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# Isolation and Characterization of a Cellular Protein-Lipid Complex from Ascites Fluid Caused by Various Neoplasms

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## ABSTRACT

High concentrations of lipids in ascites fluid caused by peritoneal carcinomatosis have been described recently. Since their nature has not yet been clarified, we isolated ascitic lipids from 25 patients with various neoplasms for further characterization. After chromatography on Sephadex G-100 gels, the ascitic lipids were fractionated on a Biogel A-5m column in three peaks. The second and third peaks were identified as low and high density lipoproteins, which were most likely of plasmatic origin, and represented the major amounts of ascitic lipids. The first peak was eluted in the void volume, indicating a molecular weight over 5 million. It consisted, on the average, of 65.3% protein, 16.2% triglycerides, 7.4% phospholipids, and 7.0% cholesterol. In a CsCl gradient, this protein-lipid complex floated in the density range from 1.128 to 1.181 g/ml. Sodium dodecyl sulfate: polyacrylamide gel electrophoresis separated up to 11 protein subunits ($M_r$ 29,000 to 97,000), and electron microscopy revealed globular particles of 36 to 64 nm in diameter. The macromolecular complex showed no immunological reaction against anti-$\alpha$- and anti-$\beta$-lipoproteins, but a single precipitation line against anti-liver-specific lipoprotein was seen.

The biochemical characteristics of this protein-lipid complex proved to have a close relationship to liver-specific lipoprotein. It is most likely derived from cell membranes of the peritoneum detached by carcinomatosis.

## INTRODUCTION

Ascitic lipids may form complexes with proteins, and considerable amounts of sodium deoxycholate (115 mg/mg protein) are necessary to separate cholesterol and triacylglycerol from the protein moiety by gel filtration (1). All density classes of lipoproteins corresponding to their plasmatic counterparts could be isolated from ascites fluid (2–5). Suzuki *et al.* (1) separated human ascites plasma LDL[2] by ultracentrifugation in two subclasses and determined quantitatively their relative protein and lipid composition. They differed from plasma LDL in that high contents of triacylglycerol of about 20% were found. Molecular weight and antigenic activities were similar to those of plasmatic lipoproteins. From corresponding electrophoretic patterns between Ehrlich ascites tumor and plasma apoproteins (2), it was concluded that ascitic lipoproteins may be derived from plasmatic lipoproteins (3) synthesized in the liver and intestine of the tumor-bearing host (4, 5). However, distinct differences to corresponding plasma lipoproteins have been observed by the same authors (4).

Recently high lipid concentrations have been described in tumor ascites fluid (6, 7). The nature and origin of these lipids have not yet been investigated. Therefore, it was the aim of the present study to isolate and to characterize lipids from ascitic fluid caused by various neoplasms.

## MATERIALS AND METHODS

**Patients.** We studied a total of 25 patients from 38 to 74 yr of age with peritoneal carcinomatosis and ascites fluid. The group consisted of three male patients with adenocarcinoma of the stomach, two patients each with gallbladder (female) and pancreas cancers (male), and nine female patients each with advanced carcinoma of the ovary (Stages III and IV; Fédération Internationale de Gynécologie et d'Obstétrique) and metastatic breast cancer ($T_3N_1M_{1a}$ to $T_4N_3M_{1b}$; International Union against Cancer). Diagnoses were confirmed by radiological, sonographic, endoscopic, computer tomographic, histological, and cytological methods or by autopsy.

Ascites fluid was collected from living patients before any chemotherapy was started. One patient with chylous and four patients with macroscopically hemorrhagic ascites were excluded from the study.

**Chemical Assays.** Protein was determined with the Biuret reagent (8), and microquantities for electrophoresis were measured by the method of Lowry *et al.* (9) after precipitation of the proteins with trichloroacetic acid.

Total cholesterol and triglyceride determinations were performed enzymatically with commercial test kits (Boehringer, Mannheim, Federal Republic of Germany) (10, 11).

Phospholipids were determined in chloroform:methanol extracts (3:1, v/v) by colorimetric phosphorous determination (12). Further fractionation was achieved by thin-layer chromatography on 20- x 20-cm glass plates coated with silica gel (Merck, Darmstadt, Federal Republic of Germany). Chloroform:methanol:ammonia (13:5:1, v/v/v) was used as solvent system. The separated phospholipids were made visible with iodine vapor and stained individually. Cardiolipin, phosphatidylcholine, sphingomyelin, and phosphatidylinositol served as reference substances.

The accuracy was tested by Precilip E. L. standard sera (Boehringer).

**Fractionation of Ascites Fluid.** The fractionation of ascitic fluid at 4°C was started within 1 h after the collection. Samples were passed through a Selecta No. 1117½ filter (Schleicher-Schüll, Dassel, Federal Republic of Germany) in order to remove cells and cellular debris of 5 to 10 $\mu$m in diameter. To avoid bacterial growth and enzymatic degradation, 0.2 g of sodium azide ($NaN_3$) and 0.05 mmol of PMSF in 50% aqueous isopropanol were added per 1000 ml of ascites fluid. The PMSF solution was always freshly prepared before it was added to the ascitic fluid, since premature inactivation occurred unless it was immediately brought into contact with proteases. Concentration to about one-tenth of the original volume was performed in a stirred cell (Amicon, Witten, Federal Republic of Germany; Models 402 and 52) using PM 10 membranes. Four-ml specimens were incubated at 20°C with 0.4 $\mu$Ci of ethanolic [1,2-$^3$H]cholesterol (New England Nuclear, Boston, MA), which is incorporated into protein:lipid complexes (13) and serves as an easily detect-

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[2] The abbreviations used are: LDL, low density lipoproteins; HDL, high density lipoproteins; VLDL, very low density lipoproteins; LSP, liver-specific lipoprotein; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

able, highly sensitive, but not quantitative marker of the lipid moiety. Afterwards gel chromatography was performed on a 100- x 2.5-cm Sephadex G-100 column. The amounts applied to the column did not exceed 300 mg of protein, and the flow was less than 16 ml/h. The elution buffer contained 0.15 M NaCl, disodium EDTA (0.1 g/liter), $NaN_3$ (0.2 g/liter), and 0.05 mM PMSF, pH 7.0 (14). Usually seventy to eighty 8-ml fractions were collected. The absorbance at 280 nm was recorded with a spectrophotometer (Beckman, Fullerton, CA; Model 24). After addition of 5 ml of Bray scintillation solution to 0.1-ml aliquots of the fractions, the $\beta$-radiation emitted by [1,2-$^3$H]cholesterol was measured in a Betascint BF 5000 counter (BF-Vertriebs-GmbH, München, Federal Republic of Germany). All fractions containing [1,2-$^3$H]cholesterol were pooled and concentrated to 3 to 5 ml. After rechromatography on a 120- x 1.8-cm Biogel A-5m column, about fifty 8-ml fractions were collected. Their counts per minute and the absorbance at 280 nm were plotted graphically. The elution patterns revealed three peaks, each of which was pooled and concentrated to 1 to 3 ml. They were the basis of the following analysis.

After addition of $NaN_3$ and PMSF, the fractionated ascites fluid samples could be stored in a NaCl:EDTA buffer up to 6 wk at 4°C. Multiple freezing and thawing as well as lyophilization denatured the protein moiety and altered the immunological properties (15).

Recovery studies for triglycerides, cholesterol, and phospholipids revealed a loss of ascitic lipids of between 30 and 50% at the end of the fractionation procedure.

**Density Gradient Centrifugation.** One-half ml of the sample was added to 4.0 ml of CsCl solution (density, 1.070 g/ml). During 72 h of ultracentrifugation at 4°C and 50,000 rpm in a Beckman SW-60 rotor, a concave gradient from 1.025 to 1.300 g/ml was established (16). Eleven to twelve 0.4-ml fractions were obtained by pipeting from the top. Densities were calculated by weighing 0.2-ml portions. The absorbance at 280 nm and the counts per minute were measured in each fraction.

**Electrophoresis.** For SDS:polyacrylamide gel electrophoresis, aliquots of the samples were diluted (1:4, v/v) with 0.31 M Tris-HCl buffer, pH 6.8, containing SDS (2 g/dl), 2-mercaptoethanol (5 ml/dl), and glycerol (10 ml/dl) (17). After incubation for 5 to 10 min at 100°C and centrifugation for 2 min at 2000 rpm afterwards, the protein subunits were separated on discontinuous vertical polyacrylamide gels (T = 3 and 10%; C = 2.6%) at 20°C. The proteins were stained for 1 h with Coomassie Blue R 250. Molecular weights were estimated by comparison with calibration standards (Pharmacia, Freiburg, Federal Republic of Germany) according to the method of Weber and Osborn (18). Densitometric scans were performed with an Elscript 3 densitometer (Hirschmann, Unterhaching, Federal Republic of Germany).

**Electron Microscopy.** Electron micrographs were taken with a Siemens 101 electron microscope at 80 kV.

The isolated protein:lipid complex was negatively stained with 2% potassium phosphotungstate, pH 7.0 (19). A small drop of the suspension was placed on a 200 or 300 Formavar carbon-coated grid and examined after drying at room temperature.

**Immunodiffusion.** Ouchterlony immunodiffusion against anti-LSP was performed in agarose (0.8 g/dl) in 0.01 M sodium phosphate-buffered NaCl solution, pH 8.6, with and without SDS (0.5 to 1.0 g/dl). The plates were incubated 24 to 48 h at 30°C. All other immunodiffusion studies took place in Bacto-agar (2 g/dl; 0.02 M sodium diethylbarbiturate:0.03 M sodium acetate), pH 8.6, in the absence of SDS (20). The staining was done with Amidoblack-10 B and Oilred O (Sigma, München, Federal Republic of Germany). We tested against industrially produced and purified antisera against human serum, human serum albumin, and $\alpha$- and $\beta$-lipoproteins (Behring, Marburg, Federal Republic of Germany) identical to apo-HDL and apo-LDL, respectively. Special antibodies against cellular lipoproteins of the liver (anti-LSP) were made available by U. Behrens, Veterans Administration Medical Center, Bronx, NY. LSP was isolated 4 to 8 h postmortem from normal human liver that showed neither autolysis nor any other significant histological changes (21). Preparation was carried out at 4°C, observing exactly the instructions of McFarlane *et al.* (15). Antisera against the purified antigens were produced in rabbits by repeated injections and absorbed with lyophilized human plasma and tissue extracts at 37°C for 30 min (21). Organ specificity was tested by double immunodiffusion, indirect immunofluorescence, and immunoenzyme techniques.

## RESULTS

After the determination of the protein and lipid concentrations in ascites fluid (Table 1), the lower molecular proteins (Fig. 1, *second peak*) were separated from protein-bound lipids (Fig. 1, *first peak*) by gel filtration on a Sephadex G-100 column. The elution profile showed rising absorbance at 280 nm coinciding with elevated counts per minute in the first peak and high protein absorbance without any evidence of radioactivity in the second peak.

The calibration of the columns was performed with plasma lipoproteins isolated by ultracentrifugation (22, 23). On Biogel A-5m, VLDL eluted in the void volume, LDL represented a second lower molecular peak, and HDL formed a third (Fig. 2*a*). Only a slight overlap between LDL and HDL was detected by immunodiffusion.

Rechromatography of the pooled and concentrated ascitic protein:lipid fractions (Fig. 1, *first peak*) on the Biogel A-5m column and recording the absorbance and the counts per minute revealed three peaks resembling the calibration curve (Fig. 2*b*). The first appeared in the void volume, indicating a molecular weight of more than 5 million, compatible with very low density lipoproteins. However, immunodiffusion studies were negative against anti-$\alpha$- and anti-$\beta$-lipoproteins, suggesting that the pres-

Table 1

*Ranges and median values of lipid and protein concentrations in ascites fluid listed in categories of neoplasms*

| | Carcinoma of the | | |
|---|---|---|---|
| | Gastrointestinal tract ($n$ = 7)[a] | Ovary ($n$ = 9) | Breast ($n$ = 9) |
| Triglycerides (mg/dl) | 46.7–105.5 (60.3)[b] | 36.0–104.1 (75.4) | 44.5–124.6 (73.8) |
| Total cholesterol (mg/dl) | 47.3–139.2 (67.0) | 66.6–140.8 (131.0) | 51.1–214.9 (84.9) |
| Phospholipids (mg/dl) | 21.6–77.8 (62.4) | 24.6–103.2 (66.6) | 10.8–83.2 (55.4) |
| Total protein (g/dl) | 1.6–7.4 (4.1) | 3.5–5.6 (4.4) | 2.7–6.0 (3.8) |

[a] Number of subjects.
[b] Numbers in parentheses, median.

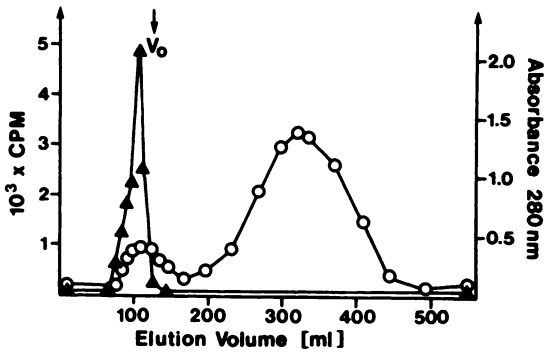


Fig. 1. Typical gel chromatographic elution profile of a concentrated ascites fluid sample from a patient with carcinoma of the stomach on Sephadex G-100. Column size, 100 x 2.5 cm. *Points* represent cpm emitted by [1,2-$^3$H]cholesterol (▲) and the absorbance at 280 nm (○) recorded in each collected fraction. $V_o$, void volume.

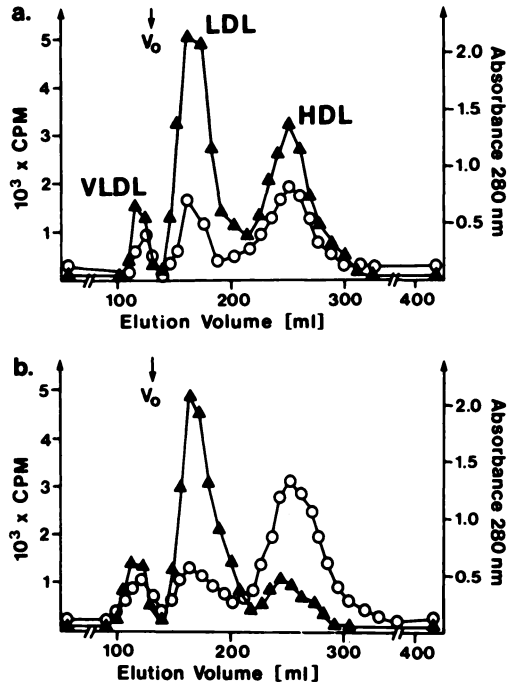


Fig. 2. *a*, calibration curve of the Biogel A-5m column with ultracentrifugally isolated lipoproteins. *b*, typical gel chromatographic elution profile of the pooled and concentrated ascitic lipid:protein peak (Fig. 1) from a patient with carcinoma of the stomach on Biogel A-5m. Column size, 120 x 1.8 cm. *Points* represent cpm emitted by [1,2-$^3$H]cholesterol (▲) and the absorbance at 280 nm (○) in each collected fraction. $V_o$, void volume.

Table 2

*Ranges and median values of the percental lipid and protein composition of the isolated ascitic protein-lipid complex listed in categories of neoplasms*

| % | Carcinoma of the | | |
|---|---|---|---|
| | Gastrointestinal tract ($n$ = 7)[a] | Ovary ($n$ = 9) | Breast ($n$ = 9) |
| Triglycerides | 12.9–21.0 (15.7)[b] | 16.4–23.1 (18.1) | 12.8–20.9 (15.0) |
| Total cholesterol | 5.3–13.0 (7.2) | 1.8–8.2 (3.2) | 5.9–14.5 (9.2) |
| Phospholipids | 9.1–14.9 (9.8) | 7.9–14.3 (8.5) | 2.9–11.4 (3.2) |
| CL[c] | 3.9 | 1.2 | 2.3 |
| PE | 29.1 | 13.8 | 17.2 |
| PC | 12.2 | 30.8 | 34.3 |
| PI | 21.5 | 17.3 | 11.5 |
| SM | 26.9 | 32.6 | 29.8 |
| Total protein | 61.3–68.4 (65.1) | 59.3–67.8 (64.1) | 62.8–69.7 (66.5) |

[a] Number of subjects.
[b] Numbers in parentheses, median.
[c] CL, cardiolipin; PE, ethanolamine glycerophospholipids; PC, phosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin.

ence of plasma VLDL was unlikely.

The chemical analysis of the pooled and concentrated first peak fractions showed the presence of 65.3% protein, 16.2% triglycerides, 7.4% phospholipids, and 7.0% cholesterol (median values). The relative composition of these components listed in categories of neoplasms is illustrated in Table 2. The phospholipid moiety was further fractionated by quantitative thin-layer chromatography in cardiolipin, ethanolamine glycerophospholipids, (lyso-)phosphatidylcholine, phosphatidylinositol, and sphingomyelin.

An average density of 1.128 to 1.181 g/ml was determined by CsCl gradient ultracentrifugation for the macromolecular protein:lipid complex (Fig. 3).

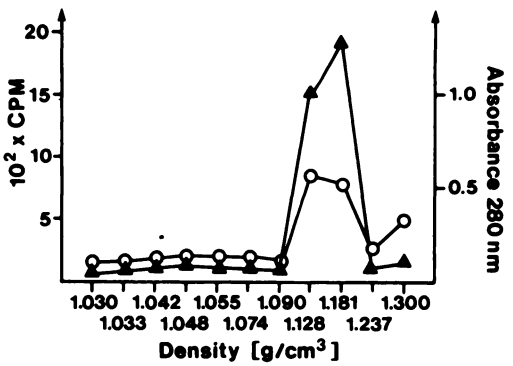


Fig. 3. Ultracentrifugal density determination of the isolated ascitic protein-lipid complex from a patient with carcinoma of the gallbladder in a CsCl gradient (50,000 rpm for 72 h). *Points* represent cpm emitted by [1,2-$^3$H]cholesterol (▲) and the absorbance at 280 nm (○) recorded in each fraction pipeted from the top.

For further characterization, proteins were separated by SDS:PAGE, since the macromolecular complex did not penetrate the gel completely in the absence of SDS. Up to 11 protein subunits in a molecular weight range of 29,000 to 97,000 were detected (Fig. 4). *Band 5* showed identical mobility to human serum albumin and was reduced by ultracentrifugation. The main difference between various ascites fluid samples was varying concentrations of corresponding subunits determined semiquantitatively by densitometric scans. No protein band specific for certain types of carcinoma could be observed. Electron microscopy revealed lipoprotein-like globular macromolecules with diameters of 36 to 64 nm (Fig. 5). An average molecular weight of approximately 45 million was calculated.

In 21 of the 25 specimens there was a single precipitation line against anti-LSP, suggesting identical antigenic determinants of the macromolecular protein:lipid complex and cell membrane-associated LSP (Fig. 6a). In the second peak, double radial immunodiffusion showed precipitation lines against antiapo-LDL. In the third peak a positive reaction with antiapo-HDL was seen (Fig. 6, *b* and *c*). Table 3 presents the median values and ranges of the percental lipid distribution on the three gel chromatographically separated peaks. The major amount of ascitic lipids was found as low and high density lipoproteins.

## DISCUSSION

The origin of high lipid concentrations in ascites fluid of patients with neoplastic processes has not yet been clarified.

In our study the predominant part of ascitic lipids was recovered as low and high density lipoproteins. On Biogel A-5m columns, 61.1% of cholesterol, 49.7% of triglycerides, and 47.0% of phospholipids were eluted in the LDL fraction, and 21.9% of cholesterol, 29.5% of triglycerides, and 32.8% of phospholipids appeared in the HDL molecular weight range (median values). Antigenic identity with plasmatic apo-LDL and apo-HDL was confirmed by Ouchterlony immunodiffusion. In accordance with these findings, protein-associated ascitic lipids similar to plasma lipoproteins have been described by Mathur and Spector (2) and Malmendier (4). Abnormal capillary permeability is the main process of ascites fluid formation in peritoneal carcinomatosis (24). Additional mechanisms are obstruction of draining lymphatics or venous blockade. In this way plasma

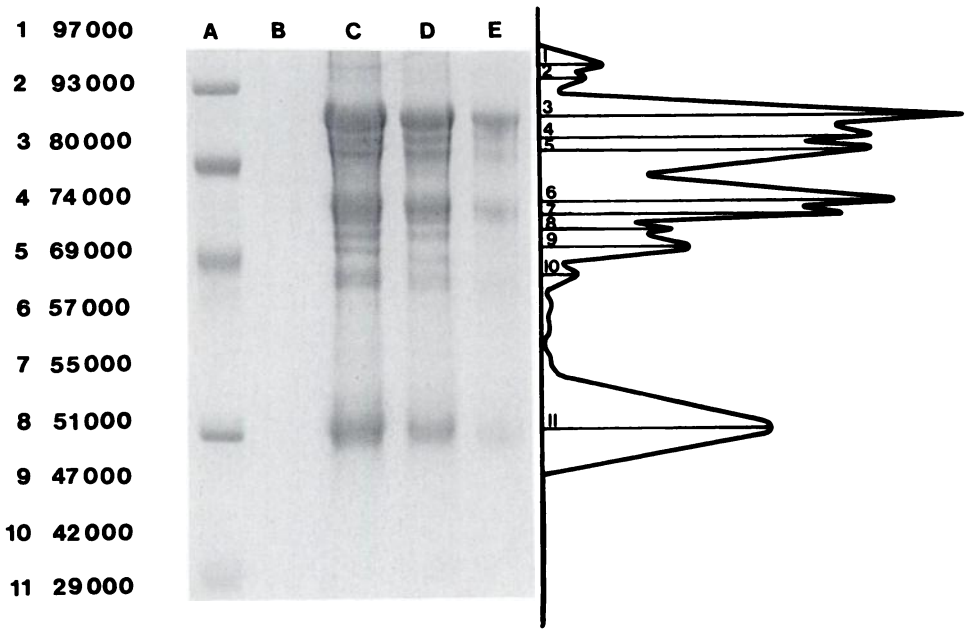


Fig. 4. SDS:PAGE of the isolated macromolecular ascitic protein-lipid complex from a patient with carcinoma of the pancreas in 3 different sample concentrations (*lanes C* to *E*) on a 1.5-mm discontinuous polyacrylamide gel (C = 2.6%; T = 3 and 10%). The standard calibration kit (phosphorylase *b*, $M_r$ 94,000; albumin, $M_r$ 67,000; ovalbumin, $M_r$ 43,000; carbonic anhydrase, $M_r$ 30,000; and trypsin inhibitor, $M_r$ 20,100) is shown in *Lane A. Left*, molecular weights of subunits; *right*, semiquantitative densitometric scan.

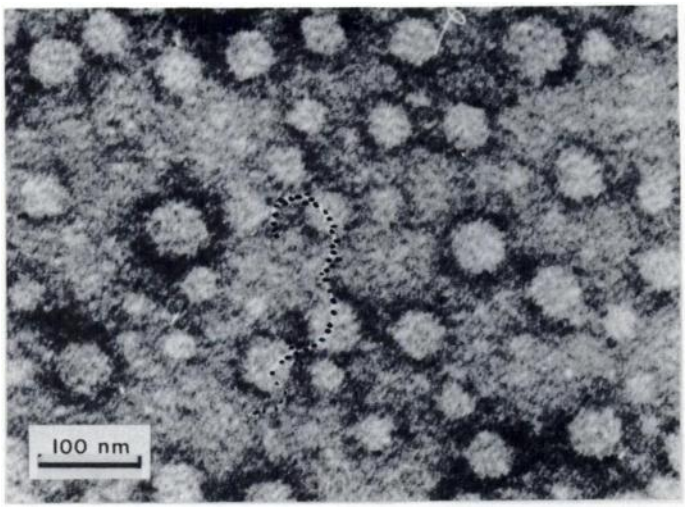


Fig. 5. Electron microscopy of the isolated macromolecular ascitic protein-lipid complex from a patient with carcinoma of the breast using a Siemens 101 electron microscope and a negative staining technique with 2% potassium phosphotungstate, pH 7.0. × 140,000.

lipoproteins originating in the liver and intestine may be precursors of part of the ascitic lipoproteins (4, 5).

In addition to lipoproteins which were most likely of plasmatic origin, we isolated a macromolecular protein-lipid complex resembling VLDL with respect to its molecular weight of about 45 million. However, in immunodiffusion studies, there was no proof of anti-$\alpha$- and anti-$\beta$-lipoproteins. Identity with plasmatic very low density lipoproteins could be excluded. The complex consisted of 59.3 to 69.7% protein, 12.8 to 23.1% triglycerides, 2.9 to 14.9% phospholipids, and 1.8 to 14.5% cholesterol. Its varying chemical composition is most likely caused by different extents of partial delipidation during the isolation procedure. Most of the lipids were lost during the ultrafiltration, probably due to adherence or direct passage of free molecules originating from the ascitic protein:lipid complex through the PM 10 membrane. The permeation of the intact complex is not likely because of its size. Less than 5% of lipids was retained in the included volume of the gel chromatography columns. Ascites fluid filtration with a Selecta No. 1117½ filter did not have a significant influence on the lipid recovery of 50 to 70%.

In accordance with the high protein content shown by CsCl gradient ultracentrifugation, the ascitic protein-lipid complex floated in the density range of 1.128 to 1.181 g/ml. SDS:PAGE revealed up to 11 protein subunits. One of them was present in about 40% of the samples and could be identified as albumin. It was looked upon as a nonspecific contaminant, for its concentration was reduced considerably by ultracentrifugation. The first peak electrophoretic patterns resembled each other regardless of the underlying disease. The main differences were varying concentrations of certain components. The total absence of single bands in some specimens was probably caused by quantities below the detection limit. No subunits specific for certain types of carcinomas were found. The possible reason is the general peritoneal carcinomatosis common to every patient of the study.

The macromolecular protein-lipid complex showed a single precipitation line with anti-LSP. Twenty-one of 25 ascites fluid samples reacted immunologically with anti-LSP. This might be explained by the lability of the protein:lipid complex (15) or by concentrations not detectable by the methods applied.

LSP is present in the 105,000 × *g* supernatant of human liver homogenates. Using molecular sieving techniques, a molecular weight of more than 4 million was determined. The existence of higher molecular aggregates could not be excluded (15). In the ultracentrifugation gradient, LSP floated in a density range of 1.107 to 1.188 g/ml (21). Protein analysis by SDS:PAGE revealed 8 to 13 subunits ranging from 40,000 to 96,000. One of them showed the same electrophoretic mobility as albumin. Its concentration could be diminished by rechromatography but was not eliminated completely. Most likely it is not part of the apopro-

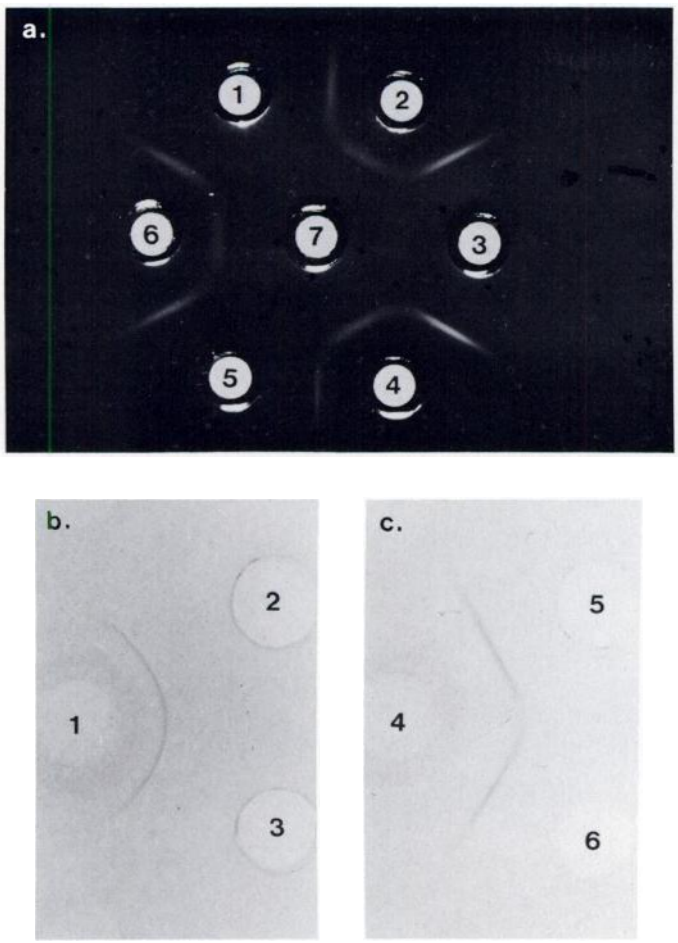


Fig. 6. *a*, immunodiffusion of the gel chromatographically isolated ascitic protein-lipid complex from a patient with carcinoma of the ovary (*Wells 2*, *4*, and *6*) in agarose (0.8 g/dl) in 0.01 M sodium phosphate-buffered NaCl solution, pH 8.6, with SDS (0.5 to 1.0 g/dl) against anti-LSP (*Wells 1*, *3*, *5*, and *7*). *b*, immunodiffusion of the gel chromatographically isolated, pooled, and concentrated second peak fractions (Fig. 2*b*) from a patient with carcinoma of the ovary (*Well 1*) against antiapo-LDL (*Wells 2* and *3*); and *c*, that of the pooled and concentrated third peak fractions (Fig. 2*b*) from a patient with carcinoma of the ovary (*Well 4*) against antiapo-HDL (*Wells 5* and *6*). Immunodiffusion was performed in Bacto-agar (0.2 g/dl), 0.02 M sodium diethylbarbiturate, and 0.03 M sodium acetate, pH 8.6. The lipoproteins were stained with Amidoblack-10B and Oilred 0 (*b* and *c*).

Table 3

*Ranges and median values of the percental lipid distribution on the three gel chromatographically fractionated peaks on Biogel A-5m (n = 25 patients)*

| % | Macromolecular protein-lipid fraction (Peak 1) | LDL fraction (Peak 2) | HDL fraction (Peak 3) |
|---|---|---|---|
| Total cholesterol | 2.3–30.9 (11.4)[a] | 32.1–79.9 (61.1) | 11.2–43.9 (21.9) |
| Triglycerides | 1.7–37.5 (24.2) | 32.4–81.3 (49.7) | 17.4–43.5 (29.5) |
| Phospholipids | 1.5–28.7 (12.4) | 29.2–79.7 (47.0) | 13.0–47.6 (32.8) |

[a] Numbers in parentheses, median.

tein (15). In contrast to former opinions (25), LSP was found not to be organ specific. Behrens and Paronetto (21) produced anti-LSP sera in rabbits which reacted with membranes of liver cells and also of intestine, smooth muscle, spleen, and kidney. These findings were supported by Murakami *et al.* (26) who were not able to establish a cell line producing monoclonal antibodies exclusively against liver cell membranes. Indirect immunofluorescence and immunoenzyme light microscopy on cryostat sections allowed the identification of LSP as a part of the surface membrane of the cell (21, 27).

Considering the many similarities despite the different isolation procedures and method-related inaccuracies, a close relationship of the ascitic protein:lipid complex and LSP is very likely. The presence of LSP in ascites fluid could be caused by peritoneal carcinomatosis, which led to accumulation of cell membrane proteolipids in the peritoneal effusion. This is no proof that the macromolecular protein:lipid complex is especially associated with cancer. In other body fluids, comparable proteolipids of cellular origin have been described in the absence of neoplastic processes (28). However, in ascitic fluid of patients with chronic liver disease, this complex could not be found, possibly due to minor concentrations not detectable with the methods used.

In conclusion, major amounts of ascitic lipids were found as low and high density lipoproteins probably originating from plasma. However, macromolecular protein-lipid complexes derived from cell membranes of the peritoneum by carcinomatosis contribute to the total lipids in malignant ascites fluid.

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