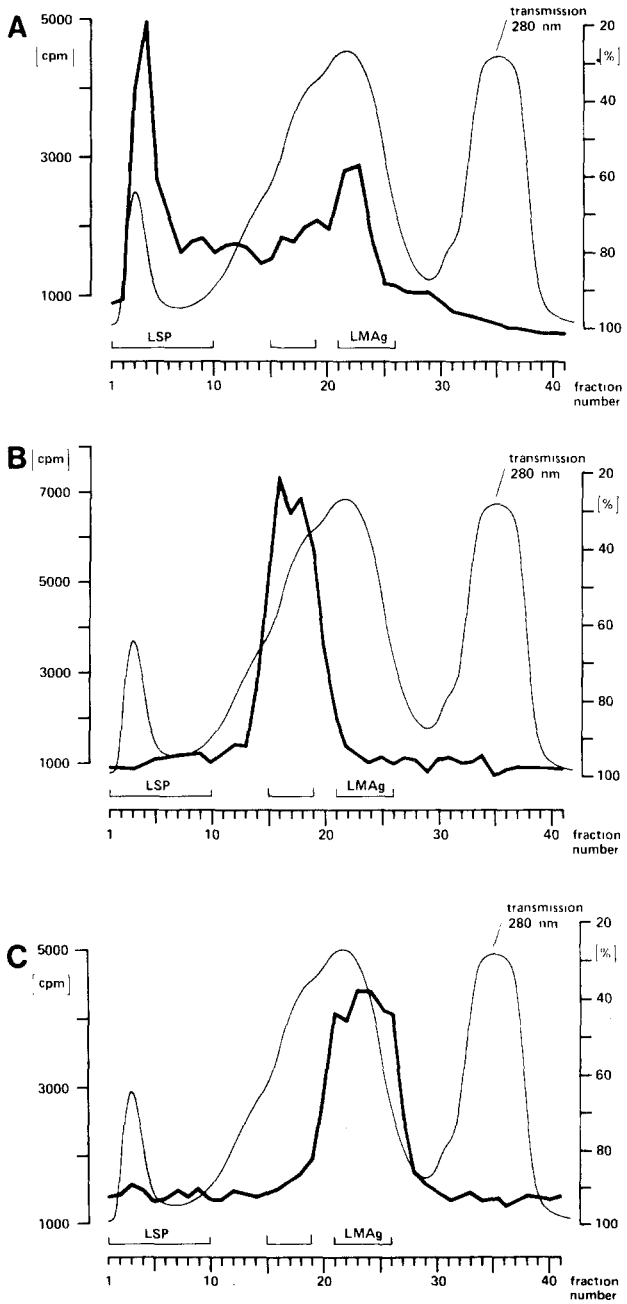

Fig. 5 A-C. Sepharose 6B chromatography of human soluble liver proteins. Fraction 1 represents the first fraction after a void volume of 124 ml. —: RIA for detecting liver membrane antigens, results are presented as counts per minute (cpm). —: protein content indicated as optical transmission at 280 nm (%). **A** Serum from a patient reacting with the LSP- and LM-AG-fraction. **B** Patient reacting with the third antigen fraction. **C** Patient with antibody to LM-Ag. With rabbit liver proteins similar results were obtained for all patients tested

donors. All these sera were tested by the LMA-test as well; LMA-test was positive in 9/14 patients with HBsAg negative CAH, all other sera were negative. The antibody activity detected by RIA could be absorbed by isolated liver cell membranes. No correlation between the RIA results and SMA, ANA, or AMA was observed. Supernatants (100,000 *g*) were chromatographed on Sepharose 6B and all fractions obtained after Sepharose 6B column were tested by RIA. The corresponding antigens were found in three different antigen fractions of Sepharose 6B (Fig. 5). Besides the first peak, resembling LSP, autoantibodies reacted with a fraction that exhibited the characteristics of LM-Ag. A third antigen fraction was localized at fractions with a molecular weight of approximately 2×10^5–3×10^5 daltons. Tests for organ-specificity showed that the RIA positive sera were reacting with equivalent kidney protein fractions, only antibodies reacting with the first peak of Sepharose 6B so far were found to be organ-specific. These data demonstrate a heterogeneity of liver membrane autoantibodies and it may be concluded that in inflammatory liver diseases we are not dealing with one target antigen-antibody system. Coating – cross – and blocking tests revealed an individual-specificity and species-cross-reactivity of many of the detected liver membrane autoantibodies. An interaction with HLA-antigens could be ruled out in these studies. A further characterization and purification of these liver membrane antigens will be the subject for further studies. Well defined autoantibodies from patients' sera should be used in these studies. In addition further investigations will have to deal with the evaluation of the organ-specificity of liver membrane target antigens.

4. Cellular Immune Reactions Against Liver Membrane Antigens

Studies on cellular immunity against liver membrane antigens have relied predominantly on in vitro studies with either crude liver homogenates or LSP preparations as antigens. Three types of assay systems have been employed, namely leucocyte migration inhibition, lymphocyte stimulation, and various cytotoxicity assays.

Each of these assays measures different functions of the immune response. The production of certain leucocyte kinins, such as leucocyte migration inhibition factor (LIF) and monocyte migration inhibition factor (MIF) in response to specific antigens, is the basis for the leucocyte migration inhibition test in its various modifications. The lymphocyte stimulation assay measures the early steps of antigen recognition with proliferation of antigen-specific immunocytes and blast transformation. The cytotoxicity assay systems measure the cytotoxic potential of lymphocytes or lymphocyte subpopulations sensitized against target antigens present on the plasma membrane of target cells. Human hepatocytes, rabbit hepatocytes, cultured liver cells, and antigen coated red cells have been used in the past. The results are influenced by the complex processes of antigen recognition by sensitized immunocytes and the differentiation into cytotoxic effector cells.

4.1. Leucocyte Migration Inhibition

We have used a two stage leucocyte migration inhibition assay to study cellular immune reactions in patients with CAH [35]. Twenty nine of 34 (85%) of untreated

patients with hypergammaglobulinaemic, autoimmune CAH and 8/19 patients with kryptogenic cirrhosis showed significant leucocyte migration inhibition. A subsequent study expanded these findings on defined subgroups of CAH (38). Seventeen of 20 (85%) of untreated patients with autoimmune CAH, 46% of patients with type B CAH, and 29% of patients with HBsAg negative, autoimmune marker negative CAH showed significant inhibition of leucocyte migration.

Bacon et al. investigated cellular immunity in 32 patients with chronic active hepatitis and obtained evidence for cellular sensitization in 75% of patients against a crude liver extract [2]. Miller et al. studied 16 patients with CAH and found leucocyte migration inhibition in 11 patients (40). Four patients treated with immunosuppressive agents failed to show migration inhibition. Lee et al. reported leucocyte migration inhibition in 67% of HBsAg negative, anti-HBs negative patients with CAH, and a reduction of migration inhibition in response to immunosuppressive therapy [28].

4.2. Lymphocyte Stimulation

The first report of lymphocyte stimulation in response to soluble liver homogenate came from Tobias et al. who observed significant lymphocyte stimulation in two of four patients with PBC and in one of two patients with CAH [46]. Thestrup-Petersen et al. studied lymphocyte stimulation in response to LSP in 34 patients with various liver diseases. Eight of ten patients with CAH or CPH and two of five patients with non-alcoholic cirrhosis showed a positive in vitro reactivity to LSP. There was no significant correlation between the stimulation with LSP and the presence or absence of HBsAg or other biochemical abnormalities [44].

4.3. Cytotoxicity Assay Systems

Cytotoxicity of peripheral blood lymphocytes against a number of target cells has been reported in the past. There is a lack of agreement of the results, which is due to several factors, such as methodological differences and differences in the target cells studied. The results obtained with different target cells and the likely target antigens in these assays will be discussed below. The topic of effector cell mechanisms is dealt with in the paper by Wands published in this issue.

4.3.1. Rabbit Hepatocytes. Rabbit hepatocytes as target cells have been extensively studied. The hepatocytes were isolated by perfusion and the use of collagenase. Cytotoxicity was assessed by determining the percentage of viable plastic adherent hepatocytes after incubation with patient lymphocytes. A high effector-target cell ratio of 400:1 was necessary for optimal cytotoxicity. Increased cytotoxicity was observed in 20/22 patients with CAH, HBsAg positive or HBsAg negative. The cytotoxicity could be blocked by addition of small amounts of human LSP, thus suggesting that LSP had been the target antigen of the cytotoxic attack [45]. In a subsequent study all 15 untreated cases of CAH showed increased cytotoxicity, which became negative in four of nine patients studied over a prolonged period. Patients who had a negative cytotoxicity under immunosuppressive therapy had a better prognosis than those patients under therapy with positive cytotoxicity. There

was a positive correlation between cytotoxicity and the histological grading of disease activity, in particular with the extent of piece-meal necrosis [7]. There was no correlation between cytotoxicity and the presence or absence of HBsAg.

Facchini et al., using the same method, found increased cytotoxicity against rabbit hepatocytes in patients with CAH [8]. These authors observed no correlation with the presence or absence of HBsAg, autoimmune markers, gammaglobulins, or enzyme elevation. Patients under steroid therapy had a decrease of cytotoxic activity.

Kawanishi et al. studied antibody dependent cellular cytotoxicity of peripheral blood lymphocytes from normal subjects against rabbit hepatocytes in the presence of HBsAg positive and HBsAg negative CAH sera [25]. Two patterns of IgG binding could be distinguished: sera from HBsAg negative CAH patients showed a diffuse linear membrane pattern and sera from HBsAg positive patients showed both a granular and a weaker diffuse immunofluorescence. The immunofluorescence could be abolished by prior absorption of these sera with lyophilized human liver homogenate. Human embryonal intestinal cells did not show IgG binding. A significant cytotoxicity of peripheral blood lymphocytes against rabbit hepatocytes in the presence of CAH serum but not in the presence of normal serum was observed in HBsAg negative and HBsAg positive CAH sera.

4.3.2. Human Hepatocytes. When autologous human hepatocytes are used as target cells in cytotoxic assays the problems of HLA restriction of T cell cytolysis can be avoided. These advantages are, however, offset by the fact that human hepatocytes have to be isolated by enzymatic methods, that the cell yield is usually low, and that these cells have only a limited viability. The studies using human hepatocytes have yielded discrepant results. Wands et al. found an increased cytotoxicity of peripheral blood lymphocytes toward autologous liver cells, which returned to normal values in patients under prednisolone therapy [51]. In a similar study, increased cytotoxicity was found in 53% of patients with CALD, but in 32% there was a significantly decreased cytotoxicity when compared to normal controls. The remaining patients had a normal cytotoxicity. Patients exhibiting cytotoxicity against hepatocytes had a serum factor which was able to inhibit the phytohaemagglutinin-induced lymphocyte transformation of normal lymphocytes [10]. Paronetto et al. studied cytotoxicity of lymphocytes against cultured autologous hepatocytes and observed increased cytotoxicity in eight of ten patients with CAH. Cytotoxicity was seen in both HBsAg positive and HBsAg negative patients [41].

Vergani et al. studied cellular cytotoxicity against autologous hepatocytes after 48–96 h in culture [48]. They found significantly increased cytotoxicity in 10/16 patients. All six untreated patients showed cytotoxicity, but only 4/10 patients under immunosuppressive treatment were cytotoxic to autologous hepatocytes. There was a statistical association between cytotoxicity and the extent of histologically assessed liver damage. The cytotoxicity could be blocked in all cases by the addition of human LSP preparation, thus suggesting that LSP was the target antigen.

One of the problems with this approach is, for obvious ethical reasons, the control group was not studied in an autologous system but consisted of normal

lymphocytes studied with CAH hepatocytes. Hepatocytes which were killed by patients' lymphocytes were also killed to some degree by normal lymphocytes, which may have been due to decreased viability or to membrane fixed immunoglobulin by these hepatocytes.

4.3.3. Cultured Cells. Chang liver cells have been used by several investigators with different results [1, 16, 24, 49, 51, 52]. This may be due to the fact that some Chang cells are contaminated with, or are in fact, HeLa cells. We have studied the membrane expression of LSP on several Chang cell strains and have observed differences of membrane expression [18]. This could explain some of the discrepancies reported in the literature and underlines the importance to control the expression of the putative target antigens.

Increased cytotoxicity in eight patients with CAH against Chang cells was observed by Wands et al. [52]. Different results were obtained by Vierling et al. who studied spontaneous (SCMC) and antibody dependent (ADCC) cytotoxicity against Chang cells and a mouse sarcoma cell line [49]. There was no difference in SCMC and ADCC in patients with CAH when compared to controls; patients with PBC showed a decreased cellular cytotoxicity. When comparing the cytotoxic effect on Chang cells or a mouse sarcoma line, no differences were encountered, suggesting that the cytotoxicity was not organ-specific. We have studied SCMC and ADCC in HBsAg positive CAH and non-A, non-B CAH. SCMC and ADCC were increased in patients when compared to normal controls. Sera of patients were inhibitory to SCMC and ADCC when compared to homologous AB serum [16]. The cytotoxicity was partially inhibited by the addition of human LSP. An inhibitory effect of autologous CAH serum was also observed by Kakumu et al. [23]. A study by ourselves of patients with non-A, non-B CPH disclosed increased SCMC but normal ADCC when compared to normal controls [16]. Kawanishi studied the cytotoxicity of CAH serum on Chang sells by lymphocytes from normal subjects. He found a significantly increased cytotoxicity by CAH serum when compared to normal serum. The cytotoxicity could be blocked by addition of heat-aggregated IgG and prior absorption of sera with liver homogenate [24].

4.3.4. *LSP-Coated Target Cells.* Vogten et al. developed a test system, in which human LSP was non-covalently bound to avian erythrocytes [50]. The observed increased cytotoxicity in approximately 50% of 62 CAH patients, whereas only 5/100 normal persons and 2/8 patients with PBC showed cytotoxicity. The cytotoxic effect could be blocked by addition of human LSP and heat-aggregated IgG.

Behrens et al. coated a mastocytoma line either with human LSP or the kidney equivalent KSP and studied cytotoxicity of human granulocytes from normal persons in the presence of CAH serum [4]. Several sera from patients contained antibodies against liver and kidney antigens. Inhibition experiments revealed that the cytotoxicity could be blocked by addition of the appropriate coating antigen but in many cases also by other antigens. Cross-inhibition of LSP coated target cells with KSP was not always observed, suggesting that the LSP preparation contained an organ-specific component. The authors concluded, that the cytotoxicity against LSP coated target cells is not always an organ-specific phenomenon.

5. Animal Models for Autoimmune CAH

Evidence for a pathogenetic role of LSP in chronic active hepatitis originated from experiments in which rabbits were long-term immunized with human liver subfractions containing organ-specific determinants. In these experiments the first peak of a Sephadex G-100 column from 100,000 *g* supernatants of human liver (HLP) or rabbit liver (RLP) were used [12, 14, 26, 27, 33, 34]. Animals immunized with HLP over a period of 143 weeks i. p. with complete Freund's adjuvant developed CAH or cirrhosis after this time. The combined administration of HLP together with RLP caused development of histological lesions in only 13% of rabbits. There was a correlation between the histological liver lesions and cellular immunity against allogeneic liver proteins as assessed by skin testing [14, 33]. As in addition only rabbits that had developed CAH after long-term immunization had circulating autoantibodies against allogeneic rabbit LSP, it was suggested that the loss of tolerance against a species-non-specific determinant of LSP was responsible for the development of CAH [31]. More recently the development of inflammatory liver lesions in rabbits was demonstrated after long-term immunization with purified LSP, using the first peak obtained after Sepharose 6B chromatography as immunizing antigen [8, 47].

6. Conclusion

The reported data on liver membrane antigens demonstrate that these antigens are targets for humoral and cell mediated immunity in human inflammatory liver diseases. Undoubtedly some of these determinants are organ-specific. Future work will have to concentrate on a further purification of these antigens. Special interest should concentrate on the attempt to separate organ-specific from non-organ-specific determinants. If these determinants are located on identical molecules, then organ-specific determinants can only be identified and characterized by monospecific autoantibodies. At present studies for CMI and humoral immunity against liver membrane antigens have to include appropriate control experiments with equivalent protein preparations from organs other than liver.

The described liver membrane antigens are constituents of normal liver cell membranes. Nothing is known about their functional integrity. Are they associated with membrane enzymes, or linked to receptors of the liver cell membrane, i. e. hormone receptors, receptors for drugs or toxins? Are they located at the sinusoidal or biliary poles of the hepatocyte membrane?

Further purification and definition of these antigens will facilitate the development of more sensitive and more reproducible assays enabling a more precise evaluation of the clinical relevance of liver membrane autoantibodies. It has still to be investigated whether special types of liver membrane autoantibodies are characteristic for subgroups of chronic active liver diseases. Furthermore prospective studies will be needed to determine whether liver membrane autoantibody titres decline under immunosuppressive treatment.

There are several problems with the cytotoxicity studies reported in patients with chronic liver diseases. The use of autologous hepatocytes is limited by the low viability and low yield of cells from these preparations. In the published studies the

Table 2. Putative target antigens of immune reactions in various forms of chronic active hepatitis

Hepatitis	Immune reactions	
	Humoral	Cellular
CAH Type B	anti-HBs, anti-HBc, anti-LSP	LSP, HBsAg (?)
CAH Type NANB	anti-LSP(?) anti-NANB-antigen	LSP(?), viral antigens (?)
Autoimmune CAH	anti-LSP, LMA	LSP, LM-Ag
Drug-induced CAH	(?)	Hapten-modified membrane antigens (?)
Alcohol hepatitis	anti-LSP anti-hyalin (?)	LSP alcoholic hyalin

cytotoxicity in patients was studied in an autologous system but was compared with normal lymphocytes against allogeneic patient hepatocytes. This fact limits the usefulness of this system, since control experiments are thus not performed in a syngeneic system. Another problem is the use of collagenase for the isolation of hepatocytes, since membrane alterations cannot be excluded.

The use of cultured cells of hepatic origin presents different problems. It has to be assessed critically, whether cultured cells express liver specific antigens on the cell membrane including LSP. Most of these cell lines are rather dedifferentiated and have lost most of their liver specific biochemical functions. Also the problem of contamination with mycoplasms must be remembered. Our results using Chang cells have stressed this problem. The discrepant results using Chang cells as target cells of cytotoxicity assays by different authors might well be explained by differences in membrane expression of liver specific antigens or contamination by other cell types.

An alternative to the use of autologous hepatocytes and cultured cells is the use of red blood cells coated with viral antigens or with liver specific protein. In this case the specificity of the cytotoxicity assay is controllable by inhibition experiments with either purified LSP, unrelated membrane proteins, or other cell types. However, since LSP probably bears non-organ-specific as well as organ-specific determinants the inhibition by LSP is insufficient to demonstrate organ specificity. In this area more work on the specificity of the target antigens is important.

These various test systems used to study CMI in inflammatory liver diseases indicate that the immune phenomena measured are important factors in the immunopathogenesis. Nevertheless the present assays are rather complicated and therefore restricted to special research laboratories. For many of the described assay systems little is known about the target antigens involved or the exact effector mechanisms. In addition, in many cases the authors did not study the organ-specificity of the detected immune-phenomena. A further unanswered question is whether in a single patient humoral and CMI may coexist or are mutually exclusive.

Concerning the importance of the described immune-phenomena for the immunopathogenesis, one can state that these immune reactions are important factors for the course of inflammatory liver diseases, but we can only speculate as to

whether the origin of the disease is mediated by a primary autoimmune attack possibly mediated by a genetic predisposition. In addition, viruses and even drugs have been suggested to be involved in the primary loss of tolerance against liver membrane antigens.

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