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The relationship between liver-specific lipoprotein and the hepatocyte plasma membrane

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SUMMARY

Liver-specific lipoprotein (LSP) has been the subject of intense investigation as a candidate target antigen in chronic active hepatitis. Fundamental to the interest in LSP has been the belief that it is an antigen complex of hepatocyte plasma membrane origin. In this study the physical, biochemical and antigenic relationships between LSP and isolated hepatocyte plasma membrane (HPM) were investigated. Electron microscopic examination of LSP showed it to be devoid of plasmalemma sheets that were abundant in HPM. The plasma membrane marker enzyme 5′-nucleotidase was enriched 11-fold in HPM relative to liver homogenate, whereas the enzyme activity in LSP was 17% of that found in liver homogenate and only 1·5% of that found in HPM. The antigenic relationship between LSP and HPM was assesed using sera from rabbits immunized with either mouse LSP or mouse HPM. By filtration ELISA, antibody to LSP reacted poorly with entrapped HPM, relative to antibody to mouse HPM. Antisera to LSP and HPM were both effectively absorbed by the immunizing antigen, however antibody to LSP was not absorbed with HPM, and minimal cross-absorption of HPM antibody with LSP was found. By immunoblot of SDS–PAGE separated LSP and HPM, it was shown that antigenic cross-reactivity between LSP and HPM at the polypeptide level was rare. By immunofluorescence, antibody to LSP failed to react with the surface of viable mouse hepatocytes, whereas antibody to HPM showed linear fluorescence. The data show that the two preparations, LSP and HPM, are dissimilar antigen complexes. HPM may be a more appropriate preparation for the study of autoimmune liver disease than LSP.

INTRODUCTION

Liver-specific lipoprotein (LSP) has been the subject of intense investigation as a candidate target antigen for autoimmune hepatocyte injury in chronic active hepatitis (reviewed by McFarlane, 1984; Meyer zum Buschenfelde & Manns, 1984). LSP is a macrolipoprotein complex prepared from a 105,000 *g* supernatant of liver homogenate by two-stage gel filtration (Meyer zum Buschenfelde & Miescher, 1972; McFarlane *et al.*, 1977). Fundamental to the interest in LSP has been the assumption that it is plasma membrane associated, and therefore accessible to immune mediators. Recent reviews of autoimmunity in liver disease refer to LSP as liver-specific membrane lipoprotein (McFarlane, 1984; Meyer zum Buschenfelde & Manns, 1984), promulgating the view first expounded in 1972 by Meyer zum Buschenfelde and Miescher that LSP was a soluble protein of membrane origin. In seminal studies, Meyer zum Buschenfelde and co-workers (Meyer zum Buschenfelde & Miescher, 1972; Hopf, Meyer zum Buschenfelde & Freudenberg, 1974) and McFarlane *et al.* (1977) found that antisera to a Sephadex G100 fraction of human liver proteins, containing LSP, reacted with the surface of isolated hepatocytes by immunofluorescence. Further evidence for the membrane association of LSP was provided in subsequent studies by Meyer zum Buschenfelde and co-workers, where antisera to human LSP prepared in sheep and rabbits were reported to produce a linear membrane fluorescence with isolated human hepatocytes (Meyer zum Buschenfelde *et al.*, 1979; Manns *et al.*, 1980), and by Chisari *et al.* (1981) and Lebwohl & Gerber (1981) who demonstrated membrane fluorescence with some cultured human hepatocellular carcinoma cells using rabbit antisera to human LSP. Only one study has attempted to determine the antigenic relationship between LSP and isolated hepatocyte plasma membrane; De Kretser *et al.* (1980) demonstrated that guinea-pig antiserum to human LSP reacted with a Triton-X100 extract of rabbit hepatocyte plasma membrane by gel diffusion. In contrast, this group found that the same antisera failed to react by immunofluorescence with the surface of isolated rabbit hepatocytes (McFarlane, Wojcicka & Williams, 1980).

LSP is prepared by methods that would be expected to enrich for cytoplasmic macromolecules and small organelles, rather than for plasma membrane. Membrane fragments have been demonstrated within LSP by electron microscopy (Lebwohl & Gerber, 1981; Jensen, Hall & Majewski 1983); however, the quantitative contribution of membrane fragments to the

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LSP preparation was not reported. For LSP to remain a candidate target antigen in autoimmune chronic active hepatitis (CAH), a strong immunological relationship between LSP and the hepatocyte plasma membrane should be demonstrated. Furthermore, if the objective is to measure immune reactivity to antigens associated with the hepatocyte plasma membrane, then good reason should be offered as to why LSP is used as a source of such antigens in the alternative to isolated hepatocyte plasma membranes. In this study the physical, biochemical and antigenic relationships between isolated hepatocyte plasma membranes and LSP were investigated. The data suggest that future studies of autoimmunity in CAH should employ isolated hepatocyte plasma membranes (HPM) as a source of relevant antigens.

MATERIALS AND METHODS

Animals

Specific pathogen-free young adult BALB/c mice and Dutch Belted rabbits were supplied by the Animal Resources Centre, Murdoch, Western Australia.

Preparation of LSP

LSP was prepared from a 105,000 *g* supernatant of homogenized mouse (BALB/c) and rabbit (Dutch Belted) liver by two-stage gel filtration through Sephadex G100 and Sepharose 6B (Pharmacia, Uppsala, Sweden) by the method of McFarlane *et al.* (1977). Mouse and rabbit liver was homogenized in 0·25 M sucrose buffer (pH 8·0) and centrifuged at 105,000 *g* for 60 min, the supernatant was recovered and stored at −20°. Twenty ml of 105,000 *g* liver supernatant were thawed and centrifuged at 30,000 *g*; the supernatant was then applied to Sephadex G100. The first protein peak from Sephadex G100 filtration was concentrated and applied to Sepharose 6B; the first protein peak from Sepharose 6B was designated LSP. The LSP was stored at 4° at 1 mg/ml in Tris/EDTA buffer (0·1 M Tris HCl, 0·2 M NaCl, 1 mM disodium EDTA, pH 8) containing antibiotics (penicillin 200 IU/ml, gentamicin 10 μg/ml) and used within 2 months of preparation.

Preparation of hepatocyte plasma membranes (HPM)

HPM were prepared from mouse and rabbit liver after the method of Lesko *et al.* (1973) as described by Swanson *et al.* (1985). HPM were adjusted to a concentration of 1–2 mg membrane protein per ml in 0·25 M sucrose in 50 mM Tris buffer (pH 7·5) with penicillin (200 IU/ml) and gentamicin (10 μg/ml) added, and stored at 4°. HPM stored in this manner were used within 1 month of preparation. Where necessary, HPM were concentrated from sucrose/Tris buffer by centrifugation at 2400 *g* for 10 min.

Preparation of control kidney plasma membranes (KPM)

Mouse kidney plasma membranes (KPM) were prepared as described by Swanson *et al.* (1985) using an aqueous two-phase polymer system after the method of Glossman & Gips (1974) for rat kidneys. KPM were stored at 4° as described for HPM.

Electron microscopy

HPM and LSP were fixed in 2·5% glutaraldehyde, washed in phosphate buffer (pH 7·4), resuspended in 10% bovine serum albumin for 15 min and then centrifuged at 13,400 *g* for 10 min into a compact pellet. The pellet was fixed in 2·5% glutaraldehyde and processed using a Sakura automatic ultraprocessor (Tokyo, Japan). Specimens of HPM and LSP were embedded in araldite (Fluka, Eindhoven, The Netherlands) and viewed using a Phillips 410 electron microscope (Fig. 1a and b).

Isolation of mouse hepatocytes

Viable mouse hepatocytes were isolated by perfusion with the chelating agent ethylene-glycol-bis-aminoethyl ether-*N*-tetra-acetate (EGTA, Sigma Chemical Co., St Louis, MO) followed by perfusion with collagenase (Cooper Biomedical, NJ) in RPMI-1640 medium after the method of Seglen (1972). The RPMI-1640 medium (Gibco, Grand Island, NY) used for hepatocyte isolation was supplemented with 10 mM HEPES (Sigma Chemical Co.), penicillin 100,000 IU/l and streptomycin sulphate 100 mg/l, and the osmolality adjusted to mouse osmolality (333 mOsm). The enzymically isolated hepatocyte preparation was enriched for viable hepatocytes by separation on a Percoll (Pharmacia Fine Chemicals) gradient (Pertoft *et al.*, 1977). For assessment of viability of hepatocytes, one volume of suspension was added to ten volumes of 0·5% trypan blue solution in PBS; viability in 25 experiments ranged from 95 to 99%.

Immunization regimes

Rabbits received an initial intradermal injection of 500 μg of mouse LSP or HPM emulsified 1:1 with Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI). At fortnightly intervals thereafter, rabbits were injected by subcutaneous route with a further 500 μg of either LSP or HPM in phosphate-buffered saline (PBS, pH 7·4) and bled from the marginal vein of the ear at 1, 2 and 3 months after initial injection. Six rabbits were immunized with mouse HPM (Rabbits 1–6), six with mouse LSP (Rabbits 7–12), and three rabbits were injected with FCA only. The antibody studies described here were conducted with sera 3 months after initial HPM or LSP injections.

Indirect immunofluorescence

One-hundred microlitres of a suspension of 1×10^6/ml viable hepatocytes in RPMI were incubated with 100 μl of an appropriate dilution of rabbit sera in RPMI for 30 min at 37° in an atmosphere of 5% CO_2 and 95% O_2. The viability of hepatocytes by trypan blue exclusion after this incubation was between 90% and 95%. The cells were washed three times in RPMI by centrifugation at 40 *g* for 3 min, then fixed in 100% methanol for 10 min at room temperature, before two further washes in RPMI. The fixed cells were resuspended in 100 μl of RPMI and incubated with 100 μl of an appropriate dilution of fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of sheep antibody to rabbit immunoglobulins (Silenus, Melbourne, Australia) for 30 min at 37°. After three washes in RPMI, the cells were counterstained with 0·02% Evans Blue in PBS for 10 min, and washed again before resuspension in glycerol:PBS (9:1) for ultraviolet microscopy. The cells were viewed with a Zeiss Standard 14 microscope under epi-fluorescence with both blue excitation and selective fluorescein excitation and observation filters (Carl Zeiss, West Germany).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for measure-

ment of antibody to HPM and LSP bound to poly-L-lysine-treated polystyrene microtitre plates was performed as previously described by us (Swanson *et al.*, 1985) for measurement of HPM antibody. Antibody to HPM was also measured using the Millititre Filtration System (*Millipore Catalogue and Purchasing Guide*, Millipore Corp., Bedford, MA, 1985, p. 48) with HPM entrapped in a low protein binding Millititre 0·22 μm GV 96-well filtration plate. The filtration ELISA was also performed after the method of Swanson *et al.* (1985), the essential difference being filtration entrapment of HPM and washing by filtration. Prior to entrapment of HPM, the filtration plate was wet by filtration of ELISA diluent (PBS containing 0·05% Tween 20 and 10% fetal calf serum); 200 μl of HPM diluted to 20 μg/ml were applied to the wells and entrapped by immediate filtration followed by three washes with ELISA wash buffer. After blocking with ELISA diluent for 20 min at room temperature, appropriate dilutions of rabbit sera in 100 μl of ELISA diluent were added for 60 min at 37° and the plates washed three times before the addition of a 1/1000 dilution of alkaline phosphatase-labelled goat antibody to rabbit IgG (Tago Inc., Burlingame, CA) for 60 min at 37°. Following this incubation, the plates were washed three times before the addition of the chromogenic substrate (1 mg/ml *p*-nitrophenyl phosphate) for 60 min at 37°. After incubation with chromogenic substrate, the contents of the filtration plate wells were transferred with an eight-channel pipettor to a 96-well polystyrene plate for measurement of chromophore at 405 nm using a Titertek Multiscan (Flow Laboratories Inc., Helsinki, Finland).

Antibody absorption

Sera from rabbits immunized with either mouse HPM or mouse LSP were diluted 1/500 in ELISA diluent and an equal volume of diluent containing incremental amounts of absorbent mouse HPM, mouse LSP and mouse KPM was added to give a final concentration of absorbant over a range of 2·5–100 μg/ml. The sera and absorbants were incubated at 37° for 1 hr, and for a further 18 hr at 4°, and centrifuged at 11,500 ***g*** for 10 min to sediment antigen–antibody complexes. Antibody activity within the supernatant was measured by ELISA, with the antigens adsorbed to poly-L-lysine-treated polystyrene microtitre plates.

Protein determinations

Protein concentration in LSP, HPM and KPM was determined by the Ponceau-S/TCA method of Pesce & Strande (1973) using QCS Normal Control Serum (Gilford, Irvine, CA) as standards.

Measurement of enzyme activities

5′-Nucleotidase (EC 3.1.3.5) was measured by the method of Evans (1978), and the amount of phosphate liberated was subsequently measured (Fiske & Subbarow, 1925). Prior to assay of LSP, EDTA was removed by dialysis against Tris/HCl buffer (pH 8). Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed using the method of Swanson (1955), with 4 mM EDTA and 1 mM potassium fluoride included to inhibit acid or alkaline phosphatase activities, and liberated phosphate was subsequently measured. Lactate dehydrogenase (EC 1.1.1.27) activity was assayed using the method of Korzeniewski & Callewaert (1983).

Polyacrylamide gel electrophoresis and immunoblot

LSP and HPM were separated by electrophoresis in 10% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS–PAGE) and transferred to nitrocellulose for determination of antibody specificity. Five-hundred micrograms of protein were dialysed against 10 mM Tris-HCl buffer containing 1 mM EDTA, 1% SDS (specially pure, BDH, Poole, Dorset, U.K.), 5% 2-mercaptoethanol and 10 M urea (pH 8) for 18 hr at room temperature. The samples were then heated to 100° for 10 min in a boiling water bath before application of the samples to 10% polyacrylamide gel with a 3% polyacrylamide stacking gel. After electrophoresis, transfer of proteins to nitrocellulose and immunoblot was conducted after the method of Towbin, Staehelin & Gordon (1979). The polyacrylamide gels used in transfer to nitrocellulose were stained with Commassie Blue to determine the amount of residual protein; none was detected. After blocking of the nitrocellulose with 3% gelatin in Tris-buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7·5), an appropriate dilution of rabbit antibody to either LSP or HPM in 1% gelatin-TBS was applied for 1 hr at room temperature with agitation. The nitrocellulose was rinsed in distilled water and washed twice for 10 min in TBS. Peroxidase-labelled goat antiserum to rabbit immunoglobulins (Bio-Rad Laboratories, Richmond, CA) at a dilution of 1/1000 in 1% gelatin-TBS were added for 1 hr at room temperature with gentle agitation. The nitrocellulose was rinsed in distilled water and washed twice for 10 min in TBS before the addition of peroxidase substrate (containing 4-chloro-l-napthol, Bio-Rad Laboratories). PAGE gels and nitrocellulose blots were scanned with a laser densitometer (LKB Model 2202 Ultroscan, LKB, Stockholm, Sweden).

RESULTS

Physical characteristics of HPM and LSP

If left undisturbed for 6 hr at a concentration of 1 mg/ml, HPM in sucrose/Tris buffer appeared as a white sediment with a clear supernatant; LSP in Tris/EDTA buffer was opalescent with no visible sediment. In order to determine if HPM and LSP could be separated into 'insoluble' and 'soluble' components, freshly prepared mouse HPM at a concentration of 1·20 mg/ml and LSP at a concentration of 1·04 mg/ml were either centrifuged or filtered and the recovered protein measured. After centrifugation at 11,500 ***g*** for 10 min, no protein was detectable in the supernatant of HPM, whereas 0·84 mg/ml (81%) of protein was found in the supernatant of LSP. After filtration through a Millipore 0·22 μm GV filter (with low protein-binding properties, *Millipore Catalogue and Purchasing Guide*, Millipore Corp., 1985, p. 42), HPM filtrate contained no detectable protein, whereas LSP filtrate contained 1·08 mg/ml protein.

Electron microscopy

Transmission electron microscopy of HPM and LSP showed HPM to be predominantly composed of plasmalemma sheets, whereas the precipitable components of LSP were predominantly microsomes. HPM contained parallel strips of membrane of up to 8 μm in length exhibiting desmosomes and associated vesiculated profiles; bile canalicular domains encircled by membranes were also present (Fig. 1a). LSP contained no

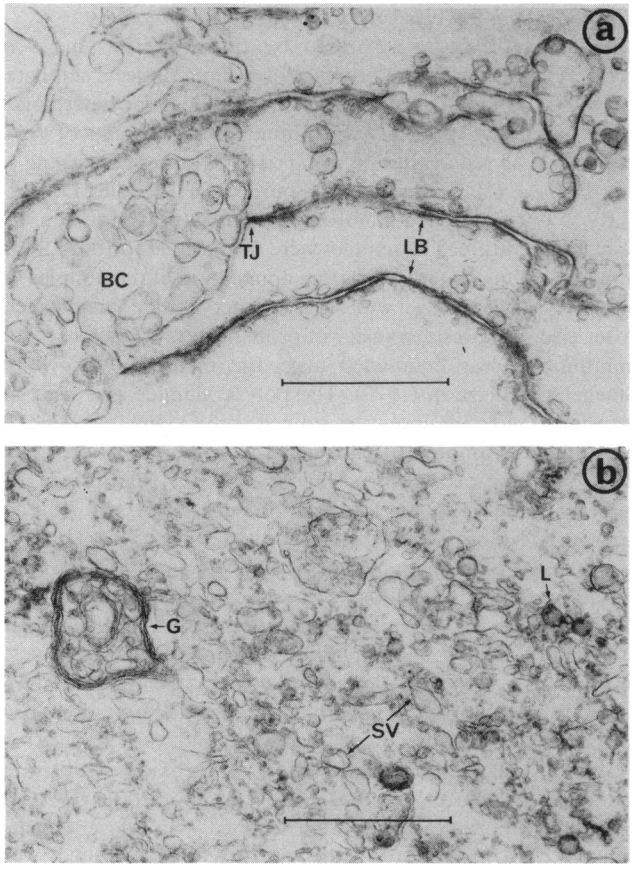

Figure 1. Transmission electron micrographs of (a) mouse HPM and (b) mouse LSP showing parallel strips of plasmalemma in HPM, and an absence of plasmalemma in LSP. The bar depicts 1·0 μm at a magnification of ×21,600. Features of HPM (a) shown include bile canalicular vesicles (BC) and lateral borders (LB) with tight junctions (TJ). Components of LSP (b) shown include smooth vesicles. (SV), lysosomes (L) and Golgi complex (G).

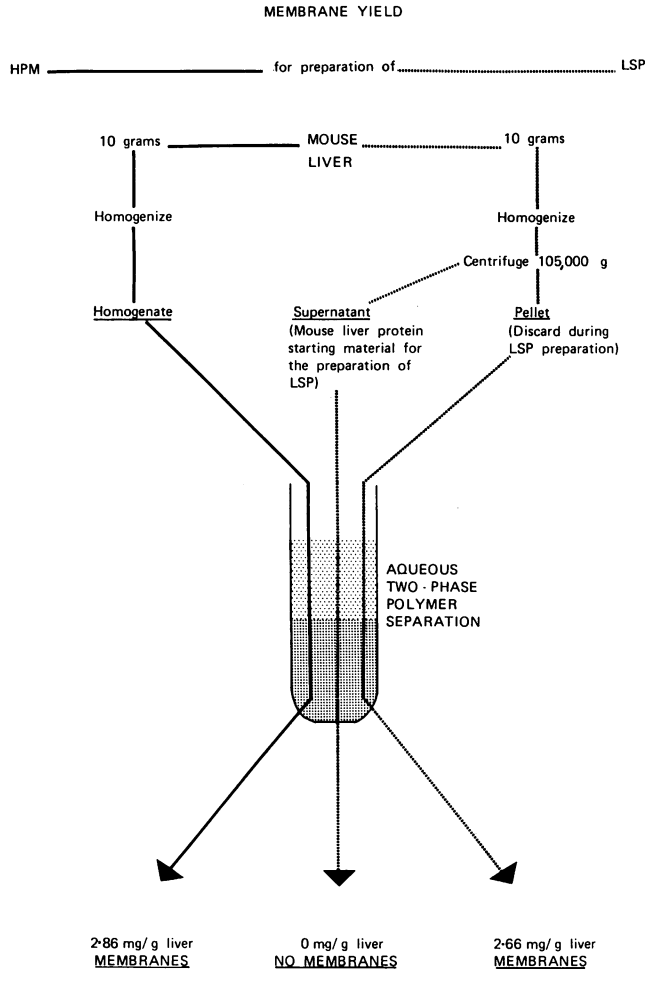

Figure 2. Aqueous two-phase polymer separation of mouse liver homogenate, and 105,000 ***g*** supernatant and pellet of mouse liver homogenate.

plasmalemma sheets; the vesicles present within LSP appeared to be smooth endoplasmic reticulum components of the hepatocyte vacuolar system; Golgi complexes and lysosomes were also present (Fig. 1b). None of the components of LSP exceeded 1 μm in size, the average vesicle size being 0·24 μm.

Nil membrane recovery during LSP preparation

HPM is prepared by an aqueous two-phase polymer separation of liver homogenate; LSP is prepared by two-stage gel filtration of a 105,000 ***g*** supernatant of homogenized liver. In order to determine the extent to which HPM could be recovered from the 105,000 ***g*** supernatant starting material for LSP, homogenate, 105,000 ***g*** supernatant and pellet were each applied separately to the aqueous two-phase polymer system. The direct application of liver homogenate to aqueous two-phase polymer resulted in a recovery of 2·86 mg of HPM protein per gram of liver. No membrane protein was recovered from the 105,000 ***g*** supernatant of homogenized mouse liver applied to aqueous two-phase polymer; the membrane protein was recovered from the pellet of 105,000 ***g*** centrifugation of homogenized mouse liver (Fig. 2). Ten milligrams of mouse LSP were also applied to aqueous two-phase polymer without recovery of membrane protein from the interface.

Enzyme levels in HPM and LSP

The enzyme 5′-nucleotidase is located primarily in the plasma membrane and has frequently been used as a marker enzyme in cell-surface isolation studies (Evans & Gurd, 1973). 5′-Nucleotidase activity was measured in mouse liver homogenate, mouse HPM, the 105,000 ***g*** supernatant of mouse liver homogenate, proteins from the first peak of Sephadex G100 separation of 105,000 ***g*** supernatant of mouse liver homogenate, and mouse LSP. 5′-Nucleotidase specific activity relative to starting liver homogenate (Evans, 1978) for each of these preparations is shown in Fig. 3. Mouse HPM prepared by aqueous two-phase polymer separation showed an 11-fold enrichment in 5′-nucleotidase activity relative to liver homogenate, whereas the 105,000 ***g*** supernatant and subsequent gel filtration products towards preparation of mouse LSP showed progressive reduction in 5′-nucleotidase activity. The specific activity of the endoplasmic reticulum (microsomal) marker glucose-6-phosphatase (Evans, 1978) relative to liver homogenate was 0·14 for

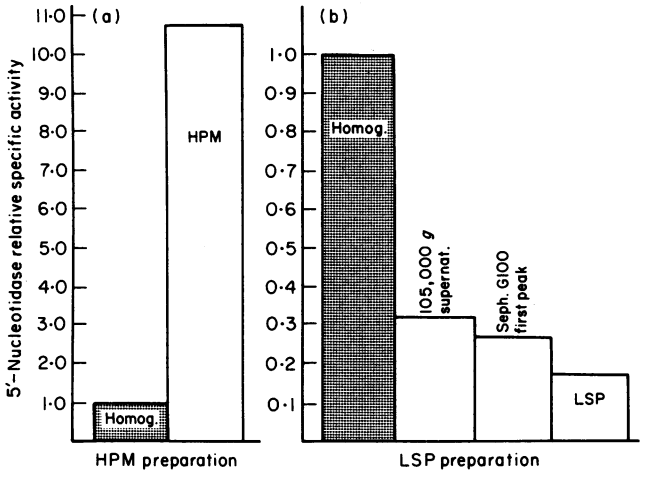

Figure 3. 5'-nucleotidase activity relative to mouse liver homogenate (a) in mouse HPM and (b) during the preparation of LSP.

HPM and 0·92 for LSP. On a unit protein basis HPM was poor in microsomes, whereas LSP had equivalent microsomes to liver homogenate. The specific activity of the soluble cytosol marker lactate dehydrogenase (Evans, 1978) relative to liver homogenate was 0·04 for HPM and 0·06 for LSP, suggesting that neither of these preparations contained significant amounts of soluble low molecular weight proteins.

Reactivity of antisera to HPM and LSP with HPM by filtration ELISA

Sera from rabbits immunized with either mouse HPM or mouse LSP were reacted with both mouse and rabbit HPM by filtration ELISA. The HPM was entrapped in low protein-binding Millipore 0·22 μm GV membrane and, as no HPM passes the filter (see above), this method enables quantitative retention of HPM in the support medium. LSP passes through the 0·22 μm GV filter. The assay proved as sensitive as the ELISA previously developed by us for measurement of antibody to HPM absorbed to poly-L-lysine-treated polystyrene plates (Swanson *et al.*, 1985). Sera from rabbits immunized with mouse HPM contained higher levels of antibody to mouse HPM and autoantibody to rabbit HPM than did sera from rabbits immunized with mouse LSP. Figure 4 shows data from individual immunized (Rabbits 3 and 7) and FCA-treated rabbits that are representative of the findings for the respective treatment groups.

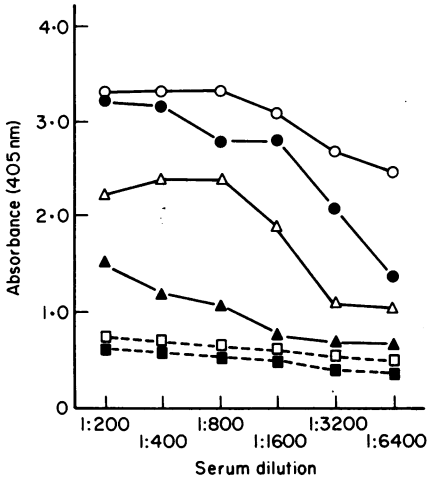

Figure 4. Antibody to mouse HPM (open symbols) and rabbit HPM (closed symbols) measured by filtration ELISA in serum from Rabbit 3, immunized with mouse HPM (○,●); Rabbit 7, immunized with mouse LSP (△,▲); and a control rabbit injected with FCA only (□,■).

Low level of cross-reactivity between HPM and LSP in absorption studies

Sera from three rabbits immunized with mouse HPM and from three rabbits immunized with mouse LSP were absorbed with mouse HPM, mouse LSP and mouse KPM over the range 5–100 μg/ml prior to measurement of antibody to the immunizing antigen by ELISA (antigens adsorbed to polystyrene microtitre plates). Representative data from these experiments are shown in Fig. 5a and b. Antibody to mouse HPM in the serum of a rabbit immunized with mouse HPM (Rabbit 3) was absorbed with mouse HPM immunogen but only minimally absorbed with mouse LSP or mouse KPM (Fig. 5a). Antibody to mouse LSP in the sera of a rabbit immunized with mouse LSP (Rabbit 7) was absorbed with mouse LSP, whereas no reduction in LSP

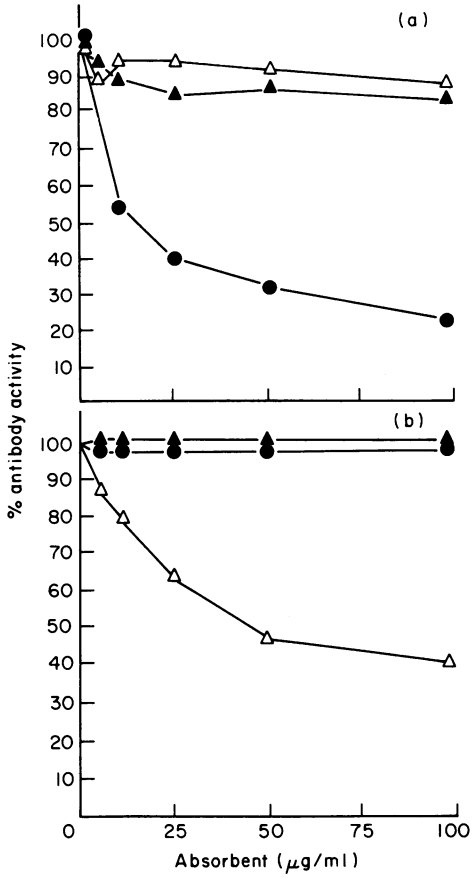

Figure 5. Sera from (a) Rabbit 3, immunized with mouse HPM, and (b) Rabbit 7, immunized with mouse LSP, absorbed with HPM (●), LSP (△), and KPM (▲) prior to measurement of antibody to (a) HPM and (b) LSP by ELISA.

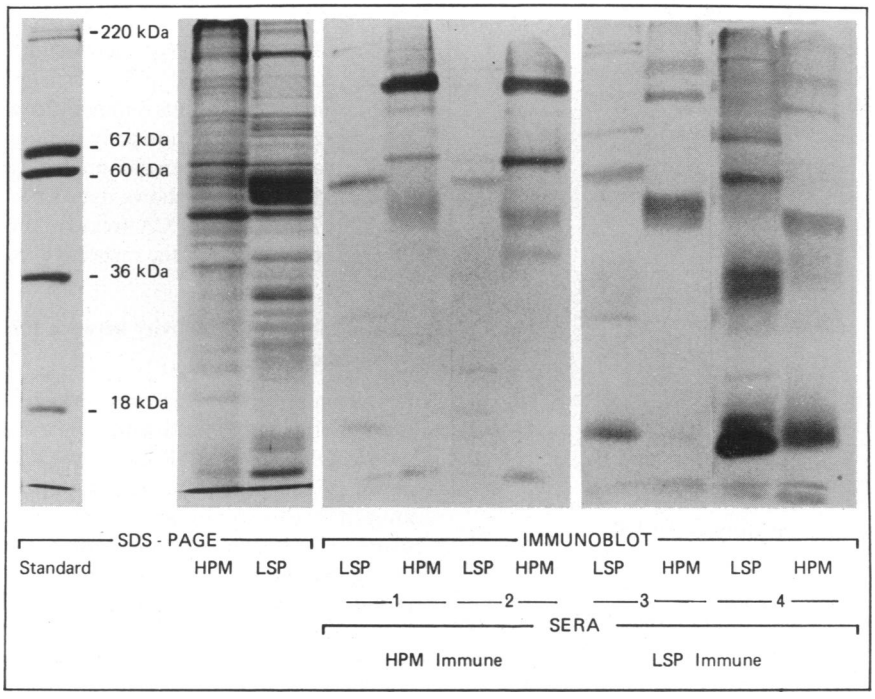

Figure 6. SDS–PAGE and immunoblot of mouse HPM and LSP using sera from Rabbit 3 (1), and Rabbit 5 (2) immunized with mouse HPM; and Rabbit 7 (3) and Rabbit 9 (4) immunized with mouse LSP.

antibody activity occurred with HPM or KPM absorbent (Fig. 5b).

Analysis by PAGE and immunoblot

PAGE of HPM and LSP revealed 22 polypeptides in HPM and 23 polypeptides in LSP of molecular weight (MW) between 5000 and 220,000. Quantitative analysis by laser densitometer of the gels shown in Fig. 6 revealed nine common polypeptides. With six of the common polypeptides the amount in HPM was less than 30% of that in LSP, with one the amount in LSP was only 22% of that in HPM, and equal amounts of a 62,000 and a 94,000 MW polypeptide were present in both HPM and LSP. Six of the antisera to mouse HPM and four of the antisera to mouse LSP were applied to HPM and LSP transferred to nitrocellulose. The essential finding was that sera from rabbits immunized with either HPM or LSP reacted with both antigen complexes but with different polypeptides within each complex (Fig. 6, sera 1, 2 and 3). Antigenic cross-reactivity between HPM and LSP at the polypeptide level was rare. Laser densitometry of blots showed that with nine sera (Rabbits 1–8, Rabbit 10) any cross-reactivity detected between HPM and LSP polypeptides was less than 5% of the major reactivity with the polypeptide. Serum from one rabbit immunized with LSP (Rabbit 9) demonstrated reactivity with a polypeptide of an estimated 12,000 MW in both LSP and HPM (Fig 6, serum 4).

Hepatocyte reactivity of antisera to HPM and LSP by immunofluorescence

Sera from rabbits immunized with either mouse LSP or mouse HPM were reacted with isolated viable mouse hepatocytes by immunofluorescence. Sera from six rabbits immunized with mouse HPM showed strong linear fluorescence with the circumference of isolated mouse hepatocytes; an example of the membrane fluorescence, with Rabbit 3 serum, is shown in Fig. 7a and b. In contrast, sera from six rabbits immunized with mouse LSP did not react with the plasma membrane of viable hepatocytes but reacted with the cytoplasm of 5–10% of hepatocytes (Rabbit 7, Fig. 7c and d). Before methanol fixation, a similar percentage of hepatocytes was found to be non-viable by Trypan Blue staining. The linear fluorescence reactivity of rabbit antibody to mouse HPM (Rabbit 3) was retained after absorption of the sera with 100 μg of mouse LSP (Fig. 7e and f), whereas 100 μg of mouse HPM absorbed all activity (Fig. 7g and h).

DISCUSSION

This study reports a qualitative and quantitative comparison of the physical, morphological, biochemical and antigenic characteristics of HPM and LSP. Two different views of the physical properties of LSP have emerged from other studies. Meyer zum Buschenfelde & Miescher (1972) described LSP as a soluble protein of membrane origin; more recently, Jensen *et al.* (1983) described LSP as a heterogeneous material 'highly enriched in plasma membranes'. Both reports argued in support of LSP as a preparation of value in the study of autoimmunity to hepatocyte (plasma) membrane antigens. The first position implies that LSP is a preparation containing soluble components of plasma membrane origin; the second a preparation containing, amongst other things, plasma membranes of utility in the study of immune destruction of hepatocytes. Our data show a marked contrast between the physical properties of the preparations, LSP and HPM. LSP passed a 0·22 μm filter and only 19% of it was sedimented at 11,500 ***g***, whereas HPM did not pass the filter

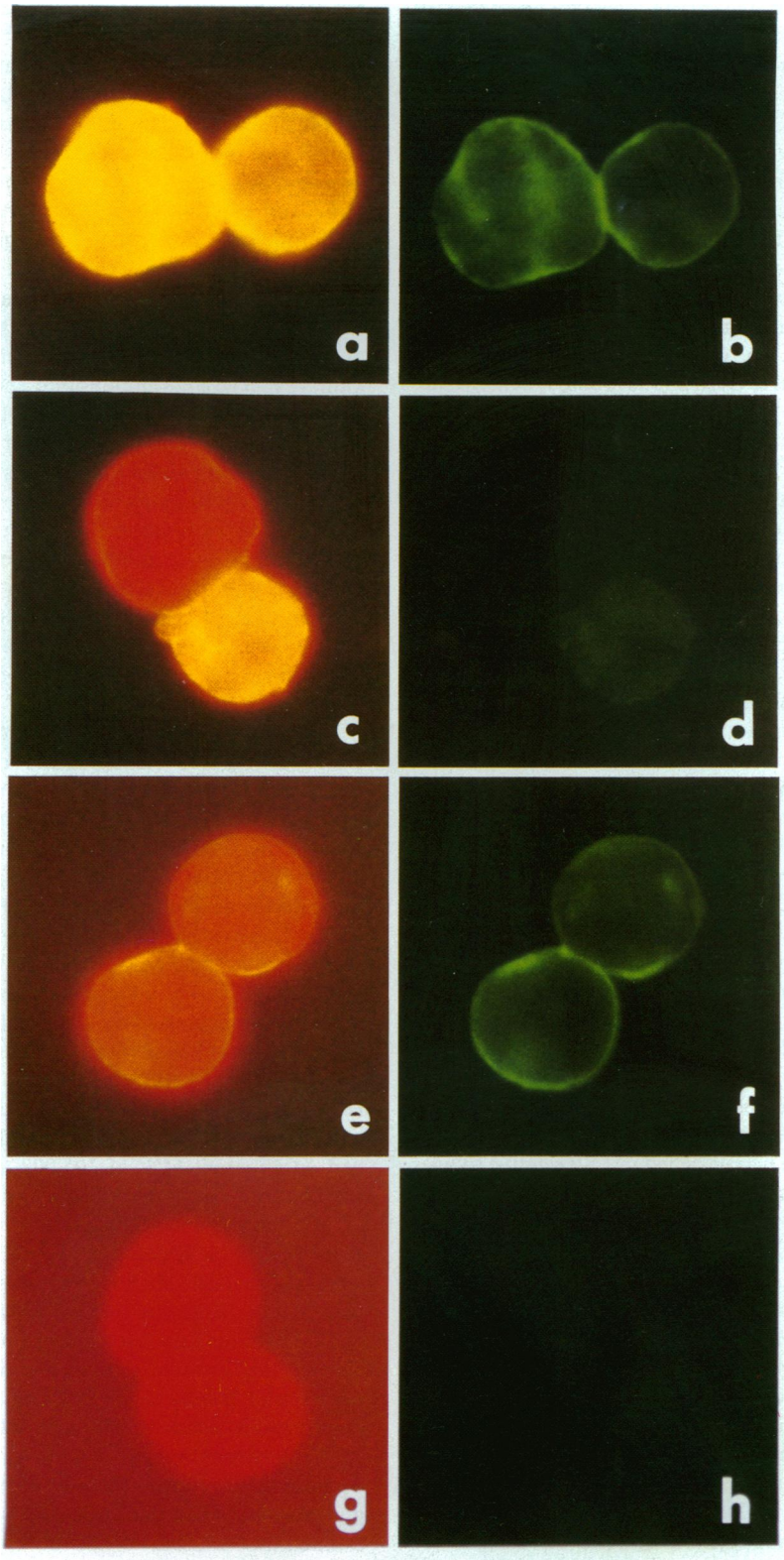

Figure 7. Indirect immunofluorescence with mouse hepatocytes (95% viable). Serum from Rabbit 3, immunized with mouse HPM: unabsorbed (a and b), absorbed with 100 μg of mouse LSP (e and f), and absorbed with 100 μg of mouse HPM (g and h). Serum from Rabbit 7, immunized with mouse LSP: unabsorbed (c and d). (a), (c), (e) and (g): blue excitation. (b), (d), (f) and (h): selective fluorescein excitation (magnification $\times 425$).

and was totally sedimented at 11,500 *g*. Clearly, LSP is a largely soluble protein preparation. Two major questions arise: to what extent does the minor insoluble component of LSP equate with plasma membrane, and to what degree does the LSP preparation share an antigen complement with plasma membranes from hepatocytes?

Lebwohl & Gerber (1981) described small membrane fragments and numerous small vesicles in negatively stained human LSP by electron microscopy, ranging in size from 0·4 to 1·6 μm. Jensen *et al.* (1983) reported numerous membrane vesicles of 0·04–0·5 μm diameter and sheets in rabbit LSP revealed by transmission electron microscopy; a size range for membrane sheets was not given but none of the particles illustrated exceeded 0·5 μm in length. The electron microscopy pictures of LSP illustrated by Lebwohl & Gerber (1981) and by Jensen *et al.* (1983) are similar to those found in this study; however, we did not find that LSP contained membrane sheets as claimed by Jensen *et al.* (1983). The morphology of LSP particles is in marked contrast with that observed by us for HPM. Mouse HPM contained membranes of up to 8 μm in length in associations identifiable as the lateral borders of hepatocytes and bile canaliculi; vesicles seen were linked to larger membranes. This picture for mouse HPM is similar to that described by others for rat HPM (Hubbard, Wall & Ma, 1983). LSP is prepared by gel filtration of a 105,000 *g* supernatant of liver homogenate. When this supernatant was applied to an aqueous two-phase polymer system, no plasma membrane protein was recovered; plasma membranes were found in the pellet of the 105,000 *g* centrifugation. Similarly, LSP applied to the aqueous two-phase polymer system failed to produce recoverable plasma membrane protein. These observations suggest that membrane fragments visualized by electron microscopy in LSP (Lebwohl & Gerber, 1981; Jensen *et al.*, 1983) constitute a negligible amount of the protein in LSP.

Relative to liver homogenate, HPM was enriched 11-fold for the plasma membrane marker enzyme 5′-nucleotidase; in contrast, each stage in the preparation of LSP led to a progressive reduction in the 5′-nucleotidase activity. There are two previous reports of 5′-nucleotidase activity in LSP. Feighery & Weir (1980) found that LSP was not enriched for 5′-nucleotidase relative to liver homogenate; Jensen *et al.* (1983) found that 5′-nucleotidase was enriched in LSP relative to liver homogenate to an amount equivalent to relative enrichment in HPM. The finding by Jensen *et al.* (1983) of equivalent 5′-nucleotidase activity in LSP and HPM suggested that LSP contained an equivalent component of plasma membranes; this is irreconcilable with our results where 5′-nucleotidase activity in LSP was only 17% of that found in starting liver homogenate and only 1·5% of the activity found in HPM. In contrast with our findings on 5′-nucleotidase levels, HPM had low levels of the microsomal enzyme glucose-6-phosphatase, whereas LSP had levels six-fold greater than HPM. Both the glucose-6-phosphatase levels and electron microscopy suggest that LSP is rich in microsomes. Lahav *et al.* (1982) reported that microsomes were sedimented from liver homogenate in 0·25 M sucrose by centrifugation at 105,000 *g*; however, the supernatant contained a minor microsomal component. During Sephadex G100 and Sepharose 6B gel filtration of liver homogenate 105,000 *g* supernatant the microsomal component would be excluded from the gels and elute with the first protein peak, and thus be enriched in LSP.

Antisera to HPM had greater reactivity with plasma membranes by filtration ELISA than did antisera to LSP. In terms of the value of HPM and LSP in the study of experimental autoimmune hepatitis, it is significant that injection of rabbits with mouse HPM elicited high levels of autoantibody to rabbit HPM, whereas injection of mouse LSP did not. Further evidence of the antigenic dissimilarity between HPM and LSP was obtained in absorption studies using antisera against mouse HPM and LSP in a sensitive ELISA. HPM and LSP absorbed their respective antisera but negligible cross-absorption with these antigen complexes was found. Kidney plasma membrane failed to reduce significantly the levels of antibody to either HPM or LSP, suggesting that the antibodies measured were organ specific at the level of discrimination between liver and kidney. PAGE and immunoblot showed that antigenic cross-reactivity betwen HPM and LSP at the polypeptide level was rare. Sera from rabbits immunized with either HPM or LSP reacted with both antigen complexes but with different polypeptides within each complex. Thus, there are components of HPM in LSP and vice versa. The immune system amplified the perception of these minor components within the respective antigen complexes. Serologically by immunoblot, the antibody product of the amplification process reacted with a rich source of the minor components, but showed little or no reactivity with those determinants at the low concentrations found in the immunogen.

Our data suggest that LSP is rich in microsomes. Gurd, Evans & Perkins (1972) found only low antigenic cross-reactivity between mouse HPM and mouse hepatocyte microsomal membranes; a rabbit antiserum to mouse HPM had only 14% of the reactivity by radioimmunoassay with microsomal membranes that it had with HPM. By immunoprecipitation and SDS–PAGE of solubilized mouse HPM, Gurd, Evans & Perkins (1973) showed that mouse HPM contained about seven antigenic components, constituting 20% by weight of membrane protein; the major antigen component was a 120,000 MW glycoprotein expressed on the membrane surface. With the six rabbit antisera to mouse HPM employed in immunoblot in our study, six to nine antigenic polypeptides were found in mouse HPM, including five common antigenic polypeptides found with each sera. The major antigenic determinant in mouse HPM had an apparent MW of 105,000, and may be related to the 120,000 component described by Gurd *et al.* (1973).

We have found that antibody to HPM reacted with the surface of viable hepatocytes by immunofluorescence, whereas antibody to LSP did not. No previous studies have simultaneously compared the relative efficacy of antibodies to HPM and LSP in this way. Meyer zum Buschenfelde and co-workers claimed that antisera to liver proteins containing LSP (Hopf, Meyer zum Buschenfelde & Freudenberg, 1974) and to LSP (Meyer zum Buschenfelde *et al.*, 1979; Manns *et al.*, 1980) showed a strong linear fluorescence with isolated hepatocytes. Against these reports, the group that developed methods for purification and stabilization of LSP (McFarlane *et al.*, 1977) claimed that antisera to LSP do not react with the surface of isolated hepatocytes (McFarlane *et al.*, 1980). They attributed their earlier report of hepatocyte surface reactivity of antibody to LSP (McFarlane *et al.*, 1977) to the crudity of LSP preparations used in raising antisera.

These data suggest physical, biochemical and antigenic dissimilarity between the preparations LSP and HPM. Plasma

membrane presentation of target antigens is likely to be of fundamental importance in the destruction of hepatocytes by immune mechanisms. The hepatocyte plasma membrane constitutes the subcellular region towards which autoimmunity in liver disease should be measured; it is also the relevant immunogen for experimental studies of autoimmune liver disease in animals. We submit that HPM may be a more appropriate preparation for the study of autoimmune liver disease than LSP.

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