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Identification of the hepatic asialo-glycoprotein receptor (hepatic lectin) as a component of liver specific membrane lipoprotein (LSP)

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SUMMARY

Liver specific membrane lipoprotein (LSP), the target for anti-LSP antibodies in various liver diseases, is thought to be comprised of fragments of the hepatocellular plasma membrane. In the present study, therefore, evidence has been sought for the presence in LSP of the hepatocyte surface receptor (hepatic lectin) that binds desialylated glycoproteins. Eight guinea-pig anti-LSP antisera (four anti-human and four anti-rabbit LSP) were found to react by ELISA and/or RIA against affinity purified human and rabbit hepatic lectin. Binding of the antisera to ^{125}I-hepatic lectins was inhibited by the unlabelled lectins, by human and rabbit LSP and by purified rabbit liver plasma membranes but not by a 50,000-fold excess of kidney homogenate. The results indicate that hepatic lectin is a liver specific, species cross-reactive antigen comprising about 0·25% of the protein in LSP.

Keywords liver specific protein anti-LSP asialoglycoprotein receptors liver plasma membranes

INTRODUCTION

Cellular and humoral immune reactions against the liver specific membrane lipoprotein complex (LSP) have been widely reported in patients with liver disease and there is currently much discussion about the significance of these findings in relation to the pathogenesis of immunologically-mediated parenchymal liver damage (Chisari, 1980; Klingenstein & Wands, 1981; Eddleston & Williams, 1981; Vergani & Eddleston, 1981). LSP is a large, lipid associated complex containing several antigens, including species specific and species cross-reactive determinants, of which at least two seem to be liver specific, but their precise identity has not been established (McFarlane, Wojcicka & Williams, 1980; Manns *et al.*, 1980; Manns & Meyer zum Büschenfelde, 1982; Uibo, Helin & Krohn, 1982). Furthermore, the exact nature of LSP itself is unclear, although immunochemical and electron microscopical evidence suggests that it is derived from the liver cell plasma membrane (Hopf, Meyer züm Buschenfelde & Freudenberg, 1974; de Kretser *et al.*, 1980; Lebwohl & Gerber, 1981). If this is the case, it should contain one or more of the many receptors (for vitamins, hormones and other metabolites) known to be present on the plasma membrane.

In the present study, therefore, we have examined whether LSP contains a component immunochemically similar to the hepatic receptor for desialylated glycoproteins since this receptor, known as hepatic lectin, is not only associated with the plasma membrane but also is specific to the liver (Ashwell & Morell, 1974; McFarlane, 1983).

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MATERIALS AND METHODS

Preparation of the standard LSP. Normal human liver was obtained from renal transplant donors and rabbit livers were taken from overnight fasted normal animals. A portion of each liver was immediately placed in liquid nitrogen and the remainder processed according to McFarlane *et al.* (1977). Briefly, the tissues were washed for 5 h at 4°C with 0·25 M sucrose, pH 8·0, homogenized (50% wt/vol.), centrifuged for 1 h at 105,000*g* and the supernatants stored at −20°C. LSP was prepared from liver supernatants by Sepharose 6B gel filtration as previously described (McFarlane *et al.*, 1977) using 0·1 M boric acid/sodium borate, pH 8·5, containing 1mM disodium ethylene diamine tetra-acetic acid (borate/EDTA buffer) in place of Tris/EDTA. Five separate preparations each of human and rabbit LSP were pooled and used throughout the study.

Animal antisera. Anti-LSP antisera were raised in guinea-pigs by immunization with human or rabbit LSP (4 × 1 mg over 6 weeks) in Freund's complete adjuvant according to McFarlane *et al.* (1977). Control antisera were raised in an identical manner against normal human plasma (McFarlane *et al.*, 1979). Antiserum from each animal was stored separately at −20°C and all were absorbed with pooled normal human plasma (McFarlane *et al.*, 1979) before use. Between 10% and 20% of the liver specific anti-LSP activities of these antisera were directed at species cross-reactive antigens as determined by ELISA against human and rabbit LSP (McFarlane *et al.*, 1983).

Preparation of liver plasma membranes. Rabbit liver plasma membranes were prepared by the aqueous two phase polymer system of Lesko, Donlon & Marinetti (1973) and characterized by electron microscopy, marker enzymes and biochemical composition as previously described (de Kretser *et al.*, 1980).

Preparation of hepatic lectin. Rabbit and human hepatic lectin were prepared by affinity chromatography according to Hudgin *et al.* (1974) and Baenziger & Maynard (1980), respectively. Briefly, acetone powders of livers that had been stored in liquid nitrogen were extracted with 1% Triton X-100 in a Ca^{++} containing buffer and the extracts passed over an asialo-orosomucoid/Sepharose affinity column. Hepatic lectin was eluted in a Ca^{++} free buffer and stored at 4°C in 1 mM Tris-HCl, pH 7·0 (lectin buffer). Protein concentrations were measured by the Coomassie blue procedure of Bearden (1978). Yields of the purified rabbit and human lectins were 37·3 μg and 1·6 μg per gram liver, respectively. The purified lectins were characterized by kinetics of binding of ^{125}I-asialo-orosomucoid (ASOR) according to Hudgin *et al.* (1974) and Baenziger & Maynard (1980). Binding of ASOR by the lectins was found to be Ca^{++}-dependent with a Ka of $2{\cdot}0 \times 10^9$ 1 mole. In addition, the rabbit lectin showed the typical 40,000 and 48,000 mol.wt subunits on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Owing to the relatively low yield there was insufficient human lectin to permit its characterization by SDS-PAGE.

Radioiodination of hepatic lectin. Sixteen and a half micrograms, 5·3 μg and 20 μg, respectively, of rabbit and human hepatic lectin and human LSP were labelled with Na^{125}I (Amersham) using immobilized lactoperoxidase and glucose oxidase (Enzymobeads, Bio-Rad) according to Schwartz *et al.* (1981). The labelled products were stored at 4°C in the lectin buffer or borate/EDTA (as appropriate), with 1% bovine serum albumin, 0·02% sodium azide, 10 μg/ml gentamycin and 200 iu/ml penicillin added. Protein bound radioactivities of the products were greater than 98% as determined by precipitation with 3% perchloric acid and specific radioactivities were 14,600 and 7,600 ct/min/ng for the rabbit and human lectins, respectively, and 14,500 ct/min/ng for the human LSP.

Radioimmunoassay (RIA). For examining the binding of anti-LSP antisera to purified hepatic lectin, the double antibody radioimmunoprecipitation anti-LSP assay of Kakumu *et al.* (1979) was employed with modifications derived from preliminary experiments. Thus, all dilutions of anti-LSP antisera were made up in the lectin buffer with normal guinea-pig serum (Wellcome) added as a carrier when necessary so that a constant final dilution of 1:100 guinea-pig serum was maintained. Twenty-five microlitres (5 ng) of ^{125}I-lectin was incubated with 25 μl anti-LSP antiserum (at appropriate dilution) or lectin buffer (as a blank) for 3 h at 4°C in 1·5 ml polypropylene centrifuge tubes (Sarstedt) then 100 μl of rabbit anti-guinea-pig IgG (Miles), diluted 1:20 with lectin buffer,

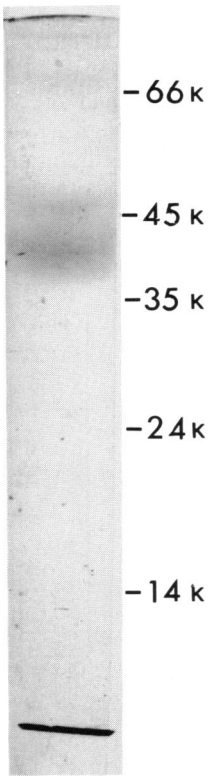

Fig. 1. SDS-PAGE of purified rabbit hepatic lectin (5 μg) showing its constituent subunits (40,000 and 48,000 mol.wt). Bars indicate positions of mol. wt markers: bovine serum albumin (66K), ovalbumin (45K), pepsin (35K), trypsinogen (24K) and lysozyme (14K). Stained with Coomassie brilliant blue R.

were added and incubation continued overnight at 4°C. Eight hundred and fifty microlitres of phosphate-buffered saline, pH 7·2, were added to each and the tubes centrifuged at 13,500*g* for 10 min. The upper 500 μl of each supernatant was transferred to a second tube and both this and the remaining infranatant (containing the immunoprecipitate) were counted in a Packard PRIAS Model PGD autogamma counter. The percentage of radiolabel in the immunoprecipitate was calculated according to the equation:

$$\text{percentage binding} = \frac{\text{ct/min I} - \text{ct/min S}}{\text{ct/min I} + \text{ct/min S}} \times 100.$$

where ct/min I and ct/min S are the counts in the infranatant and supernatant, respectively, after subtraction of the blank value. The overall coefficient of variation for the assay (determined on 16 replicates on four separate occasions) was 1·56%.

To derive titres of anti-LSP, the percentage binding values of serially diluted samples were plotted against $\log_{10}$ serum dilution. The linear portions of the resulting curves were extrapolated to zero and titres defined as the serum dilutions at points of intercept on the abscissa.

Competitive inhibition studies were carried out as above with the inhibitor (at the required concentration) added to the ^{125}I-lectin, before addition of the anti-LSP, in such a way that the final volume and total ct/min remained constant.

Enzyme linked immunosorbent assay (ELISA). The anti-LSP microELISA was applied as previously described (McFarlane *et al.*, 1983) except that the optimal concentration of hepatic lectin for coating the microtitre plates was found to be 0·2 μg/ml.

RESULTS

Initial studies with one anti-human LSP (GP2) and one anti-rabbit LSP (GP8) antiserum in the ELISA revealed that both antisera reacted strongly against purified human and rabbit hepatic lectins (Fig. 2a). Binding of the antisera to the lectins in the ELISA was found to be inhibited by absorbing the sera with either lectin or LSP. None of the four control guinea-pig antisera reacted against the lectins and unabsorbed anti-LSP sera did not show significant binding to uncoated plates. In additon, both anti-LSP antisera showed typical antibody titration curves (Fig. 2b).

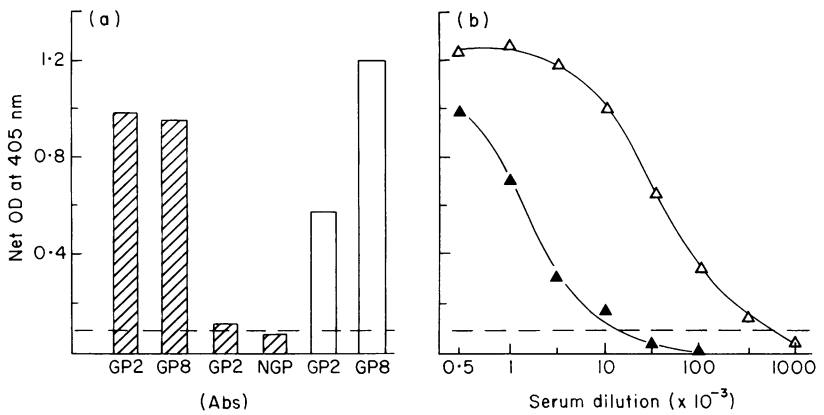

Fig. 2. ELISA results showing binding of guinea-pig anti-LSP antisera to hepatic lectin. In (a) anti-human LSP (GP2), anti-rabbit LSP (GP8) and control guinea-pig (NGP) sera were used at 1:500 against human (▨) and rabbit (□) hepatic lectin; the effect of absorbing GP2 (GP2 Abs.) with 1 μg human hepatic lectin per ml antiserum is also shown. In (b) the titration curves for GP2 (▲) and GP8 (△) against human and rabbit lectin, respectively, are shown. Each value is the mean of triplicate measurements. Broken line indicates maximum binding obtained with unabsorbed antisera on uncoated wells.

Table 1. Binding of guinea-pig anti-LSP antisera to ^{125}I-labelled rabbit and human hepatic lectin and human LSP

	Percentage binding		
Antiserum	Rabbit lectin	Human lectin	Human LSP
Anti-human LSP			
GP1	66·0	40·4	63·5
GP2	33·8	11·9	43·5
GP3	61·5	64·0	64·0
GP4	75·7	69·3	61·7
Anti-rabbit LSP			
GP5	84·5	14·1	22·0
GP6	91·2	62·9	53·7
GP7	91·5	27·4	51·2
GP8	82·0	11·3	20·9
Control	1·0	0·2	9·4

Antisera were tested at 1:100 dilution by radioimmunoassay as described in the text. Each value is the mean of triplicate measurements.

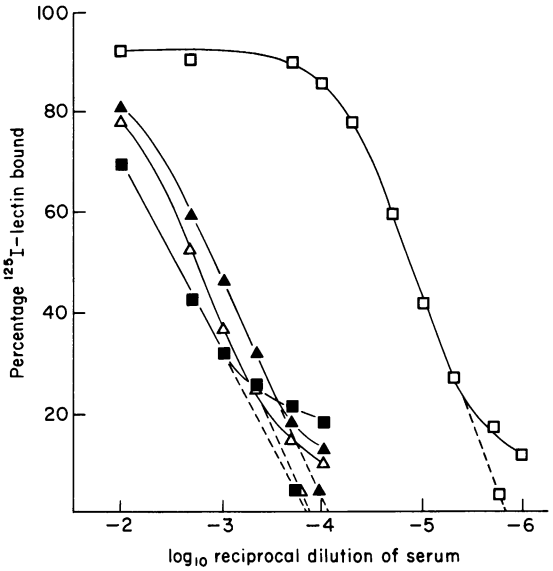

Fig. 3. Titration of guinea-pig anti-human LSP (GP4, △, ▲) and anti-rabbit LSP (GP6, □, ■) against human (▲, ■) and rabbit (△, □) hepatic lectin in the radioimmunoassay. Broken lines indicate extrapolation to zero to obtain titre values as described in the text.

These findings were confirmed when all eight anti-LSP antisera were screened by radioimmunoassay against human LSP and rabbit and human hepatic lectin (Table 1). There was considerable variation in the reactivity of the different antisera against the different antigens but GP4 (anti-human LSP) and GP6 (anti-rabbit LSP) showed high reactivity against all three antigens and were, therefore, selected for further study. Upon titration (Fig. 3), GP6 was found to have a high titre (1:500,000) of antibodies against rabbit lectin and a much lower titre (1:5,000) against

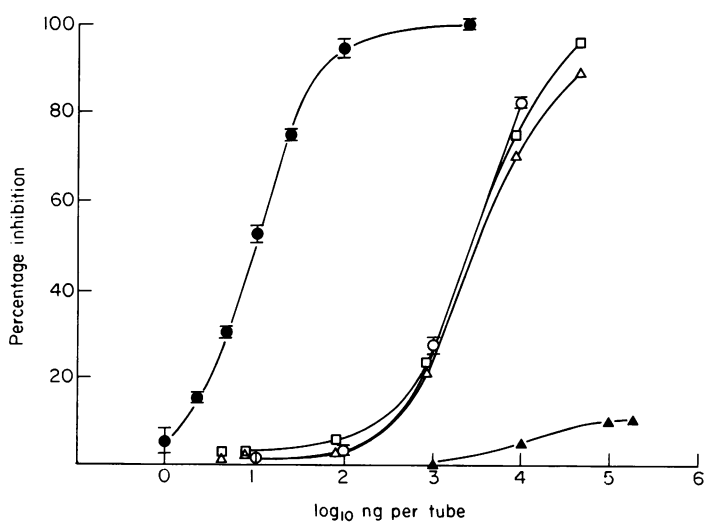

Fig. 4. Inhibition of binding of anti-rabbit LSP (GP6) to ^{125}I-rabbit lectin. Results are mean ± s.e. of four estimations with unlabelled rabbit lectin (●) and rabbit LSP (○) and means of duplicate measurements with rabbit kidney homogenate (▲) and each of two separate preparations of rabbit liver plasma membranes (□, △). GP6 was used at a dilution of 1:20,000 ($\log_{10}$ −4·3, Fig. 3).

human lectin, while GP4 had titres of 1:6,300 and 1:12,500 against rabbit and human lectin, respectively.

It was found that 50% inhibition of binding of GP4 or GP6 to ^{125}I-human or -rabbit lectin in the radioimmunoassay could be achieved by addition of about a two-fold excess of unlabelled lectin but much larger amounts (about 500-fold) of LSP were required to achieve the same effect. From the inhibition curves shown in Fig. 4, it was estimated that 0·25% (wt/wt) of the protein in LSP is hepatic lectin. The corresponding values for two separate preparations of purified liver plasma membranes were 0·25% and 0·28%, respectively.

Binding to ^{125}I-lectin could not be inhibited by up to 50,000-fold excess of kidney homogenate (Fig. 4) and since it had been shown, in a separate study (McFarlane *et al.*, 1983), that after absorption with kidney homogenate the same antisera did not recognise antigens in homogenates of lung, spleen, heart or pancreas, this finding suggests that the reactions against the hepatic lectins were directed principally against liver specific determinants.

DISCUSSION

The finding, in the present study, that LSP contains a protein immunochemically similar to the hepatic asialo-glycoprotein receptor is, to our knowledge, the first time that any component of LSP has been positively identified. This receptor protein (hepatic lectin) has been extensively studied in rat, rabbit and man and, *in vivo*, is responsible for clearance from the circulation of a large number of desialylated glycoproteins that have exposed terminal galactosyl residues (McFarlane, 1983). In functional terms, the hepatic lectin is liver specific and species cross-reactive and the present results suggest that it conforms to this description also on immunochemical criteria. Studies with animal antisera (McFarlane *et al.*, 1980; Manns *et al.*, 1980) and with anti-LSP antibodies in sera from patients with liver disease (Manns & Meyer zum Büschenfelde, 1982) indicate that LSP contains at least one liver specific, species cross-reactive antigen. The hepatic lectin might be that antigen for, as all eight anti-LSP antisera contained antibodies against it, the lectin does seem to be a component of LSP that is very immunogenic—at least in guinea-pigs.

Whether this finding can be regarded as evidence supporting the plasma membrane origin of LSP is uncertain. Only about 10% of the hepatic lectin in the liver cell is associated with the plasma membrane (Pricer & Ashwell, 1976; McFarlane, 1983). The remaining 90% is located in the membranes of a variety of subcellular organelles, including the Golgi complex, lysosomes and the smooth endoplasmic reticulum, and is thought to represent newly synthesized receptor protein *en route* to the plasma membrane. Furthermore, purified hepatic lectin has a tendency to form large aggregates in solution (Kawasaki & Ashwell, 1976). Thus, lectin derived from various endomembranes (and released during processing of the liver tissues) could, on subsequent gel filtration over sepharose 6B, elute in the same high mol. wt fraction as LSP. However, the close concordance observed with respect to the lectin contents of LSP and purified plasma membranes suggests that hepatic lectin is not a fortuitous 'contaminant' of LSP preparations but may be an integral part of this antigen complex.

The finding of hepatic lectin in LSP is in agreement with other evidence relating to the plasma membrane origin of the LSP complex. Previously, it was demonstrated that animal anti-LSP antisera show immunofluorescent staining of the surfaces of isolated hepatocytes (Kakumu *et al.*, 1979; McFarlane *et al.*, 1980) and react on immunodiffusion against purified plasma membranes (de Kretser *et al.*, 1980). Very recently we have obtained preliminary evidence that LSP may bear receptors for the Fc portion of IgG (McFarlane *et al.*, 1983) and it is known that such receptors are present on the surfaces of hepatocytes (Hopf, Meyer zum Buschenfelde & Dierich, 1976). In addition, Lebwohl & Gerber (1981) have found, by direct electron microscopic examination of negatively stained LSP, that it consists of numerous smooth vesicles (40–1,600 nm diameter) and membrane fragments. Similar observations (unpublished) have been made in our laboratories and clearly defined vesicles have been seen, in ultra thin sections of resin embedded LSP, by Jensen *et al.* (1981) who also found enrichments of several plasma membrane marker enzymes in freshly prepared LSP.

If, as the existing evidence seems to suggest, LSP contains fragments of the hepatocellular plasma membrane, then it must comprise multiple molecular species. It follows, therefore, that 'anti-LSP' in patients' sera may represent a population of antibodies with specificities for different molecular components of LSP. Indeed, Manns, Meyer zum Büschenfelde & Hess (1980) have shown that whereas in acute viral hepatitis the anti-LSP antibodies react mainly against human specific determinants of LSP, patients with chronic active hepatitis have anti-LSP antibodies recognizing both human specific and species cross-reactive components. It will be important now to determine whether anti-LSP antibodies in the sera of these patients recognize the hepatic lectin and, if so, whether they are specifically directed at the asialo-glycoprotein receptor site on the lectin molecule since (by analogy with the anti-acetylcholine receptor antibodies in myasthenia gravis) such antibodies could have pathogenetic implications.

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