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A Species-Non-Specific Liver Plasma-Membrane Antigen and its Involvement in Chronic Active Hepatitis

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Rabbit liver plasma membranes were isolated and purified by using an aqueous two-phase polymer system. Examination of these preparations with respect to electron-microscopical appearance, distribution of marker enzymes and gross biochemical composition revealed them to be free from contamination by intracellular components. Sera from ten patients with chronic active hepatitis, four with and six without hepatitis B viral markers (HBsAg) in their sera, produced a single precipitin line on immunodiffusion against a detergent extract of the isolated plasma membranes. Sera from HBsAg-positive and HBsAg-negative patients reacted against the same antigen. This antigen was enriched in the plasma-membrane preparations compared with whole-liver homogenates and was identical with a species-non-specific antigen in a macromolecular fraction of normal human liver, which has been previously described as liver-specific lipoprotein.

Chronic active hepatitis is a progressive disease of the liver in which a variety of aetiological factors have been implicated, but in which the underlying mechanisms of liver damage are not fully understood. Chronic infection with the hepatitis B virus is an important factor in many cases, but in these, as well as in patients without hepatitis B viral markers in their sera, several cellular and humoral immune reactions against normal liver cell antigens have been described, suggesting that autoimmune mechanisms may be involved (Miller *et al.*, 1972; Cochrane *et al.*, 1976; Hopf *et al.*, 1976; Jensen *et al.*, 1978*a*). Recently, interest has centred on the isolation and characterization of the antigens against which these autoimmune reactions are directed. In particular, a soluble macromolecular fraction of normal human liver, described as liver-specific lipoprotein, has been found to contain at least one such antigen (Meyer zum Büschenfelde & Miescher, 1972). Analysis of human liver-specific lipoprotein has shown that it is comprised of a number of proteins and some lipid (Meyer zum Büschenfelde & Miescher, 1972; McFarlane *et al.*, 1977). In addition, it contains at least one component that is species-non-specific (Hopf *et al.*, 1974; McFarlane *et al.*, 1977) and either this or some other (as yet unidentified) species-non-specific component appears to be located on the liver cell membrane (Hopf *et al.*, 1974; McFarlane *et al.*, 1977).

Progress in the characterization of liver-specific lipoprotein has been slow because of problems of stability (McFarlane *et al.*, 1977), differences in procedures used by various workers in preparing it and difficulties in detection of the antigen or antigens in it against which patients' sera react. In particular, the lack of a detectable precipitin reaction between patients' sera and liver-specific lipoprotein precludes the use of many immunochemical techniques in the characterization of these antigens.

In the present study, an alternative approach has been employed, based on the finding of reactions between sera of patients with chronic active hepatitis and surface components of isolated rabbit liver cells (Hopf *et al.*, 1976; Tage-Jensen *et al.*, 1977). Rabbit liver plasma membranes have been isolated and characterized with respect to their appearance under the electron microscope, distribution of marker enzymes and biochemical composition. The reaction of sera from both HBsAg-positive and HBsAg-negative chronic active hepatitis patients with a detergent extract of the isolated plasma membranes resulted in a single precipitin line on immunodiffusion. The relationship between the antigen so detected and human and rabbit liver-specific lipoprotein was investigated.

Abbreviation used: HBsAg, hepatitis B surface antigen.

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Methods

Isolation of rabbit liver plasma membranes

Adult rabbits were anaesthetized by intraperitoneal injection of pentabarbitone and exsanguinated by external cardiac puncture. The livers were removed and placed on ice and all subsequent steps performed at 4°C. Plasma membranes were prepared from 15g wet wt. of liver by using the aqueous two-phase polymer system of Lesko *et al.* (1973). The isolated plasma membranes were washed once in distilled water, freeze-dried and stored at 4°C.

Electron microscopy

Plasma membranes were fixed in 2.5% (w/v) glutaraldehyde/0.2M-sodium cacodylate, pH 7.4, and post-fixed in 1% (w/v) OsO_4/0.2M-sodium cacodylate, pH 7.4, for 1h at 4°C. The membranes were washed in 0.2M-sodium cacodylate, pH 7.4, dehydrated and embedded in Araldite. Ultrathin silver/gold sections (De Kretser & Livett, 1977) were cut on an LKB ultramicrotome, counter-stained with uranyl acetate and lead citrate and viewed in a Jeol 100C electron microscope.

Measurement of enzyme activities

Enzyme activities were measured both in the whole-liver homogenates and in the final plasma-membrane preparation. The activity of $(Na^+ + K^+)$-activated Mg^{2+}-dependent ouabain-sensitive ATPase (EC 3.6.1.3) was determined as previously described (De Kretser & Livett, 1977). The activity of 5'-nucleotidase (EC 3.1.3.5) was determined as described by Emmelot *et al.* (1964), and acetylcholinesterase activity (EC 3.1.1.7) as described by Ellman *et al.* (1961). Monoamine oxidase activity (EC 1.4.3.4) was measured by the method of Schnaitman *et al.* (1967), lactate dehydrogenase activity (EC 1.1.1.27) by the method of Neilands (1955) and glucose 6-phosphatase activity (EC 3.1.3.9) as described by Swanson (1955).

Other assays

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Cohn fraction V) as standard. Total lipid was determined by the method of Fringo & Dunn (1970) with olive oil as standard. Phospholipid was measured as described by Hess & Derr (1975) with sphingomyelin as standard, and cholesterol was determined as described by Rudel & Morris (1973). RNA was measured as described by Tsanev & Markov (1960).

Immunodiffusion experiments

Isolated liver plasma membranes and liver homogenate were extracted by incubation at 37°C for 60min at concentrations of 5.3 and 6.6mg dry wt./ml respectively in a 20% solution of Triton X-100 in a 0.025M-sodium barbitone, pH 8.5, containing 1000 kallikrein inhibitor units of aprotinin (Sigma)/ml. The suspensions were centrifuged at 30000*g* for 10min and the supernatants used as the source of antigen. Immunodiffusion was performed by the method of Ouchterlony (1958) on gels containing 1.0% agarose in 0.025M-sodium barbitone, pH 8.5, with 0.1% Triton X-100 and 0.1% NaN_3.

Absorption experiments were performed by incubation of sera (20μl) with various amounts of the appropriate absorbing material (see the legend to Fig. 3) for 1h at 25°C and then for 16h at 4°C. The absorbed sera were centrifuged at 30000*g* for 10min before use.

Preparation of other antigens

Human and rabbit liver-specific lipoproteins were prepared by gel filtration on Sepharose 6B (Pharmacia) as described by McFarlane *et al.* (1977), and stored at 4°C in the presence of 1000 kallikrein inhibitor units of aprotinin/ml. Human Tamm–Horsfall glycoprotein was a gift from Professor R. D. Marshall (Department of Biochemistry, University of Strathclyde).

Sera

Sera were obtained from untreated patients with chronic active hepatitis in whom the diagnosis was confirmed by liver biopsy. HBsAg was determined by passive haemagglutination (Hepatest, Wellcome, London SE13, U.K.). Titres of autoantibodies were as reported by the routine hospital service. Normal sera were obtained from the Southwest London Regional Blood Transfusion Service, St George's Hospital, London SW16, U.K. Guinea-pig anti-(human liver-specific lipoprotein) sera were raised as previously described (McFarlane *et al.*, 1977).

The immunoglobulin fraction of each serum was prepared by $(NH_4)_2SO_4$ precipitation (Heide & Schwick, 1978). The precipitated immunoglobulins were taken up in distilled water and extensively dialysed against 0.154M-NaCl/0.01M-sodium phosphate, pH 7.2. The immunoglobulin fractions were adjusted to the same volume as the original serum samples.

Chemicals

Chemicals of AnalaR grade were obtained from BDH (Poole, Dorset, U.K.), and biochemicals were from Sigma (London) Chemical Co. (Poole, Dorset, U.K.).

Results

Characterization of isolated plasma membranes

Electron microscopy of the isolated rabbit liver plasma membranes showed large sheets of mem-

branes free from visible contamination with intracellular structures (Plate 1). Clearly defined vesicles were not seen.

The specific activities of $(Na^+ + K^+)$-ATPase, 5'-nucleotidase and acetylcholinesterase, used as plasma-membrane marker enzymes, were increased in the isolated membranes compared with the homogenate. Conversely, the specific activities of marker enzymes for other intracellular organelles were decreased in the plasma-membrane fraction compared with the homogenate, except for glucose 6-phosphatase specific activity, which remained the same (Table 1).

In the plasma-membrane fraction, the recovery of $(Na^+ + K^+)$-ATPase activity was greater than the recoveries of either 5'-nucleotidase or acetylcholin-

Table 1. *Distribution of marker enzymes in rabbit liver homogenate and isolated plasma membranes*

The following enzyme activities were determined in the whole-liver homogenate and in the isolated plasma membranes by methods given in the text. Specific activities are given in nmol/h per mg of protein ± S.D., with the exception of monoamine oxidase specific activity which is given as ΔA_{250} units/h per mg of protein ± S.D. Numbers in parentheses indicate the numbers of different preparations assayed. The percentage of total activity originally found in the homogenate which remained in the plasma-membrane fractions is shown as the percentage recovery. Activities of $(Na^+ + K^+)$-ATPase in the homogenates and for monoamine oxidase, lactate dehydrogenase and β-galactosidase in the isolated plasma membranes were too low to permit accurate measurement under the conditions employed, therefore the maximum possible activity, calculated from the known limits of the assays, is given. Student's *t* test was used for statistical comparisons (n.s., not significant, i.e. $P > 0.05$).

Enzyme	Specific activity: Homogenate	Specific activity: Plasma membranes	Recovery (%)	Relative specific activity
$(Na^+ + K^+)$-ATPase	51.2 ± 11.3 (4)	732 ± 184 (3)	>12.9 ± 5.0 (3)	>13.7 ± 5.6 (3)
		$P < 0.001$		
5'-Nucleotidase	225 ± 89 (3)	733 ± 274 (3)	4.3 ± 2.3 (3)	3.4 ± 1.4 (3)
		$P < 0.05$		
Acetylcholinesterase	63.4 ± 16.0 (4)	196 ± 48.0 (4)	4.0 ± 2.2 (4)	3.1 ± 0.16 (4)
		$P < 0.002$		
Monoamine oxidase	1.26 ± 0.21 (4)	0.27 ± 0.02 (4)	<0.27 ± 0.11 (4)	<0.22 ± 0.03 (4)
		$P < 0.0001$		
Lactate dehydrogenase	10500 ± 1600 (4)	790 ± 340 (4)	<0.08 ± 0.02 (4)	<0.07 ± 0.03 (4)
		$P < 0.0005$		
β-Galactosidase	318 ± 40 (3)	27.3 ± 9.1 (4)	<0.08 ± 0.01 (3)	<0.07 ± 0.02 (3)
		$P < 0.00005$		
Glucose 6-phosphatase	2250 ± 449 (4)	2646 ± 416 (3)	1.90 ± 1.33 (3)	1.42 ± 0.33 (3)
		n.s.		
Protein	—	—	0.95 ± 0.12 (3)	—

Table 2. *Gross biochemical composition of rabbit liver homogenate and isolated plasma membranes*

The amounts of the components were determined in freeze-dried preparations by the methods given in the text. Results are shown as means ± S.D. for the numbers of preparations assayed given in parentheses. Student's *t* test was used for statistical comparisons (n.s., not significant, i.e. $P > 0.05$).

Component	Content (% dry wt.): Homogenate	Content (% dry wt.): Plasma membranes
Protein	48.5 ± 7.6 (4)	52.2 ± 4.1 (4)
		n.s.
Total lipid	13.9 ± 3.5 (3)	27.9 ± 3.7 (4)
		$P < 0.005$
Phospholipid	6.4 ± 2.3 (4)	15.7 ± 1.6 (3)
		$P < 0.001$
Cholesterol	0.34 ± 0.03 (3)	1.93 ± 0.67 (4)
		$P < 0.01$
RNA	2.62 ± 0.43 (3)	1.65 ± 0.23 (4)
		$P < 0.01$
Unaccounted	35.0	18.2

esterase activities, and the recoveries of these three plasma-membrane marker enzymes were considerably greater than those of the enzymes associated with intracellular components (Table 1). The plasma-membrane fraction was found to be enriched in lipid, but depleted of RNA compared with the homogenate (Table 2).

Reactions of patients' sera and animal antisera with rabbit liver plasma membranes

Serum from each of the ten patients with chronic active hepatitis gave a single precipitin line of varying intensity on immunodiffusion against a Triton X-100 extract of isolated rabbit liver plasma membranes. The line obtained with sera from HBsAg-positive patients showed identity with the line obtained with sera from HBsAg-negative patients (Figs. 1*a* and 1*b*). No precipitin line was observed with sera from any of four clinically normal subjects (Figs. 1*a* and 1*b*). The development of a precipitin line between the liver plasma-membrane extract and sera from patients with chronic active hepatitis was not related to the presence of anti-(smooth muscle), anti-nuclear or anti-mitochondrial autoantibodies in the sera of these patients (Table 3). The antigen detected by sera from patients with chronic active hepatitis was found to co-purify with the plasma membranes (Figs. 2*a* and 2*b*).

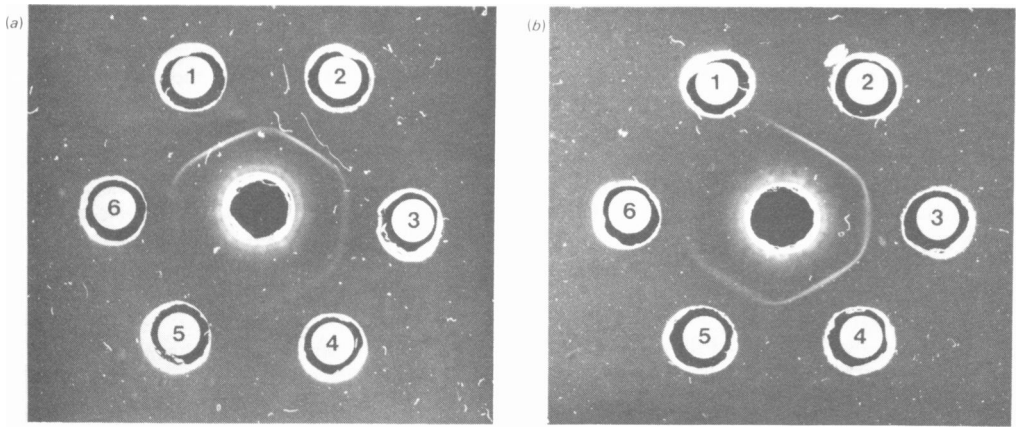
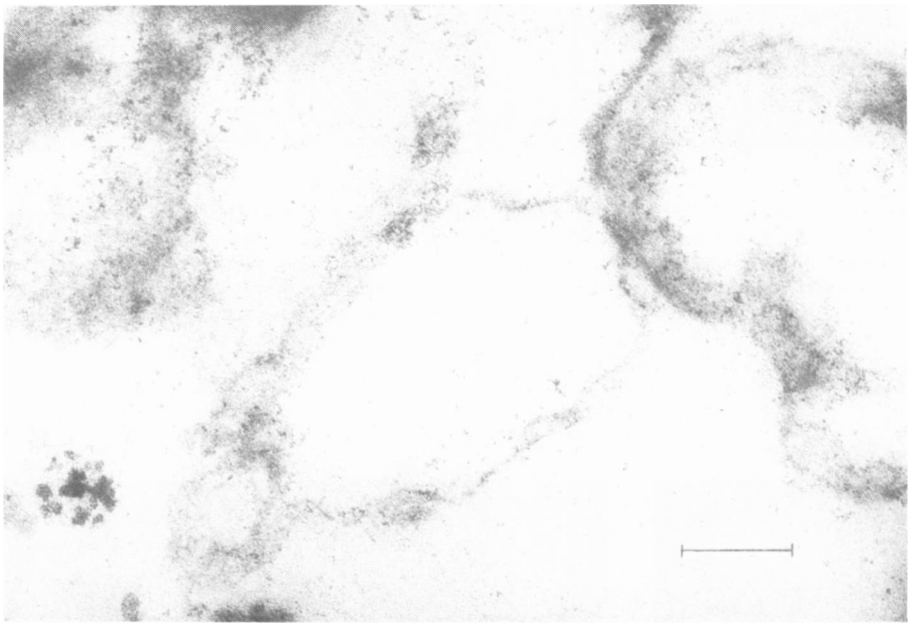


Fig. 1. *Reaction of sera from patients with chronic active hepatitis with rabbit liver plasma membranes*
Immunodiffusion plates showing precipitin lines obtained when rabbit liver plasma-membrane extracts were allowed to react against sera from HBsAg-positive (+) and HBsAg-negative (−) chronic active hepatitis patients by the methods described in the text are shown. Details relating to the patients are given in Table 3. Centre wells contained extracts from 53 μg of plasma membranes. Peripheral wells contained sera from: (*a*) 1, K (+); 2, McL (−); 3, L (+); 4, M (−); 5, normal human serum; 6, H (−); (*b*) 1, normal human serum; 2, P (+); 3, B (−); 4, A (+); 5, C (−); 6, S (−).

Table 3. *Details related to patients with chronic active hepatitis*
Age (years) and sex of each of the ten untreated patients is shown. Methods for the preparation of the patients' sera and for determining the presence (+) or absence (−) of HBsAg and titres of anti-(smooth muscle) (SMA), anti-nuclear (ANA) and anti-mitochondrial (AMA) autoantibodies are given in the text. Results not available are designated NA. The reaction with liver plasma membranes relates to the formation of a precipitin line on immunodiffusion.

Patient	Sex	Age (years)	HBsAg	Reciprocal titres of: SMA	ANA	AMA	Reaction with liver plasma membranes
K	M	17	+	40	–	–	+
L	M	49	+	NA	NA	NA	+
P	M	55	+	–	–	–	+
A	M	37	+	40	–	–	+
McL	M	39	–	–	80	–	+
H	M	48	–	–	250	–	+
M	F	53	–	160	–	–	+
B	F	56	–	10	10	5120	+
C	M	63	–	–	80	–	+
S	F	66	–	NA	NA	NA	+



EXPLANATION OF PLATE 1

Electron micrograph of isolated rabbit liver plasma membranes
The bar represents 0.1 μm.

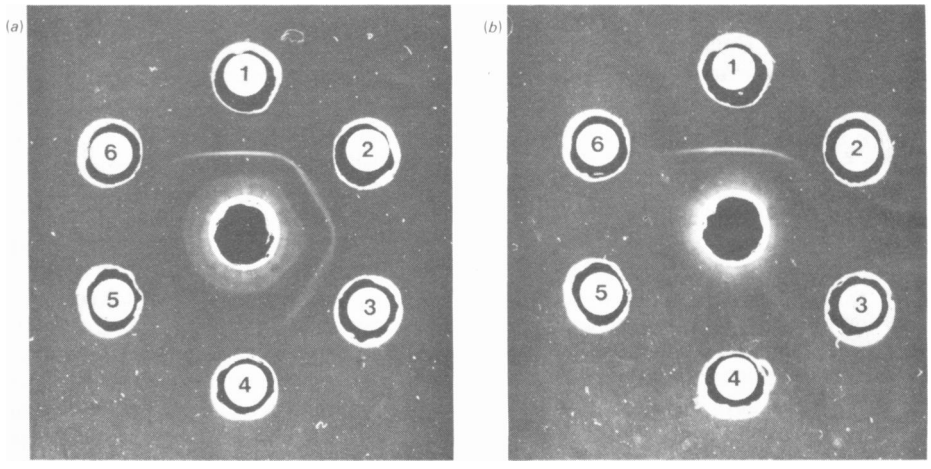


Fig. 2. *Co-purification of antigen with plasma membranes*
Immunodiffusion plates showing the co-purification, with the isolated rabbit liver plasma membranes, of the antigen detected by sera from patients with chronic active hepatitis are shown. All methods are described in the text. Centre wells contained serum from one patient (McL). Peripheral wells 2–6 contained doubling dilutions of material extracted from (*a*) 53 μg of plasma membranes in well 1 and (*b*) 66 μg of whole liver homogenate in well 1.

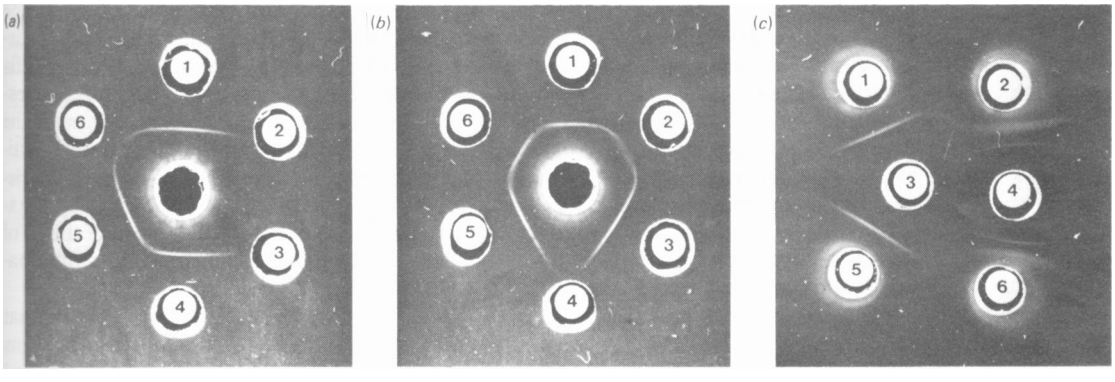


Fig. 3. *Relationship between liver-specific lipoprotein and liver plasma membrane-associated antigen*
Immunodiffusion plates showing the effects of absorption with human liver-specific lipoprotein, rabbit liver-specific lipoprotein and Tamm–Horsfall glycoprotein on the reaction between sera of patients with chronic active hepatitis and rabbit liver plasma-membrane extracts are shown. Also shown is the reaction of identity between rabbit liver plasma membranes and sera from patients with chronic active hepatitis and guinea-pig anti-(human liver-specific lipoprotein) serum. The identity of the precipitin line formed on reaction of guinea-pig anti-(human liver-specific lipoprotein) serum with rabbit liver plasma membranes and with human liver-specific lipoprotein can also be seen. All methods are described in the text. (*a*) Centre well: material extracted from 53 μg of rabbit liver plasma membranes. Peripheral wells contained serum from a patient with chronic active hepatitis (McL) absorbed as follows: 1, unabsorbed; 2, human liver-specific lipoprotein (40 μg of protein); 3, rabbit liver specific lipoprotein (37 μg of protein); 4, unabsorbed; 5, Tamm–Horsfall glycoprotein (40 μg of protein); 6, extract made from 53 μg of rabbit liver plasma membranes. (*b*) Centre well: material extracted from 53 μg of rabbit liver plasma membranes. Peripheral wells contained: 1, guinea-pig anti-(human liver-specific lipoprotein) (GpLSP) serum no. 1; 2. GpLSP serum no. 2; 3, chronic active hepatitis patient (H) serum; 4, GpLSP serum no. 3; 5, GpLSP serum no 4; 6, chronic active hepatitis patient (McL) serum. (*c*) 1, chronic active hepatitis patient (McL) serum; 2, GpLSP serum no. 1; 3, extract from 53 μg of rabbit liver plasma membranes; 4, human liver-specific lipoprotein (40 μg of protein); 5, chronic active hepatitis patient (H) serum; 6, GpLSP serum no. 4.

The precipitin reaction against the liver plasma-membrane extract could be abolished by previously absorbing the sera with human liver-specific lipoprotein, rabbit liver-specific lipoprotein or plasma-membrane extract. However, when similar amounts of these absorbing agents were used, the human and rabbit liver-specific lipoprotein preparations absorbed more effectively than did the plasma-membrane extract (Fig. 3*a*). The reactivity of the sera was not affected by prior absorption with Tamm–Horsfall glycoprotein.

Three of the four guinea-pig anti-(human liver-specific lipoprotein) sera (nos. 1, 2 and 4), when tested by immunodiffusion against the rabbit liver plasma-membrane extract, gave a single precipitin line that was identical with the line obtained with sera from patients with chronic active hepatitis (Fig. 3*b*). This line showed identity with one of the two lines obtained when the guinea-pig antisera were allowed to react against human liver-specific lipoprotein (Fig. 3*c*).

Discussion

The isolated rabbit liver plasma-membrane fraction was of sufficient purity, as assessed by electron microscopy, marker-enzyme distribution and biochemical composition, to be designated as representative of liver plasma membranes. However, it is known that liver plasma membranes are not homogeneous with respect to either function or composition (Wisher & Evans, 1975; Evans, 1977). The high recovery of $(Na^+ + K^+)$-ATPase activity compared with 5′-nucleotidase and acetylcholinesterase activities, and the preponderance of membrane sheets rather than vesicles observed in the electron microscope, suggest that this plasma-membrane fraction is derived primarily from the contiguous surface of the liver cell (Wisher & Evans, 1975; Evans, 1977; Blitzer & Boyer, 1978).

Sera from all patients with chronic active hepatitis studied contained antibodies reacting against an antigen in a detergent extract of this plasma-membrane preparation. This antigen co-purified with the plasma membranes (10-fold enrichment as compared with liver homogenate) and would appear to be a constituent of the liver cell membrane. The precipitin reaction between the plasma-membrane antigen and patients' sera was not related to the presence of anti-mitochondrial, anti-nuclear or anti-(smooth muscle) antibodies in the sera. Therefore the antigen seems to be distinct from the internal cell constituents against which these other autoantibodies are directed. In addition, the plasma-membrane antigen is not related to the renal-tubular antigen, Tamm–Horsfall glycoprotein, which has been shown to cross-react immunologically with components of human liver cell membranes (Tsantoulas *et al.*, 1974).

The plasma-membrane antigen detected by patients' sera is clearly a species-non-specific antigen, since its presence could be demonstrated in human liver-specific lipoprotein. Recently there has been some controversy over whether patients with HBsAg-positive chronic active hepatitis have autoantibodies against species-non-specific liver plasma-membrane antigens (Tage-Jensen *et al.*, 1977; Dienstag & Isselbacher, 1978). It has been shown by radioimmunoassay that sera from patients with either HBsAg-positive or HBsAg-negative chronic active hepatitis contain antibodies directed against antigen in human liver-specific lipoprotein (Jensen *et al.*, 1978*a*), which is known to contain at least one species-non-specific antigen (Meyer zum Büschenfelde & Miescher, 1972; McFarlane *et al.*, 1977). On the other hand, immunofluorescence studies have indicated the presence of a liver plasma-membrane antigen, which is species-non-specific and which is detected principally by sera from patients with HBsAg-negative chronic active hepatitis (Hopf *et al.*, 1976; Tage-Jensen *et al.*, 1977). The relationship between this antigen and the antigenic components of human liver-specific lipoprotein is unclear. In the present study, however, sera from HBsAg-positive and HBsAg-negative patients gave a reaction of identity against a rabbit liver plasma-membrane extract. This provides clear evidence of the existence, in HBsAg-positive chronic active hepatitis patients, of an immune reaction against a species-non-specific liver membrane antigen. Although the occurrence of differential reactions with other antigens cannot be excluded, it was not possible to distinguish between HBsAg-positive and HBsAg-negative chronic active hepatitis patients on the basis of the reactivity of their sera against the liver plasma-membrane extract.

The earlier studies, in which it was reported that immune reactions against liver membrane antigens could be demonstrated in both HBsAg-positive and HBsAg-negative patients, gave little information as to whether the two groups of patients were reacting against the same antigen(s) (Cochrane *et al.*, 1976; Jensen *et al.*, 1978*a*). The present study establishes that both HBsAg-positive and HBsAg-negative patients have a common immune reaction against at least one liver plasma-membrane antigen. Furthermore, this antigen was detected by a guinea-pig anti-(human liver-specific lipoprotein) serum which was used in earlier studies to develop the radioimmunoassay for antibodies to human liver-specific lipoprotein (Jensen *et al.*, 1978*b*). This indicates that at least some of the antibodies detected in patients' sera by the radioimmunoassay are directed against the species-non-specific antigen described here.

In conclusion, a species-non-specific liver plasma-

membrane antigen against which sera of patients with chronic active hepatitis react has been identified in a form amenable to study by immunochemical methods. The relevance of immune reactions to this antigen in the pathogenesis of chronic active hepatitis will remain uncertain until its localization within the plasma membrane has been established. However, further detailed characterization of antigenic constituents in the hepatocyte plasma membrane should be of value in resolving the current controversy over the existence and possible implications of autoimmune reactions in chronic liver disease.

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