Clin. exp. Immunol. (1981) **46**, 435–442.

Characterization and demonstration of human liver-specific protein (LSP) and apo-LSP

N. LEBWOHL & M. A. GERBER *The Lillian and Henry M. Stratton-Hans Popper Department of Pathology, The Mount Sinai School of Medicine of the City University of New York, New York, USA*

(Accepted for publication 22 May 1981)

SUMMARY

Liver-specific protein (LSP) prepared by standard methods from five normal human livers showed significant variations in terms of quantitative yield, lipid/protein ratio and migration characteristics of different components on SDS–polyacrylamide gel electrophoresis. This heterogeneity is probably related to varying amounts of different molecular species in the LSP preparations. Delipidation and re-chromatography of the LSP preparation appeared to result in relative enrichment of apo-LSP which showed immunological identity with LSP. Rabbit antiserum to LSP gave a precipitin line of identity with standard antisera to human LSP (anti-LSP) from two other laboratories. After extensive absorption, anti-LSP showed selective reactivity with a surface membrane antigen on a human hepatocellular carcinoma cell line (PLC/PRF/5) that exhibits functional and morphological characteristics of differentiated hepatocytes. The antiserum did not react with cell lines derived from other organs as determined by the indirect fluorescent antibody technique. The surface staining of viable PLC/PRF/5 cells was eliminated by absorption with LSP and apo-LSP, but not with the equivalent kidney fractions. These findings support the concept of a liver-specific antigen and suggest that the PLC/PRF/5 cell line may serve as a source of homogeneous LSP.

INTRODUCTION

Liver-specific protein (LSP), first described by Meyer zum Büschenfelde & Miescher in 1972, contains multiple antigenic specificities, of which at least one represents an organ-specific, macromolecular, hepatocellular plasma membrane antigen with partial species-specificity (McFarlane, Wojcicka & Williams, 1980). Stabilization of LSP in Tris or borate buffers containing EDTA by McFarlane and co-workers (1977) facilitated further characterization of LSP and development of a sensitive radioimmunoassay for antibody to LSP (anti-LSP) (Jensen *et al.*, 1978a). Several clinical studies demonstrated anti-LSP in sera of patients with HBsAg-negative and -positive acute and chronic hepatitis and with primary biliary cirrhosis (Jensen *et al.*, 1978b; Kakumu *et al.*, 1979; Gerber *et al.*, 1979; Tsantoulas *et al.*, 1980), although our investigation suggested that the development of anti-LSP may not be entirely specific for liver disease. In addition, difficulties have been encountered in the efforts to identify and characterize human LSP, which may be related in part to the heterogeneity of LSP preparations derived from different livers (Chisari, 1980). Therefore, we attempted to purify LSP further and to demonstrate LSP in continuous cell lines.

Correspondence: Michael A. Gerber, MD, Department of Pathology, The Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029, USA.

0009-9104/81/1100-0435$02.00

MATERIALS AND METHODS

Preparation of LSP and apo-LSP. LSP was prepared from five normal human livers obtained at autopsy within 6 hr of death. The method of McFarlane *et al.* (1977) was used with the following modifications: molecular exclusion chromatography was performed on a Sepharose 6B column, connected in series with a Sepharose 4B column (Pharmacia Fine Chemicals, Piscataway, New Jersey). The columns had gel beds of 90 × 2·5 and 50 × 2·5 cm, respectively, and had been equilibrated with 0·1 M boric acid/sodium borate–1 mM disodium EDTA–0·02% sodium azide, pH 8·5, buffer (borate buffer). LSP was eluted in the void volume as a distinct peak, clearly separated from a second peak (presumably representing LP2). Protein content was determined by a modification of the method of Lowry *et al.* (1951), and the total lipid content by the sulpho-phospho-vanillin reaction (Frings & Dunn, 1970). The same procedure used for the preparation of LSP was employed to prepare lipoprotein fractions from normal human serum (SP), and kidney (KP), lung and spleen and from liver of normal adult New Zealand white rabbits obtained under ether anaesthesia.

LSP was delipidated by a modification (McFarlane *et al.*, 1977) of the method of Helenius & Simons (1971) using 22 mg solid sodium deoxycholate per mg protein. The mixture was incubated at 4°C for 1 hr and then chromatographed on Sepharose 4B (gel bed 40 × 2·5 cm), equilibrated and eluted with half-strength borate buffer containing 10 mM sodium deoxycholate.

Electron microscopy. LSP, KP and SP were examined under the electron microscope on collodion-coated copper grids after negative staining with 2% phosphotungstic acid (Hirschman, Gerber & Garfinkel, 1974).

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE). A discontinuous system adapted from O'Farrell *et al.* (1975) was employed. Rod or slab gels were prepared from acrylamide and methylene-*bis*-acrylamide (stacking gel 4·5% T, 2·67% C; running gel 7·5% T, 2·67% C). Samples containing 30–150 μg protein were heated at 100°C for 5 min in the presence of 2,3-dithiothreitol, electrophoresed and stained with Coomassie brilliant blue. For determination of molecular weights, well-characterized protein standards (Pharmacia) were used.

Preparation of antiserum to LSP. Adult New Zealand white rabbits were given footpad injections of 0·6 mg LSP in Freund's complete adjuvant. Subcutaneous and intramuscular booster inoculations of 0·5–1·0 mg LSP in Freund's complete adjuvant were performed 2 and 4 weeks after the first injection. The rabbits were bled prior to the first injection and weekly after the last injection. All antisera were initially absorbed with normal human plasma blood group AB, linked to cyanogen bromide-activated Sepharose 4B (Wilchek *et al.*, 1971). For this purpose, equal volumes of antisera and insolubilized normal human plasma were incubated at 37°C for 30 min and at 4°C overnight with gentle agitation. All subsequent absorptions were done according to this procedure. Double immunodiffusion was performed in 0·6% agarose in borate buffer. Prior to use, 10 μl of 5% sodium deoxycholate was added to 50 μl of antigen at a protein concentration of 0·35 mg/ml, unless stated otherwise.

Immunohistochemical staining of cell lines. The principal cell line used for detection of LSP, PLC/PRF/5, was derived from an HBsAg-positive human hepatocellular carcinoma (Alexander *et al.*, 1976). The cells were cultured and stained by the indirect fluorescent antibody method on eight-chambered tissue culture slides (LabTek Products, Westmont, Illinois), as described previously (Gerber *et al.*, 1981). For control purposes, the following cell lines were studied: Chang cells (American Type Culture Collection, Rockville, Maryland), HeLa cells, acute lymphocytic leukaemia cells RPMI 8402, human embryonic fibroblasts IMR 90 and WI 38, human hepatoblastoma cells Sk-H-MA and fetal Rhesus monkey kidney cells FRhK-4. The viability of the cells after the staining procedure was determined by trypan blue exclusion. In order to reduce non-specific staining, all antisera were appropriately diluted and the fluorescein-conjugated sheep antiserum to rabbit gammaglobulin was absorbed with apo-LSP linked to Sepharose 4B. The acute lymphocytic leukaemia cells were stained by incubation with antisera in suspension. In several experiments, PLC/PRF/5 cells, HeLa cells, WI 38 cells and Rhesus monkey kidney cells were stained in suspension after harvest with a rubber policeman. Although the staining results were

similar by the two procedures, the slide method proved to be more satisfactory due to better cell viability.

Normal human liver and kidney obtained at surgery were also tested for the presence of LSP. Frozen sections were dried in a vacuum and stained without fixation.

RESULTS

Preparation and characterization of LSP. LSP prepared by the same method from five normal human livers showed significant variations. The yield of LSP preparations differed from 3·1 to 34 mg per 10 ml liver supernatant. The total lipid/protein ratio of LSP ranged from 0·24 to 1·09. The number (up to 13) and migration of bands on SDS–PAGE varied for LSP from different livers, although several major bands were common to all LSP preparations (Fig. 1). KP prepared from the same patient as LSP shared some, but not all, bands with LSP (Fig. 1).

Sepharose 4B chromatography of delipidated LSP resulted in three peaks (Fig. 2). The first peak eluted in the void volume and represented incompletely delipidated or aggregated LSP. The second peak contained only trace amounts of lipid and was designated apo-LSP. The third peak presumably represented mixed lipid–detergent micelles. SDS–PAGE of apo-LSP showed a major band at a position corresponding to a molecular weight of approximately 45,000 to 50,000 daltons. This band was much stronger than the corresponding band of LSP, when the same amounts (30 μg) of LSP and apo-LSP were applied to the gel. This band was not seen when apo-KP (prepared by the same method as apo-LSP) was electrophoresed even when 135 μg were loaded onto the gel.

Electron microscopy of LSP after negative staining revealed numerous smooth vesicles ranging in diameter from 40 nm to 1·6 μm, lipid globules, membrane fragments and fine granules (Fig. 3). Nuclei, mitochondria and rough endoplasmic reticulum were not seen. KP had a similar appearance while SP showed only lipid globules under the electron microscope.

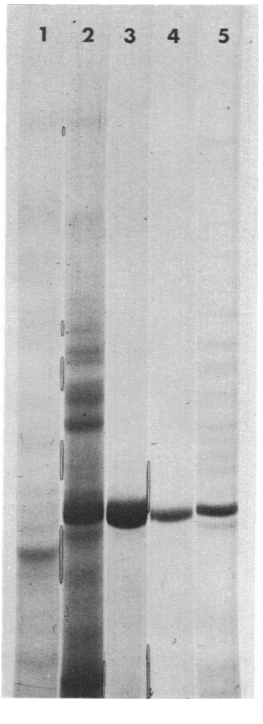

Fig. 1. SDS polyacrylamide gel electrophoresis of (1) 150 μg kidney lipoprotein, (2) 110 μg LSP (liver A), (3) 30 μg apo-LSP (liver B), (4) 30 μg LSP (liver B), and (5) 50 μg LSP (liver C).

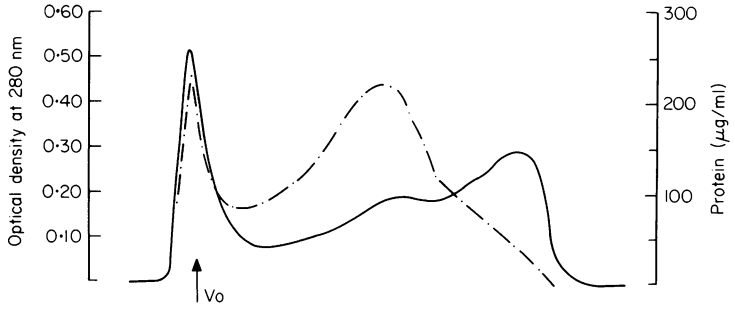

Fig. 2. Gel filtration of delipidated LSP on Sepharose 4B. The second protein peak represents apo-LSP. (———) Optical density, (– · – · –) protein concentration.

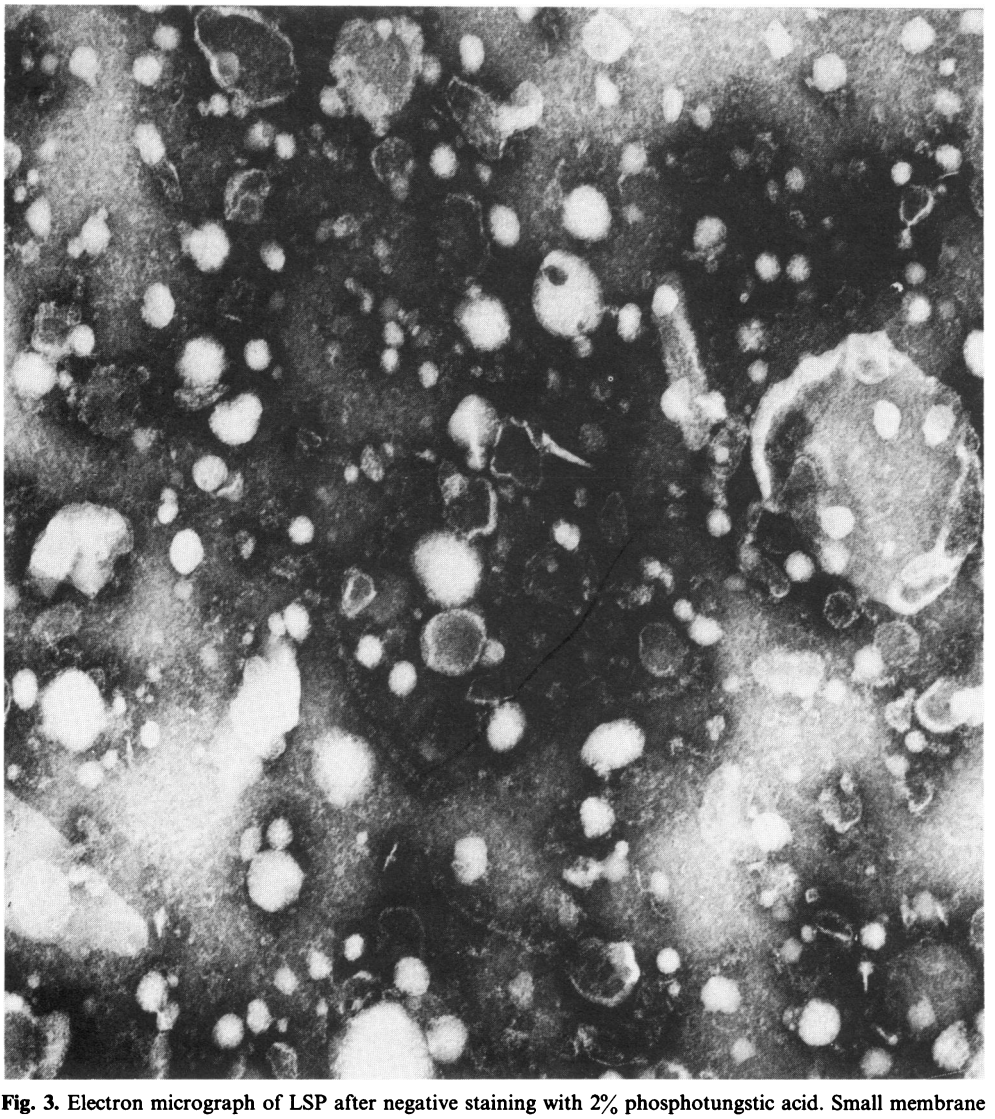

Fig. 3. Electron micrograph of LSP after negative staining with 2% phosphotungstic acid. Small membrane fragments, numerous smooth vesicles of different sizes and lipid globules are seen. (Original × 41,000.)

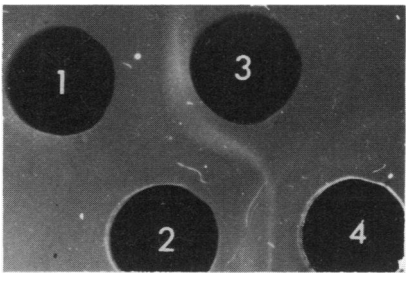

Fig. 4. Agarose gel immunodiffusion using human apo-LSP in well 1, human LSP in well 2, our rabbit anti-LSP in well 3 and sheep anti-LSP (from K.-H. Meyer zum Büschenfelde) in well 4.

Characterization and purification of anti-LSP

Rabbit antisera to LSP were absorbed with normal human plasma linked to Sepharose 4B. On agarose gel double immunodiffusion the absorbed antiserum showed a line of identity with sheep and guinea-pig anti-LSP (kindly provided by Drs K.-H. Meyer zum Büschenfelde, Berlin, and I. G. McFarlane, London, respectively) when incubated with human LSP (Fig. 4). Thus the rabbit antiserum was operationally defined as anti-LSP. This antiserum reacted with apo-LSP by immunodiffusion (Fig. 4), but it did not react with normal human serum, albumin, SP, rabbit LSP or fibronectin. The rabbit antiserum gave two precipitin lines when incubated with some preparations of human LSP and apo-LSP, but not with others. In addition, it gave a line of

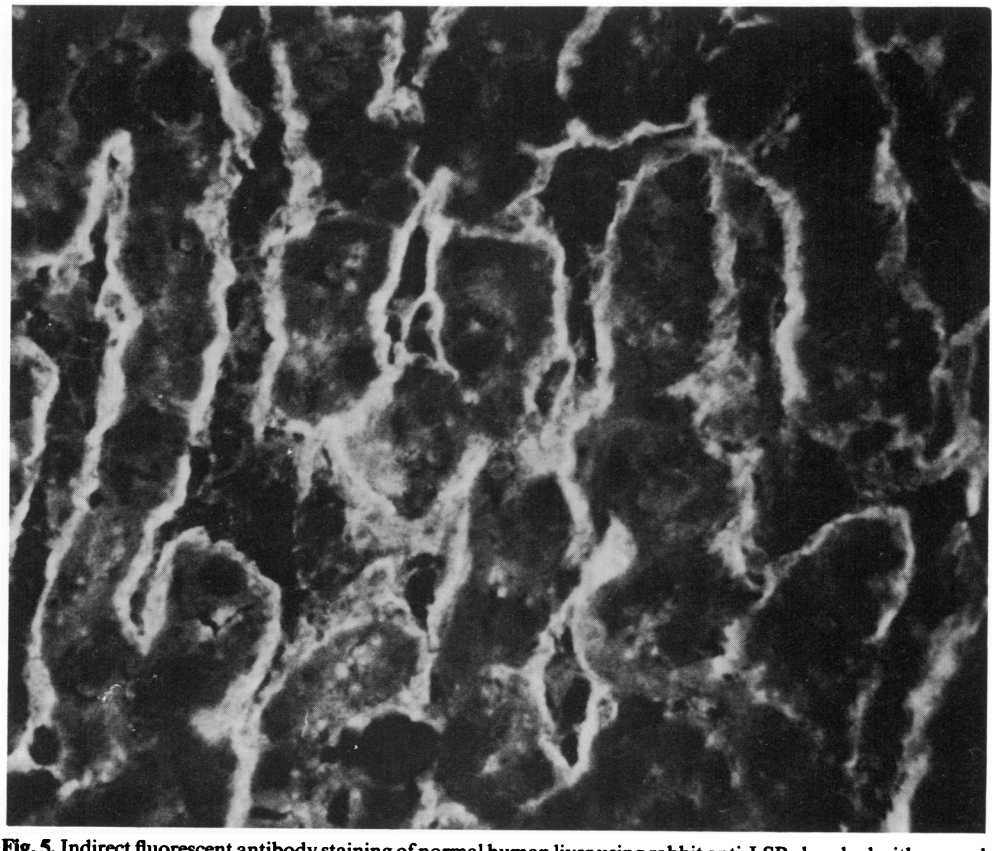

Fig. 5. Indirect fluorescent antibody staining of normal human liver using rabbit anti-LSP absorbed with normal human plasma only. The linear staining of the sinusoidal border of hepatocytes is due to an organ-non-specific antigen unrelated to LSP. (Original × 250.)

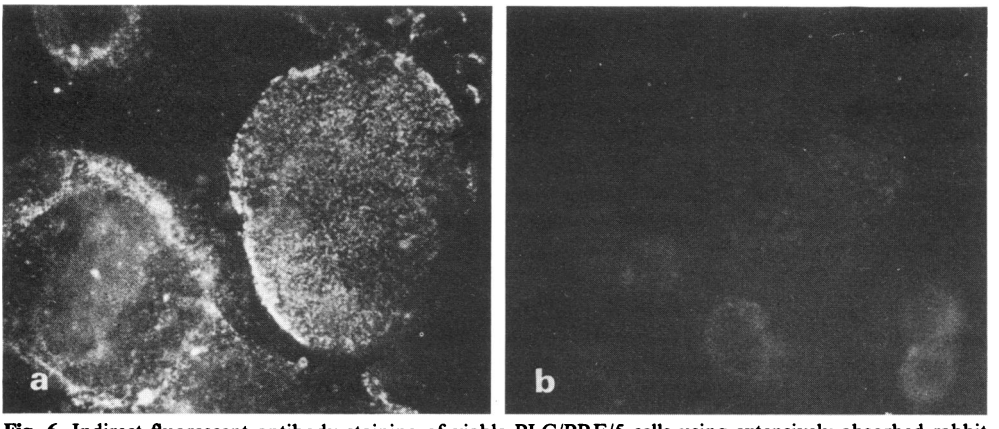

Fig. 6. Indirect fluorescent antibody staining of viable PLC/PRF/5 cells using extensively absorbed rabbit anti-LSP. The surface of the tumour cells shows fine granular staining in (a) which is abolished by absorption of the antiserum with apo-LSP in (b). (a) Anti-LSP absorbed with kidney apoprotein; (b) anti-LSP absorbed with apo-LSP. (Original × 400.)

non-identity with KP when this fraction was used at a protein concentration of 0·7 mg/ml or higher. Therefore, anti-LSP was absorbed repeatedly with KP linked to Sepharose 4B. On indirect immunofluorescence of frozen sections, the absorbed antiserum showed linear staining along the sinusoidal border of hepatocytes (Fig. 5) and of renal interstitium. The surface of viable human fibroblasts (WI 38 and IMR 90) showed a string-like staining pattern resembling the reaction with antiserum to fibronectin. However, absorption studies with fibronectin revealed that the reaction was not due to contaminating antibody to fibronectin. Therefore, we absorbed anti-LSP repeatedly with viable HeLa cells in suspension and insolubilized lung and spleen preparations.

Immunohistochemical demonstration of LSP

The extensively absorbed rabbit anti-LSP bound to the surface of viable PLC/PRF/5 cells in a finely granular pattern (Fig. 6a). Incubation of the tumour cells with preimmune rabbit serum or PBS followed by fluorescein-conjugated sheep anti-rabbit antiserum did not result in any staining. Anti-LSP did not react with sections of normal human liver or kidney, with the cytoplasm or nuclei of fixed PLC/PRF/5 cells after permeabilization of the plasma membrane, or with viable human hepatoblastoma cells SK-H-MA, Chang cells, HeLa cells, acute lymphocytic leukaemia cells RPMI 8402, human embryonic fibroblasts WI 38 and IMR 90 or Rhesus monkey kidney cells. The surface staining of the PLC/PRF/5 cells was abolished by absorption of anti-LSP with LSP or apo-LSP (Fig. 6), but not by KP or apo-KP using the same antibody/antigen ratios and protein concentrations. Furthermore, the staining was eliminated by incubation of anti-LSP with PLC/PRF/5 cells in suspension, but not when an equal number of viable Rhesus monkey kidney cells were used. Sequential studies of PLC/PRF/5 cells with anti-LSP revealed that LSP was present on the surface of virtually all tumour cells as early as 2 days and as late as 16 days after subculture when the cells had reached confluence.

DISCUSSION

The findings reported here indicated that LSP prepared by standard methods (McFarlane *et al.*, 1977) from different human livers showed significant variations in terms of quantitative yield, lipid/protein ratio and components as revealed by SDS–PAGE. It appears unlikely that the 10-fold variation in yield of LSP from different livers was only related to the time elapsed between death and

removal of the organ since the delay did not vary more than 2 and 3 hr for the five livers which were processed. We suggest that preparations of LSP derived from different livers contain varying amounts of constituents unrelated to LSP, including organ-non-specific antigens. The level of contamination of a given LSP preparation by shared antigens may account for the variations described here. For this reason it may be difficult to distinguish LSP from the corresponding kidney fraction prepared by the same method (Behrens & Paronetto, 1979). Our ultrastructural studies showed that LSP consisted of membrane fragments. The smooth vesicles observed under the electron microscope either may represent microsomes or may have been formed spontaneously from isolated plasma membrane fragments. Clearly, a preparation of whole membranes must contain multiple molecular species. This was confirmed by SDS–PAGE of LSP which revealed multiple bands as previously reported by McFarlane *et al.* (1977). Delipidation of LSP and re-chromatography on Sepharose 4B appeared to result in relative enrichment of apo-LSP. This preparation showed antigenic identity with LSP as determined by immunodiffusion. Apo-LSP still showed several bands on SDS–PAGE, but delipidation may be a useful step in the purification of LSP. Apo-LSP was different from apo-KP in antigenic reactivity and migration characteristics in SDS-PAGE. Apo-LSP may be superior to LSP in investigations of the role of an autoaggressive hepatocytotoxic immune response in patients with liver disease.

Xenogeneic antisera prepared by injection of LSP contained multiple populations of antibodies to a variety of antigens including organ-non-specific proteins. Our rabbit antiserum showed a precipitin line of identity on immunodiffusion with antisera to human LSP obtained from two highly experienced investigators. This indicated that we are dealing with the same antigen–antibody system. The rabbit antiserum reacted with the species-specific determinant of LSP as has been reported after short-term immunization of rabbits with LSP (Manns *et al.*, 1980b). Extensive absorption of the antiserum was required to obtain an antibody with selective reactivity with the hepatocellular carcinoma cell line PLC/PRF/5 (Alexander *et al.*, 1976). Because long-term cultures of adult human hepatocytes are not readily available, we chose this cell line as a substrate which shows functional and morphological characteristics of differentiated hepatocytes (Knowles, Howe & Aden, 1980; Gerber *et al.*, 1981). A surface antigen, presumably LSP, was detected on the PLC/PRF/5 cells, but not on a variety of cultured cells from different sources. While this manuscript was in preparation, Chisari *et al.* (1981) reported that PLC/PRF/5 cells expressed LSP on the cell surface. LSP was absent from Chang cells as has been described previously for some strains of Chang cells but not for others (Mutchnick, Kawanishi & Hopf, 1978; Hütteroth & Meyer zum Büschenfelde, 1978). The organ-specificity of LSP (Meyer zum Büschenfelde & Miescher, 1972; McFarlane *et al.*, 1977; Kakumu *et al.*, 1979; Manns *et al.*, 1980a; Ortona *et al.*, 1980) was supported by absorption studies using equivalent LSP preparations and apoproteins from liver and kidney.

It is of interest that the extensively absorbed anti-LSP did not react with frozen sections of normal human liver (and kidney). Linear staining along the sinusoidal border of hepatocytes seen prior to absorption did not represent LSP, but an organ-non-specific antigen probably derived from mesenchymal cells. Cultured human fibroblasts showed similar staining, and absorption of anti-LSP with medium conditioned by fibroblasts eliminated the reaction with liver sections and cultured fibroblasts, but not with PLC/PRF/5 cells. The amount of LSP exposed on the plasma membrane in sections of hepatocytes is probably too small to be detected by our antiserum. The presence of LSP on PLC/PRF/5 cells suggests that these cells or conditioned medium may serve as a source of homogeneous LSP for further characterization and purification.

Part of this work was done during a course by Nathan Lebwohl who is a medical student at Harvard Medical School, Boston. Dr Gerber is a recipient of Research Career Development Award AI00035 from the US Public Health Service. We are grateful to Dr S. Z. Hirschman, Dr A. Sun, Dr S. Waxman and E. Garfinkel, The Mount Sinai School of Medicine, New York, for supplying the cultured cells used in this study. We wish to thank Dr K.-H. Meyer zum Büschenfelde, Berlin, and Dr I. G. McFarlane, London, for the generous gifts of sheep and guinea-pig antisera to human LSP, respectively. Dr S. Puszkin, The Mount Sinai School of Medicine, New York, kindly provided purified fibronectin and rabbit antiserum to fibronectin. We wish to thank Mr N. Katz for photographic assistance and Mr T. Panagiotatos for technical assistance.

REFERENCES

ALEXANDER, J., BEY, E., GEDDES, E.W. & LECATSAS, G. (1976) Establishment of a continuously growing cell line from primary carcinoma of the liver. *S. Afr. Med. J.* **50,** 2124.

BEHRENS, U.J. & PARONETTO, F. (1979) Studies on 'liver-specific' antigens. I. Evaluation of the liver specificity of 'LSP' and 'LP-2'. *Gastroenterology*, **77,** 1045.

CHISARI, F.V. (1980) Liver-specific protein in perspective. *Gastroenterology*, **78,** 168.

CHISARI, F.V., BIEBER, M.S., JOSEPHO, C.A., XAVIER, C. & ANDERSON, D.S. (1981) Functional properties of lymphocyte subpopulations in hepatitis B virus infection. *J. Immunol.* **126,** 45.

FRINGS, C.S. & DUNN, R.T. (1970) A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *Am. J. clin. Pathol.* **53,** 89.

GERBER, M.A., GARFINKEL, E., HIRSCHMAN, S.Z., THUNG, S.N. & PANAGIOTATOS, T. (1981) Immune and enzyme histochemical studies of a human hepatocellular carcinoma cell line producing hepatitis B surface antigen. *J. Immunol.* **126,** 1085.

GERBER, M.A., LEBWOHL, N., THUNG, S.N., BODENHEIMER, H. & SCHAFFNER, F. (1979) Antibodies to liver-specific membrane protein in liver and kidney disease. *Gastroenterology*, **76,** 1281 (abstract).

HELENIUS, A. & SIMONS, K. (1971) Removal of lipids from human plasma low-density lipoprotein by detergents. *Biochemistry*, **10,** 2542.

HIRSCHMAN, S.Z., GERBER, M.A. & GARFINKEL. E. (1974) Purification of naked intranuclear particles from human liver infected by hepatitis B virus. *Proc. Natl. Acad. Sci. USA*, **71,** 3345.

HÜTTEROTH, T.H. & MEYER ZUM BÜSCHENFELDE, K.-H. (1978) Antigenic relationship between Chang liver cells and human hepatocytes. *Klin. Wochenschr.* **56,** 525.

JENSEN, D.M., MCFARLANE, I.G., NICHOLSON, A., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1978a) The development of a radioimmunoassay for the detection of antibodies to a liver-specific membrane lipoprotein (LSP). *J. clin. Lab. Immunol.* **1,** 31.

JENSEN, D.M., MCFARLANE, I.G., PORTMANN, B.S., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1978b) Detection of antibodies directed against a liver-specific membrane lipoprotein in patients with acute and chronic active hepatitis. *N. Engl. J. Med.* **299,** 1.

KAKUMU, S., ARAKAWA, Y., GOJI, H., KASHIO, T. & YATA, K. (1979) Occurrence and significance of antibody to liver-specific membrane lipoprotein in sera of patients with liver diseases. *Gastroenterology*, **76,** 665.

KNOWLES, B.B., HOWE, C.C. & ADEN, D.P. (1980) Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*, **209,** 497.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurements with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

MCFARLANE, I.G., WOJCICKA, B.M. & WILLIAMS, R. (1980) Antigens of the human liver. *Clin. exp. Immunol.* **40,** 1.

MCFARLANE, I.G., WOJCICKA, B.M., ZUCKER, G.M., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1977) Purification and characterization of human liver-specific membrane lipoprotein (LSP). *Clin. exp. Immunol.* **27,** 381.

MANNS, M., MEYER ZUM BÜSCHENFELDE, K.-H., HÜTTEROTH, T.H. & HESS, G. (1980a) Detection and characterization of liver membrane autoantibodies in chronic active hepatitis by a solid-phase radioimmunoassay. *Clin. exp. Immunol.* **42,** 263.

MANNS, M., MEYER ZUM BÜSCHENFELDE, K.-H., HÜTTEROTH, T.H. & HOPF, U. (1980b) The liver specific protein: evidence for species-specific and non-species-specific determinants *J. clin. Lab. Immunol.* **3,** 9.

MEYER ZUM BÜSCHENFELDE, K.-H. & MIESCHER, P.A. (1972) Liver specific antigens. Purification and characterization. *Clin. exp. Immunol.* **10,** 89.

MUTCHNICK, M.G., KAWANISHI, H. & HOPF, U. (1978) Absence of a liver-specific membrane protein in a strain of Chang cells. *Clin. Immunol. Immunopathol.* **10,** 398.

O'FARRELL, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. biol. Chem.* **250,** 4007.

ORTONA, L., LAGHI, V., CAUDA, R. & NERVO, P. (1980) Immune response to rabbit liver-specific lipoprotein in acute viral hepatitis. *Clin. exp. Immunol.* **42,** 436.

TSANTOULAS, D., PERPERAS, A., PORTMANN, B., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1980) Antibodies to a human liver membrane lipoprotein (LSP) in primary biliary cirrhosis. *Gut*, **21,** 557.

WILCHEK, M., BOCCHINI, B., BECKER, M. & GIVOL, D. (1971) A general method for the specific isolation of peptides containing modified residues, using insoluble antibody columns. *Biochemistry*, **10,** 2828.