Spur cells in patients with alcoholic liver cirrhosis are associated with reduced plasma levels of apoA-II, HDL_3, and LDL

Gilles Duhamel,[1] Patricia Forgez, Bertrand Nalpas,* Pierre Berthelot,* and M. John Chapman

Equipe de Recherche sur les Lipoprotéines, Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 9, Pavillon Benjamin Delessert, Hôpital La Pitié, 83, Boulevard de l'Hôpital, 75013 Paris, France, and Unité d'Hépatologie, Hôpital Laennec, 42, rue de Sèvres, 75007 Paris, France*

Abstract The precise nature and origin(s) of the abnormalities in lipoprotein and apolipoprotein profile associated with severe hepatic dysfunction and the presence of spur cells remain poorly defined. To shed light on this question, we have analyzed the plasma lipoprotein and apolipoprotein profiles in five patients with alcoholic cirrhosis and spur cells, and compared them with those of a group with similar hepatocellular dysfunction, but lacking spur cells, and with that of a control group. Lipoproteins were subfractionated by density gradient ultracentrifugation and their physicochemical properties were determined; apolipoprotein A-I, A-II, and B contents in plasma and the respective subfractions were quantitated by radial immunodiffusion, while the complement of low molecular weight apolipoproteins in each subfraction was analyzed by isoelectric focusing and electrophoresis in alkaline-urea polyacrylamide gels. Spur cell plasma was distinguished by reduced levels of apoA-II and elevated ratios of apoA-I/apoA-II (~13:1 as compared to 3.3–3.9:1 in the other two groups), and by reduced concentrations of HDL_3. Gradient fractionation showed the apoA-II content of HDL_3 to be dramatically and significantly diminished in spur cell plasma; in addition, apoA-II content was reduced relative to apoA-I in this subclass (4.7:1 as compared to 1:1 in cirrhotics lacking spur cells and 1.9:1 in controls). Spur cell HDL_2 was similarly deficient in apoA-II, with elevated ratios of apoA-I:apoA-II (9.8:1 in comparison with 1.9–2.5:1 in the two other groups). Nonetheless, high HDL_2 concentrations were seen in both series of cirrhotic patients, irrespective of red cell morphology. Spur cell HDL_2 thus appears to consist primarily of particles possessing only apoA-I, with a minor population containing both apoA-I and apoA-II. The free cholesterol content of all lipoprotein subfractions from spur cell plasma was increased, as indeed was the molar ratio of free cholesterol to phospholipid, in comparison with that of corresponding fractions from alcoholic cirrhotics lacking spur cells and of control subjects. LDL levels were reduced in spur cell plasma, thereby distinguishing this group from the cirrhotics without spur cells who displayed elevated LDL levels. Markedly reduced plasma levels of apoA-II, HDL_3, and LDL appear characteristic of alcoholic cirrhotics presenting with spur cells. Our findings suggest that apoA-II may be essential to the normal function and metabolism of HDL, one aspect of which may be the transport of free cholesterol and thereby the direct or indirect maintenance of red cell morphology.—**Duhamel, G., P. Forgez, B. Nalpas, P. Berthelot, and M. J. Chapman.** Spur cells in patients with alcoholic liver cirrhosis are associated with reduced plasma levels of apoA-II, HDL_3, and LDL. *J. Lipid Res.* 1983. **24:** 1612–1625.

Supplementary key words apoA-I • apoB • free cholesterol • isopycnic density gradient ultracentrifugation • radial immunodiffusion • alcohol

Acanthocytes are red blood cells of markedly altered morphology characteristically found in individuals with the genetic disorder abetalipoproteinemia (1). Spur cells, which are morphologically similar but chemically distinct from acanthocytes, occasionally occur in patients with severe liver disease and notably in alcoholics with cirrhosis (2, 3). In alcoholics with this syndrome, the abnormal shape of the red blood cells is acquired and usually reversible (4, 5); this disorder is generally associated with a hemolytic anemia of mild to marked severity and premature destruction of red cells, which occurs predominantly in the spleen (2–4, 6).

Spur cells may be distinguished from acanthocytes typical of abetalipoproteinemia by the absolute and striking increase in membrane free cholesterol content of the spur cells, and by an elevated cholesterol:phospholipid ratio (7–11). Such abnormalities in lipid composition appear to result in an increase in the surface area of the cells (2–4). The fluidity of spur cell membrane lipid is dimin-

Abbreviations: VLDL, very low density lipoproteins, density as defined; IDL, intermediate density lipoproteins, density as defined; LDL, low density lipoproteins, density as defined; HDL, high density lipoproteins; HDL_2, a subclass of HDL of density 1.066–1.100 g/ml, unless otherwise defined; HDL_3, a subclass of HDL of density 1.100–1.140 g/ml, unless otherwise defined; VHDL, very high density lipoproteins of d > 1.154 g/ml; apo, apolipoprotein; EDTA, ethylenediaminetetraacetic acid; TMU, tetramethylurea; TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); IEF, isoelectric focusing.

[1] To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 18, 2012

ished, leading to a decrease in the ability of the cell to deform (5, 6, 12). This mechanism is similar to one that occurs in acanthocytes. Flamm and Schachter (13) have recently shown that the lipid fluidity of the outer membrane leaflet of both acanthocytic red cells and of normal cells enriched with cholesterol is significantly decreased.

Despite significant progress in understanding the physiopathology of severe hepatic dysfunction and the manner in which lipoprotein (VLDL, LDL, and HDL)-free cholesterol contributes to spur cell formation (7), we remain largely ignorant of the precise nature and origin(s) of abnormalities in the profile of plasma lipoproteins and apolipoproteins in patients with spur cells and advanced liver disease. Such data could provide new insight into the pathogenesis of the disorder, particularly in cases in which plasma lipoprotein abnormalities might result in modified surface interactions with the erythrocyte membrane. To this end, we have evaluated the quantitative and qualitative characteristics of the plasma lipoproteins and apolipoproteins in five patients with alcoholic cirrhosis and spur cells, and compared them with those of a group with similarly severe hepatocellular dysfunction, but who lacked spur cells. In this study, we applied a density gradient ultracentrifugal procedure (14) whose resolution and precision allow essentially quantitative isolation of VLDL, LDL, HDL_2, HDL_3, and intermediate subfractions in a single spin.

METHODS

Subjects

Five Caucasian patients with alcoholic cirrhosis and spur cells were studied. Three were females (aged 50 to 55 years), all of whom had amenorrhoea for more than a year; the two males were 54 and 57 years old. Clinically they presented jaundice, and liver and spleen enlargement. With the exception of one patient, they lacked any hepatic encephalopathy. Laboratory data at admission are summarized in **Table 1.** Transvenous or percutaneous liver biopsies revealed micronodular cirrhosis, as defined by Anthony et al. (15), in all patients, with evidence of superimposed alcoholic hepatitis in two. None of the patients displayed acute viral A or B hepatitis by clinical, biological, or immunological analyses. Spur cells accounted for more than 50% of the erythrocytes within any given field when fresh wet preparations of venous blood were examined by phase contrast microscopy.

Ten Caucasian patients with alcoholic cirrhosis but lacking spur cells were studied for comparative purposes; all were males, aged 28 to 60 years. Their clinical, biochemical, and histological features were quite similar to those of the spur cell group above, with the exception that their erythrocytes were of normal morphology and

TABLE 1. Laboratory data at admission on alcoholic cirrhotic patients with and without spur cells

Patient	Age	Sex	Serum Hemoglobin	Reticulocytes	White Cells	Bilirubin Total/Conjugated	Serum Aspartate Aminotransferase	Serum Alanine Aminotransferase	Alkaline Phosphatase	Serum Albumin	Prothrombin Ratio	Immunoglobulin A
	yr		*g/dl*	*per mm³*	*per mm³*	*μmol/dl*	*IU/l*	*IU/l*	*IU/l*	*g/dl*	*%*	*g/dl*
Cirrhotics with spur cells												
1	55	F	12	500,000	5,100	18.0/7.0	18	15	110	3.6	36	0.66
2	50	F	10	294,000	19,500	25.5/12.2	28	12	143	2.8	67	0.49
3	50	F	9	170,000	8,900	18.6/11.1	16	19	72	2.8	40	1.10
4	57	M	13	340,000	6,500	28.8/14.7	76	55	163	2.5	42	1.00
5	54	M	11.2	30,000	6,000	15.3/9.0	33	40	160	3.3	28	0.90
Cirrhotics without spur cells												
1	51	M	14.8	83,000	4,600	5.6/2.9	21	12	74	2.5	37	0.94
2	42	M	12.6	ND	8,100	7.5/4.8	50	13	87	3.7	35	0.52
3	28	M	17	ND	7,500	0.7/-	40	43	50	4.1	89	0.25
4	59	M	15.2	ND	5,500	7.6/4.6	30	12	121	3.9	47	0.41
5	44	M	13.6	ND	6,600	9.5/6.0	54	28	246	3.8	50	0.72
6	50	M	11.2	240,000	9,700	6.4/5.0	129	41	163	3.4	80	0.33
7	60	M	13	ND	4,900	3.7/1.8	29	19	50	4.1	73	0.60
8	56	M	14.3	ND	9,600	7.0/3.5	16	11	82	3.0	36	0.31
9	37	M	10.6	450,000	9,000	7.6/4.7	60	35	73	3.0	44	1.10
10	44	M	12	ND	8,400	5.6/3.7	57	11	327	3.3	26	0.92
Normal value			13–16	25,000–100,000	3,500–9,000	0.8–1.7/<0.25	<18	<18	<90	3.5–4.5	80–100	<0.30

ND, not determined.

Downloaded from www.jlr.org by guest, on June 18, 2012

that they displayed no evidence of hemolytic anemia (Table 1). Seven of these patients exhibited histological features typical of moderate to severe alcoholic hepatitis in addition to those of cirrhosis.

None of the patients in these two groups were obese; their diet could not be specified but was presumably irregular given their high alcohol intake. Their alcohol consumption had exceeded 100 g of ethanol per day for more than 5 years. None had received any drug known to affect lipoprotein metabolism when studied at admission.

Nine healthy, nonalcoholic, normolipidemic males, aged 28 to 52 years old were studied as controls for the above groups.

Preparative Methods

Blood samples. Subjects were bled after an overnight fast, typically on the first or second day of admission. Plasma was isolated from freshly drawn, venous blood collected on EDTA (1 mg/ml). Sodium azide (0.01% w/v) and sodium merthiolate (0.001% w/v) were added to all samples prior to lipoprotein separation.

Isolation of serum lipoproteins. A density gradient ultracentrifugal procedure was used for the preparative fractionation of plasma lipoproteins, as previously described (14). Five-step discontinuous density gradients containing 3 ml of the plasma sample (adjusted to a density of 1.21 g/ml with solid KBr) and different volumes of a series of NaCl–KBr solutions of density 1.240, 1.063, 1.019, and 1.006 g/ml, respectively, were constructed at ambient temperature in cellulose nitrate tubes ($^9/_{16}$ in diameter × 3½ in length) of the Beckman SW 41-Ti swinging bucket rotor. Gradients were centrifuged at 40,000 rpm (210,000 *g* avg) for 48 hr at 15°C in a Sorvall OTD-50 ultracentrifuge in the Reograd/ARC slow mode. Gradients were subsequently subfractionated by successive downward aspiration of fractions of 1 to 2 ml with a narrow-bore Pasteur pipette; in each case, the volume removed was adjusted to correspond as far as possible to either visible bands of lipoproteins or to lipoprotein-poor zones. The density interval to which each fraction corresponded was determined by reference to the density profile obtained from control gradients in which serum had been replaced by 3 ml of a d 1.21 g/ml NaCl–KBr solution (for a representative density profile, see Fig. 2 in ref. 14).

Typically, the major fractions obtained by this procedure are: Band I, containing VLDL of d < 1.016 g/ml;[2] IFa, intermediate fraction a of d 1.016–1.028 g/ml; Band II, containing LDL of d 1.028–1.050 g/ml; IFb, intermediate fraction b of d 1.050–1.066 g/ml; Band III, containing HDL_2 of d 1.066–1.100 g/ml; Band IV, containing HDL_3 of d 1.100–1.140 g/ml; IFc, intermediate fraction c of d 1.140–1.154 g/ml and containing HDL_3-like particles (14); and finally the ultracentrifugal residue (d > 1.154 g/ml) containing only trace amounts of lipid. All fractions were dialyzed in Spectrapor membrane tubing (mol wt cutoff 3,500; Spectrum Medical Industries, Los Angeles, CA) at 4°C against a solution containing sodium azide (0.02% w/v), merthiolate (0.001%), EDTA (0.04%), Tris (5 mM), and NaCl (50 mM) at pH 7.4. The purity of these lipoprotein fractions and their lack of contamination with serum proteins has previously been determined (14).

Analytical methods

Characterization of lipoprotein fractions; chemical analysis. Total protein concentrations were determined on all gradient subfractions except the ultracentrifugal residue by the method of Lowry et al. (16); any turbidity, particularly in VLDL, was removed from the final assay mixtures by extraction with diethyl ether. Bovine serum albumin (Sigma) was used as the working standard. Total cholesterol (TC) was estimated in all samples according to the procedure of Roeschlau, Bernt, and Gruber (17), employing the enzymatic kit of Boehringer/Mannheim GmbH; unesterified or free cholesterol (FC) was assayed by the same procedure in the absence of cholesterol esterase. Cholesteryl esters (CE) were estimated as the amount of ester cholesterol (EC) × 1.67, the former representing the difference between total and free cholesterol, and the latter the ratio of the average molecular weight of cholesteryl ester to that of free cholesterol. The working standard for cholesterol quantitation was 99% pure (Merck). Phospholipids were estimated directly on aliquots of each fraction and of the various sera with the "Phospholipids B-test Wako" (Biolyon, BP 13, 69570 Dardilly, France). This procedure (18) directly and specifically measures the choline content of all choline-containing phospholipids; such lipids represent ~95% or more of the total phospholipids of the major classes of human serum lipoproteins (19). Triglycerides were quantitated by the method of Biggs, Erickson, and Moorehead (20), using purified triolein (99%, Sigma) as standard.

The mean recoveries of total cholesterol, free cholesterol, triglyceride, and phospholipid, each analyzed in duplicate or triplicate, from the density gradient fractionation of four different plasmas were 88 ± 2.7, 93 ± 2.3, 86 ± 1.9, and 83 ± 7.6%, respectively.

The reproducibility of these analyses was examined by calculation of the technical error, this being defined as $\sqrt{d^2/2N}$, where d is the difference between duplicate estimations and N the number of duplicates. The technical

[2] In addition to VLDL of d < 1.007 g/ml, this fraction also contains lipoproteins of intermediate density (i.e., of d 1.007–1.016 g/ml).

Downloaded from www.jlr.org by guest, on June 18, 2012

errors for protein, cholesterol, triglyceride, and phospholipid were, respectively, 5.4, 1.7, 3.5, and 4.1%.

Negative stain electron microscopy. Lipoprotein fractions, at protein concentrations up to 0.2 mg/ml, were mixed with an equal volume of 2% potassium phosphotungstate and dried down on copper grids coated with formvar and carbon; this procedure is essentially that described by Forte, Nichols, and Glaeser (21). These preparations were examined at 60 KV with a Philips EM 300 electron microscope at magnifications in the range of 35,000 to 70,500×. For purposes of measurement of particle size, the instrument was calibrated with a germanium-shadowed carbon replica of a ruled diffraction grating bearing 54,864 lines per inch. Two or three grids of each fraction were examined and photographed. For evaluation of the frequency distribution of particle sizes, the diameters of a minimum of 200 freely dispersed, apparently intact particles were measured on each of at least two electron micrographs, whose final magnification was accurately known; such measurements were made with an X-Y reader. The analog output of this reader was transformed into a digital form by passage through an analog-digital converter and the resultant signal was analyzed by a computer program (Wang) designed to calculate the mean particle size ± SD and to plot the frequency of size distribution.

Lipoprotein electrophoresis. After prestaining with Sudan black, whole serum and lipoprotein subfractions were electrophoresed for 1 hr at 250 V and 15 mA on polyacrylamide gel sheets (Lipofilm, Sebia, Issy-les-Moulineaux, France) constructed to give a discontinuous gradient from 2% (at point of sample application) to 3% (running gel).

Characterization of apolipoprotein contents; electrophoretic analysis. The complement of low molecular weight, tetramethylurea-soluble (i.e., non-apoB) apolipoproteins of the major density gradient subfractions were evaluated qualitatively by electrophoresis in the alkaline urea-polyacrylamide disc gel system of Davis (22) as modified by Kane (23). Samples, containing 100 μg of tetramethylurea-soluble protein, were applied to each gel (7.5% monomer); in this procedure, lipids and apolipoprotein B are precipitated. Tetramethylurea was 99% pure (Merck-Schuchardt). Gels were fixed, stained with Coomassie Brilliant Blue R (Sigma), and destained as detailed elsewhere (24).

A semi-quantitative estimate of the ratio of the concentrations of apoA-I:apoA-II was obtained by scanning the electrophoretic patterns of the alkaline urea-containing polyacrylamide gels of HDL_2 and HDL_3 at 550 nm with a Safas 170 FD spectrophotometer. The peak areas corresponding to the stained apoA-I and apoA-II bands were then determined gravimetrically on peaks cut from the scans. This approach to the quantitation of apolipoproteins was first validated by Cheung and Albers (25).

The apolipoprotein contents of fractions IFb, HDL_2, and HDL_3 from cirrhotic patients with and without spur cells and from normal subjects were also examined by analytical IEF in the pH range 4–6.5 (as above, apoB does not migrate in this system). The polyacrylamide gels (7.5% monomer) contained 6 M urea and 2% ampholine (Pharmalyte, Pharmacia Fine Chemicals). Samples (100–150 μg of protein) were applied as described by Pagnan et al. (26); the remaining conditions were those described earlier (27), the cathodic electrolyte solution (0.1 M glycine) being brought to pH 7.0 and the anodic (0.01 M Hepes) to pH 3.5. After focusing at constant voltage (400 V) for 5 hr 30 min at 10°C, gels were stained with Coomassie Brilliant Blue as outlined by Karlson et al. (28). The pI values of stained bands were assessed by measurement of the pH of aqueous eluates of slices (0.5 cm thick) cut from unstained gels, and apolipoprotein bands were identified according to their known isoelectric points (27, 29, 30).

Immunological quantitation of apolipoproteins. The quantitation of apolipoproteins B, A-I, and A-II in whole serum and in all lipoprotein subfractions from the density gradient was performed by single radial immunodiffusion in commercially prepared agarose gel plates (EP plates; Immuno-Diagnostika, Austria). These plates contained monospecific rabbit antisera to human apoA-I and to apoA-II, and a monospecific goat antiserum to human apoB; Triton X-100 (2%) was present in the agarose gel of the apoA-I and apoA-II sections of these plates. The corresponding human apolipoproteins, as native lipoproteins in whole serum stabilized with sucrose, at concentrations of: apoA-I (106 mg/dl); apoA-II, (39.9 mg/dl); and apoB (72.3 mg/dl), were supplied as Reference Standard by Immuno-Diagnostika. These values were validated by comparison with calibration curves constructed from apolipoprotein standards prepared in our laboratory: for apoB, LDL of d 1.024–1.050 g/ml, whose protein moiety typically contains >98% apoB, and for apoA-I and apoA-II, both the isolated apolipoproteins (27) and HDL_3 of known apoA-I and apoA-II content (see ref. 14). The calibration curves obtained for our laboratory standards were essentially parallel to those for the respective apolipoproteins in the Reference Standard, with variations of 10% or less in the values given by the different curves for each protein.

The monospecificity of each of these antisera was assessed by testing it against purified apoA-I, apoA-II, apoB, apoE, and apoC's (as a mixture of C-I, C-II, and C-III) prepared in our laboratory (14, 27, 31). Each antiserum reacted specifically and exclusively with the homologous apolipoprotein upon double immunodiffusion (performed according to Ouchterlony (32)). The assay for apoB was linear over the range 4.6 to 72.3 mg/dl; the apoA-I assay was linear over a range of 1.7 to 13.2 mg/dl and that

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 2. Plasma lipid and apolipoprotein concentrations in alcoholic cirrhotic patients with or without spur cells and in nonalcoholic normolipidemic controls[a]

TC	FC	$\frac{EC}{FC}$	TG	PL	A-I	A-II	B	$\frac{A\text{-}I}{A\text{-}II}$	$\frac{A\text{-}II}{B}$
				mg/dl					
Cirrhotics with spur cells (5)									
148.1 ± 44.9	67.6 ± 21.3[b]	1.23 ± 0.3[c]	65.7 ± 30.1	175 ± 53.9	78.7 ± 54.6	6.4 ± 4.4[c]	57.4 ± 22.5	13.1 ± 5.5[d]	0.11 ± 0.04[c]
Cirrhotics without spur cells (10)									
184.3 ± 61.2	68.6 ± 26.1[e]	1.84 ± 0.9[f]	100.3 ± 38.7	215.3 ± 67	63.4 ± 49	23.1 ± 20.4	84.2 ± 36.3	3.9 ± 2.2	0.34 ± 0.43
Controls (9)									
181.1 ± 33.9	46.6 ± 11.7	3.01 ± 0.74	87.3 ± 31.8	218.4 ± 31.5	101.5 ± 19.1	31.8 ± 6.2	84.2 ± 22.7	3.26 ± 0.7	0.4 ± 0.14

[a] Results are expressed as means ± SD of the number of subjects given in parentheses.
[b] >Controls; $P < 0.05$.
[c] <Controls; $P < 0.01$.
[d] >Controls and >cirrhotics without spur cells; $P < 0.01$.
[e] >Controls; $P = 0.02$.
[f] <Controls; $P = 0.01$.

of apoA-II from 0.62 to 4.96 mg/dl plasma. Prior to immunoassay, lipoprotein samples were diluted in a 0.9% (w/v) NaCl solution; dilutions of plasma used for quantitation of apoB were 1:1, 1:2, and 1:4; and 1:8 and 1:16 for both apoA-I and apoA-II.

Using the commercially prepared immunodiffusion plates, the ranges of plasma concentrations of apoB, apoA-I, and apoA-II in a series of 19 normolipidemic subjects were 52 to 125.6, 64 to 127.3, and 22.2 to 41.1 mg/dl, respectively. Samples of whole plasma and of the lipoprotein subfractions from a given individual were normally assayed on the same plate.

Preliminary experiments were performed to determine the optimal conditions for expression of the immunological reactivity in the major gradient subfractions; typically, dilutions in the range 1:1 to 1:16 of these fractions were employed. The recoveries of apoA-I, apoA-II, and apoB from the density gradient ultracentrifugal separations were 84 ± 8, 91 ± 6, and 80 ± 7%, respectively.

The Wilcoxon paired rank test was used in the statistical treatment of the results (33).

RESULTS

Plasma lipid and apolipoprotein levels

The concentrations of plasma lipids, apoB, apoA-I, and apoA-II in cirrhotics with or without spur cells and in the control group are summarized in **Table 2.** Total plasma cholesterol levels in the cirrhotic patients with spur cells ranged from 99.8 to 272 mg/dl, and although the mean level (148.1 mg/dl) was lower than that in the other two groups (ca. 180 mg/dl), it did not differ significantly. Plasma free cholesterol concentrations in patients with spur cells (mean 67.6 mg/dl) were in the range 31.8–83.9 mg/dl, resembling that in cirrhotics lacking such abnormal erythrocytes (mean 68.6 mg/dl, range 42.3–125 mg/dl); FC levels in both groups were significantly elevated as compared to controls (mean 46.6 mg/dl; $P < 0.05$ and $P = 0.02$, respectively). The ratio of plasma levels of esterified to free cholesterol in both groups of cirrhotic patients (1.2:1 and 1.8:1 in spur cell and non-spur cell plasma, respectively) was significantly lower than that in normal subjects (3:1); indeed, the proportion of cholesterol in esterified form in the spur cell patients (54.4%) was less than that in cirrhotics lacking these cells (62.8%) and substantially less than that in the normal group (74.3%). In contrast, the plasma triglyceride and phospholipid concentrations of patients with spur cells (35.6–111 and 98.4–248 mg/dl, respectively) were not significantly different from those in the other two groups, although they tended to be the lowest.

The plasma levels of apoA-I in the three groups varied widely and particularly in spur cell plasma (range 35.2–

Downloaded from www.jlr.org by guest, on June 18, 2012

161.6 mg/dl); the lowest mean concentration was seen in cirrhotic patients lacking such red cells. On the other hand, apoA-II levels were uniformly and dramatically reduced (range 2.4–13.3 mg/dl) in spur cell plasma when compared to those of controls ($P < 0.01$), but they were not significantly lower than those seen in cirrhotics lacking spur cells ($P < 0.1$). However, the ratio of the amounts of apoA-I to apoA-II in the plasma of the spur cell patients was significantly elevated relative not only to the control subjects ($P < 0.01$), but also to the cirrhotic patients who did not present such erythrocytes ($P < 0.01$).

Despite the diminished levels of apoB in the plasma of cirrhotics with spur cells (range 36.4–93.6 mg/dl), they could not be differentiated statistically from the normal subjects ($P = 0.1$) or from the cirrhotic group lacking spur cells ($P = 0.1$). Nonetheless, the ratio of plasma apoA-II:apoB in the former patients was significantly below that in the control group ($P < 0.01$).

Plasma lipoprotein levels

Summations of the amounts of individual constituents of each lipoprotein subfraction from the density gradients afforded a quantitative estimate of their plasma concentrations (**Table 3**). This approach was validated earlier following comparison with an analytical ultracentrifugal procedure (14).

Levels of the major apoB-containing fractions (ie., VLDL and LDL) were reduced (by 48% and 24%, respectively) in the cirrhotic patients with spur cells as compared to normals; intermediate density lipoproteins (isolated as IFa) were unaltered. In contrast, the amounts of both the HDL_2 and the second intermediate fraction (IFb, of d 1.050–1.066 g/ml and containing a mixture of LDL, HDL_1, and possibly Lp(a) particles were elevated (by 51% and 43%, respectively) in spur cell plasma. The most dramatic alteration in lipoprotein profile in cirrhotic patients with spur cells was a more than fivefold diminution in HDL_3 (range 13.2–78.4 mg/dl plasma; $P < 0.01$) relative to that seen in the normal population. This was reflected in a marked and significant elevation in the ratio HDL_2:HDL_3 which was inverted in the former (3.8:1) as compared to the control group (0.35:1; $P < 0.01$). Nonetheless, these features did not distinguish the cirrhotic patients with spur cells from the second cirrhotic group, since the latter also displayed a diminution in HDL_3 levels ((range 18.6–157 mg/dl plasma; $P < 0.01$ with respect to the control group), though absolute concentrations were twofold higher (70 mg/dl) than in the former subjects (35.8 mg/dl). In addition, the cirrhotic group without spur cells displayed an elevated and inverse HDL_2:HDL_3 ratio (1.9:1). These patients were further distinct in exhibiting the highest LDL and HDL_2 levels (345 and 121.2 mg/dl, respectively) of the three groups.

Chemical and physical properties of lipoprotein subfractions from the density gradient

Chemical composition. The mean weight % chemical compositions of the lipoprotein subfractions from the cirrhotic patients presenting with or without spur cells and from the control group, are presented in **Table 4.** The FC contents of all the lipoprotein subfractions isolated from spur cell plasma (with the exception of HDL_3) were elevated as compared to those in both non-spur cell cirrhotics ($P = 0.05$) and normals. Moreover, in the latter case, such elevations attained statistical significance (Table 4). Conversely, the proportions of cholesteryl esters in the LDL, IFb, and HDL_2 of the spur cell group were significantly diminished relative to the control population; a similar tendency was noted in the corresponding subfractions from cirrhotic patients. In spite of signifi-

TABLE 3. The quantitative distribution of plasma lipoprotein subfractions in alcoholic cirrhotic patients with or without spur cells and in non-alcoholic normolipidemic subjects[a]

VLDL	IFa	LDL	IFb	HDL_2	HDL_3	$\frac{HDL_2}{HDL_3}$
			mg/dl			
Cirrhotics with spur cells (5)						
29.0 ± 28.6	61.6 ± 31.7	205.4 ± 84.1	41.5 ± 13.1	103.7 ± 90.5	35.8 ± 27.7[b]	3.8 ± 2.84[c]
Cirrhotics without spur cells (10)						
44.1 ± 25.4	61.3 ± 34.8	345 ± 173.5	44.8 ± 18.7	121.2 ± 95.6	70 ± 55.1[b]	1.9 ± 0.77[c]
Controls (9)						
55.3 ± 31.5	58.4 ± 21.8	268 ± 72	28.9 ± 11.1	68.5 ± 26.9	197.5 ± 39	0.35 ± 0.14

[a] Lipoprotein levels are expressed as means ± SD for the number of subjects given in parentheses, and represent the sum of the chemically determined weights of each component in the respective lipoprotein subfractions isolated from the density gradient.
[b] <Controls; $P < 0.01$.
[c] >Controls; $P < 0.01$.

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 4. Percent weight chemical composition of serum lipoproteins from alcoholic cirrhotic patients with and without spur cells, and from non-alcoholic normolipidemic controls[a]

Fraction	Density	CE	FC	TG	PL	Protein
	g/ml					
Cirrhotics with spur cells (5)						
VLDL	<1.016	22.4 ± 6.8	11.5 ± 2.8[b]	26.8 ± 11.2[c]	19.1 ± 1.8	20.2 ± 5.3
IFa	1.016–1.028	29 ± 4.2	12.5 ± 2.6[b]	15.8 ± 5.6	19.6 ± 3.6	23.1 ± 3.7
LDL	1.028–1.050	28.8 ± 3.6[d]	13.6 ± 3.3[e]	9.2 ± 1.5[b]	24.1 ± 1[b]	24.2 ± 2.1[f]
IFb	1.050–1.066	20.3 ± 7.5[f]	14.6 ± 5.6[b]	8.7 ± 1.9	29.3 ± 5[b]	27 ± 3.3
HDL_2	1.066–1.100	13.7 ± 3.5[d]	9.7 ± 4.6[b]	8.5 ± 3	32.2 ± 3.3[b]	35.9 ± 6[c]
HDL_3	1.100–1.140	12.2 ± 4.5	2.6 ± 2.1	6.1 ± 5.6	21.2 ± 2.5	58 ± 10.2
Cirrhotics lacking spur cells (10)						
VLDL	<1.016	17.7 ± 6.8	8.8 ± 1.9[e]	32.5 ± 8.4[f]	19.9 ± 3.7	20.8 ± 5.8
IFa	1.016–1.028	22.6 ± 6.6	9.8 ± 2.2	21.8 ± 6.1	22.3 ± 3[b]	23.3 ± 2.3
LDL	1.028–1.050	29.9 ± 8.4[c]	10.4 ± 1.6	13.4 ± 6.1[b]	22.2 ± 2.4	24.1 ± 2.4[d]
IFb	1.050–1.066	21.2 ± 8.4[d]	10.5 ± 2.7[b]	11.5 ± 4.9	26.6 ± 4.6[b]	30.2 ± 4.7
HDL_2	1.066–1.100	15.5 ± 7.4	8.1 ± 4.3[b]	7.3 ± 5	33 ± 4.8[b]	36.2 ± 5.2[d]
HDL_3	1.100–1.140	13.7 ± 3.9	2.5 ± 1.6	3.6 ± 3	26.9 ± 6.5	53.3 ± 6.4
Controls (10)						
VLDL	<1.016	15.7 ± 6.1	6.6 ± 1.6	40.5 ± 9.5	17.9 ± 3.8	19.3 ± 3.7
IFa	1.016–1.028	26.2 ± 6.2	8.3 ± 1.3	20.3 ± 7.7	19 ± 1.3	26.2 ± 2
LDL	1.028–1.050	37.6 ± 3.4	9.7 ± 0.9	5.1 ± 1.5	20.6 ± 1.4	27 ± 1.2
IFb	1.050–1.066	31.9 ± 5.9	6.9 ± 1.7	10.9 ± 10.7	19.3 ± 2.3	30.9 ± 3.6
HDL_2	1.066–1.100	21 ± 3.5	3.5 ± 0.8	5.2 ± 7.3	26.6 ± 2	43.6 ± 4.7
HDL_3	1.100–1.140	17 ± 2.1	1.6 ± 0.6	2.5 ± 1.6	23.2 ± 2.5	56 ± 2.3

[a] Values are the means ± SD of duplicate determinations on the number of preparations given in parentheses for each group.
[b] >Controls; $P < 0.01$.
[c] <Controls; $P < 0.05$.
[d] <Controls; $P < 0.01$.
[e] >Controls; $P < 0.05$.
[f] <Controls; $P < 0.02$.

cantly elevated amounts of phospholipids in LDL, IFb, and HDL_2 from the spur cell group, these fractions, as well as HDL_3, contained (total) proportions of phospholipid and protein that were comparable to those in the control population (48.3 and 47.6% in LDL, 56.3 and 50.2% in IFb, 68.1 and 69.9% in HDL_2, and 79.2% in the HDL_3 in spur cell and control plasmas, respectively).

Only the composition of HDL_3 in the cirrhotic patients with spur cells approached that of its counterpart in normals; this was also the case for the cirrhotics lacking such red cells.

Electrophoretic mobility. The behavior of the major subfractions from spur cell plasma upon electrophoresis in polyacrylamide gel films was essentially indistinguishable qualitatively from that of the corresponding subfractions from normal plasma (data not shown; for mobilities of subfractions from controls, see Fig. 6 in ref. 14). A band corresponding to Lp(a) was not revealed in any of the spur cell LDL or IFb fractions by this procedure.

Electron microscopic analysis. The morphology of the VLDL (Band I, d < 1.016 g/ml) particles from three patients with spur cells (**Fig. 1A**) strongly resembled that of VLDL from normolipidemic subjects (see Fig. 5; ref. 14). The range in particle size was rather similar in the three preparations (Fig. 1A), particles being distributed essentially symmetrically about modes of 375 and 325 Å (each of two preparations). More than 94% were in the range 200–500 Å. Mean particle diameters were 303 ± 7, 376 ± 12, and 315 ± 10 Å, respectively, and are therefore remarkably similar to VLDL typical of normal (fasting) subjects (mean 349 Å and range 200–615 Å (14)). Particles of 100–250 Å diameter may be present in VLDL (d < 1.006 g/ml) from fasting plasma, an observation (34) which is consistent with the present and earlier (14) findings, although the content of 150–250 Å particles in our VLDL of d < 1.016 g/ml may be due in part to intermediate density lipoproteins which are characteristically isolated in the density interval 1.006–1.019 g/ml. The majority (95–97%) of spur cell LDL (Band II, d 1.028–1.050 g/ml) could not be differentiated from the corresponding particles of normolipidemic individuals (Fig. 1B); (Fig. 5, ref. 14). Spur cell LDL tended to be larger than LDL from normolipidemic subjects, displaying mean diameters (±SD) of 240 ± 12, 219 ± 6, and 256 ± 23 Å, respectively, as compared to 211 Å in a representative control subject. Particles were distributed rather symmetrically about the mode in all three spur cell LDL's (Fig. 1B); the ranges in diameters were 150–

Downloaded from www.jlr.org by guest, on June 18, 2012

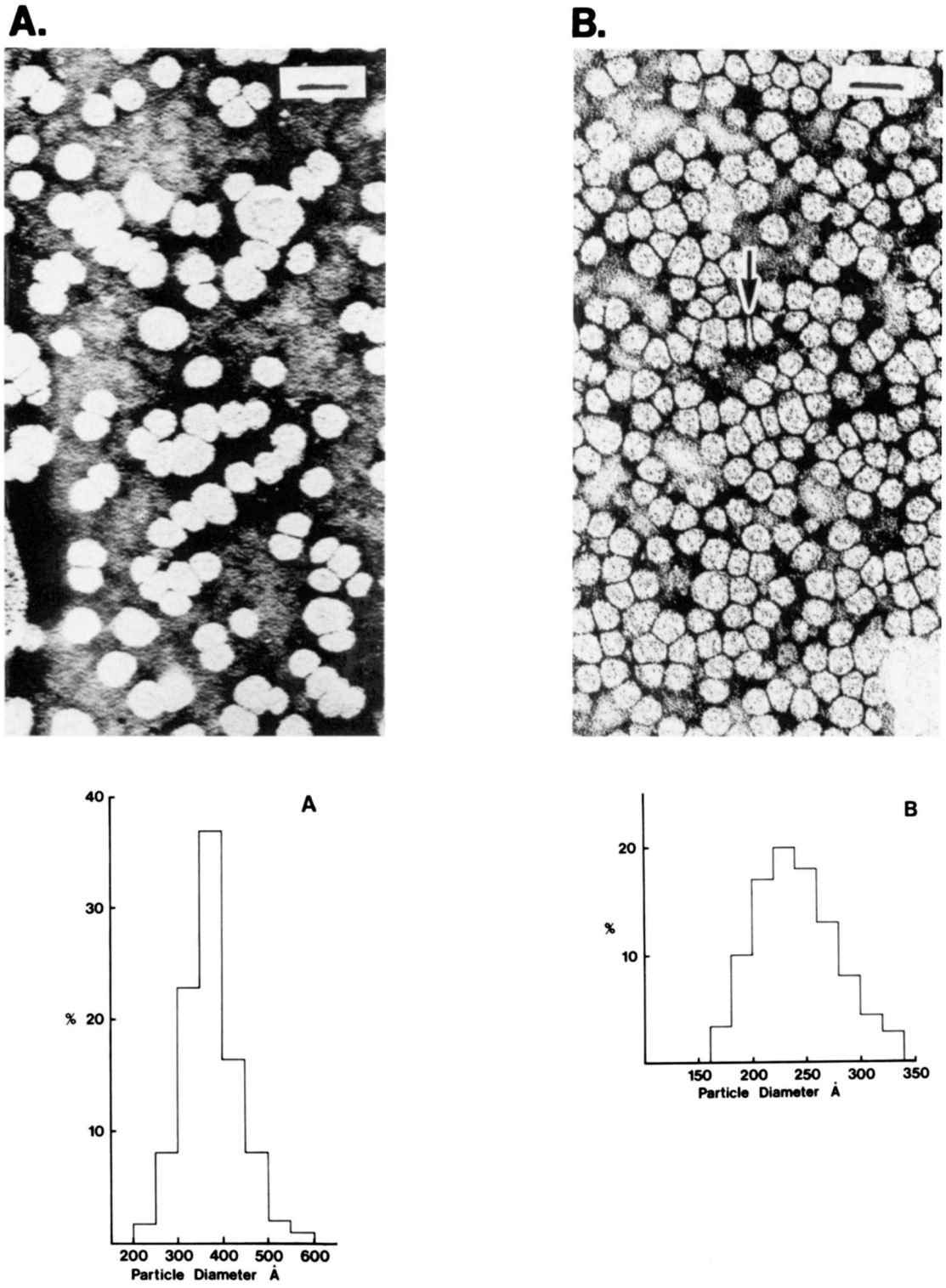

Fig. 1. Representative electron micrographs and particle size distribution of plasma lipoproteins isolated from patients with alcoholic cirrhosis and spur cells by density gradient ultracentrifugation. (A), VLDL, d < 1.016 g/ml; (B), LDL, d 1.028–1.050 g/ml. Above, electron micrographs in which the horizontal bar represents 500 Å. Below, frequency distribution of lipoprotein particle sizes, in which up to 3% of the occasional particles at each extreme of the ranges is excluded. Arrow identifies the minor population of discoid particles seen in LDL fractions (B).

300, 160–340, and 160–360 Å (normal range 160–250 Å (14)). An additional population of LDL with sizes in the range 250–300 Å was therefore present in spur cell plasma, this population representing 23, 36, and 49%, respectively, of the total in each case. A small but consistent proportion (ca. 1–3%) of particles in spur LDL

Downloaded from www.jlr.org by guest, on June 18, 2012

was discoidal (Fig. 1B). As such, they could only be positively identified in crowded fields where they were seen "on-end", presenting a thickness of ~40–60 Å, and a length of 190–300 Å.

The IFb subfraction (d 1.050–1.066 g/ml) was composed of a heterogeneous population of essentially spherical particles whose size ranged from approximately 100 to 220 Å (not shown) and as such was comparable to control IFb particles which typically showed an overall range of 125–200 Å and a mean diameter of 180 Å. The mean diameters of IFb particles from two spur cell plasmas were 141 ± 28 and 167 ± 23 Å. These particles probably correspond to denser species of LDL (14), since their size ranges overlap with the latter in the region of ~150 to 220 Å, as well as with HDL_1 of S_f 0–3 and d 1.04–1.06 g/ml (35) and with HDL_1 of d 1.08–1.09 g/ml and 130–190 Å diameter (36). A minor proportion (1–3%) of IFb particles was seen in packed fields in discoidal form with dimensions resembling those described above in LDL. It is noteworthy that LDL and IFb subfractions from two cirrhotic patients lacking spur cells similarly contained very small amounts (<5%) of LP-X-like particles by electron microscopy.

Apolipoprotein density profile. The urea-soluble apolipoproteins of low molecular weight in HDL_2 and HDL_3 were examined by electrophoresis in urea-containing polyacrylamide gels at alkaline pH (23) (**Fig. 2**). Both HDL_2 and HDL_3 from spur cell plasmas were markedly deficient in apoA-II, C-II, and C-III; only the most acidic form of apoC-III (C-III-3) was in evidence. ApoA-I clearly predominated with small amounts of apoE (Fig. 2a and b). The proportion of apoE in HDL_3 appeared slightly higher than that in the control fractions (Fig. 2b and e). The proportions of both apoA-II and the C apoproteins

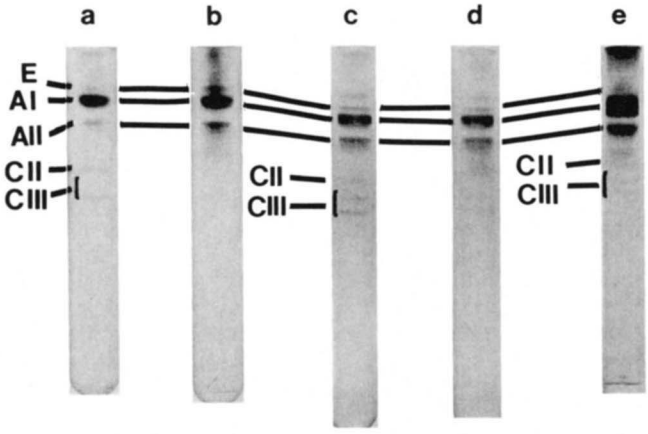

Fig. 2. Electrophoretic patterns of tetramethylurea-soluble apolipoproteins from HDL subclasses in alkaline-urea polyacrylamide gel. Samples are: a and b, HDL_2 and HDL_3 from cirrhotic subjects presenting spur cells; c and d, HDL_2 and HDL_3 from cirrhotic subjects lacking spur cells; e, HDL_3 from a control subject.

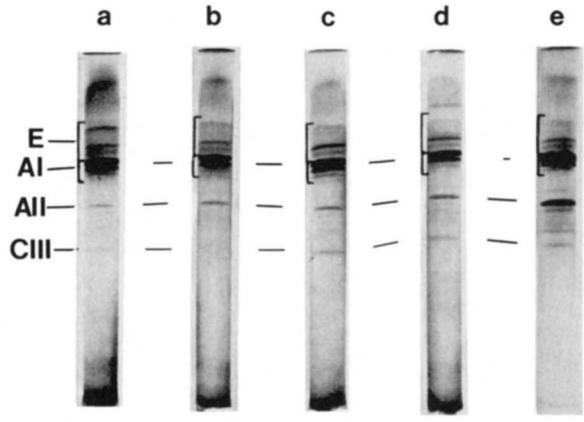

Fig. 3. Electrophoretic patterns of apolipoproteins from HDL subclasses upon analytical isoelectric focusing in the pH range 4–6.5. Samples are: a and b, HDL_2 and HDL_3 from cirrhotic subjects with spur cells; c and d, HDL_2 and HDL_3 from cirrhotic subjects lacking spur cells; e, HDL_3 from a control subject.

also appeared diminished in HDL_2 and HDL_3 from cirrhotic subjects lacking spurred erythrocytes as compared to controls (Fig. 2c and d).

The urea-soluble apolipoprotein contents of HDL_2 and HDL_3 from the three groups were also evaluated by analytical IEF in the pH range 4–6.5 (**Fig. 3**); the focusing patterns of HDL_2 and HDL_3 from our spur cell subjects confirmed the above findings in TMU-gels. Thus, apoA-II and the C-peptides were poorly represented; only apoC-III-3 was distinct among the latter (Fig. 3a and b). By contrast, apoE3 and apoE4 bands were clearly distinguishable. Similar observations were made in focusing gels of HDL_2 and HDL_3 from cirrhotic subjects lacking spurred erythrocytes (Fig. 3c and d). In certain cirrhotics, irrespective of whether they lacked or presented spur cells, an additional minor apoprotein focused at a pI of ~6.4; this may correspond to apoC-I.

The quantitative distributions of apolipoproteins A-I, A-II, and B amongst the seven principal lipoprotein subfractions, and in addition, in the "bottom" fraction or ultracentrifugal residue ($d > 1.154$ g/ml), are presented in **Table 5.** Preliminary electrophoretic studies in the alkaline urea-gel system (23) provided a semi-quantitative indication that apoA-I and apoA-II together represented more than 70% of the protein moieties of high density ($1.066 < d < 1.154$ g/ml) subfractions in all three groups. As predicted by the reduced VLDL and LDL concentrations in spur cell plasma, apoB levels were below normal (and also below those of the cirrhotic patients) in these subfractions; some reduction was also seen in the IFb density range. Such differences were not statistically significant however ($P = 0.1$).

The distribution of apoA-I between subfractions of density > 1.050 g/ml in the two groups with alcoholic

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 5. Quantitative distribution of apolipoproteins in plasma lipoprotein subfractions from alcoholic cirrhotic patients with or without spur cells and from non-alcoholic normolipidemic subjects[a]

Apolipoprotein	Subfractions							
	VLDL	IFa	LDL	IFb	HDL_2	HDL_3	IFc	Bottom[b]
				mg/dl				
Cirrhotics with spur cells (5)								
ApoA-I				4.7 ± 2.6[c]	26.6 ± 27.8	11.7 ± 10.3[d]	1.9 ± 1.6[d]	20.6 ± 11.9
ApoA-II				0.02 ± 0.04[e]	2.7 ± 1.5	2.5 ± 2.2[f]	0.2 ± 0.2[f]	
ApoB	1.7 ± 1.4	6.4 ± 5.1	32.5 ± 18.3	3.2 ± 2.7				
Cirrhotics without spur cells (10)								
ApoA-I				1.4 ± 1.7	17.7 ± 17.3	11 ± 13[d]	2.9 ± 3.4[d]	23.7 ± 13.9
ApoA-II				0.4 ± 0.6	9.4 ± 11.6	10.1 ± 9.2	1.2 ± 0.9	0.5 ± 0.9
ApoB	2.5 ± 1.3	6.2 ± 3.2	49.2 ± 25.8	6.5 ± 5.6	1.5 ± 1.3			
Controls (9)								
ApoA-I				1 ± 1.2	11.4 ± 5.8	37.7 ± 9.5	9.3 ± 2.2	25 ± 3.6
ApoA-II				0.2 ± 0.2	4.6 ± 0.7	19.4 ± 7.2	1.6 ± 0.8	2.2 ± 0.5
ApoB	3.4 ± 2.2	7.5 ± 2.6	49.8 ± 11.8	5.3 ± 2.2	1.3 ± 0.4			

[a] Values are means ± SD for the number of subjects given in parentheses.
[b] The bottom fraction corresponded to the ultracentrifugal residue of d > 1.154 g/ml.
[c] >Controls; $P < 0.05$ and >cirrhotics without spur cells; $P < 0.02$.
[d] <Controls; $P < 0.01$.
[e] <Controls; $P < 0.01$ and <cirrhotics without spur cells; $P = 0.02$.
[f] <Controls; $P < 0.01$ and <cirrhotics without spur cells; $P < 0.05$.

liver cirrhosis resembled that in controls, although all the characteristics of this pattern were more pronounced in the spur cell subjects. Thus, whereas 40.6% of total apoA-I was present in HDL_2 from spur cell plasma, only 31.2% and 13.5% were detected in this subclass in cirrhotics lacking spur cells and in normal subjects, respectively. In contrast to apoA-I, apoA-II was detected at reduced levels in HDL_2 from patients with spur cells. Furthermore, fraction IFb from spur cell plasma was almost devoid of apoA-II. Further alteration in the particles constituting spur HDL_2 was suggested by the absence of immunologically detectable apoB, which was present in small amounts (ca. 1.5 mg/dl) in both normal and non-spur cell cirrhotic groups, probably as a component of the LpB found in this density range (37).

The cirrhotic patients presenting spur cells were not only distinguished from our healthy controls by their dramatically lower levels of both apoA-I and apoA-II in HDL_3 (3.2- and 7.8-fold reductions, respectively; $P < 0.01$ in both instances), but also from the cirrhotic patients without spur cells (apoA-II, 4-fold lower; $P < 0.05$). Furthermore, the apoA-I:apoA-II ratio was elevated in both HDL_2 and HDL_3 from spur cell plasma (9.8:1 and 4.7:1, respectively) when compared to controls (2.4:1 and 1.9:1, respectively; $P < 0.01$ in each case), as well as to cirrhotic patients lacking spur cells (1.9:1 and 1.1:1, respectively; $P = 0.02$ and $P < 0.01$, respectively). Similar tendencies were seen in the denser HDL_3 subfraction, defined as IFc of d 1.140–1.154 g/ml, from spur cell plasma, since it exhibited a markedly diminished A-II content, and as a consequence, a high A-I:A-II ratio (10.5:1 as compared to 2.5:1 and 5.8:1 in the cirrhotic and normal goups, respectively). Finally only a minor proportion of the total apoA-I was present within the HDL_3 density range (d 1.100–1.154 g/ml) in spur cell plasma (20.8%) relative to that in control subjects (55.7%).

The apoA-I content of the ultracentrifugal residue (d > 1.154 g/ml) was alike in all three groups, but it is noteworthy that this represented a significant proportion of total plasma apoA-I, (31.4 and 26.6%, respectively) in patients with spur cells and normal subjects, and still more (56.7%) in cirrhotics in which such erythrocytes were not detectable. Similarly elevated levels of apoA-I have been reported in fractions of density > 1.158 g/ml upon density gradient ultracentrifugation of normolipidemic human plasma (38). In contrast, apoA-II was undetectable in the d > 1.154 g/ml residue from both normal and spur cell plasmas, but represented 2.3% of total A-II in plasma from cirrhotics lacking spurred erythrocytes (Table 5).

DISCUSSION

Our lipid and apolipoprotein analyses of whole plasma identified apolipoprotein A-II as the parameter that distinguished the spur cell subjects, since their apoA-II levels were significantly diminished (fivefold) as compared to

Downloaded from www.jlr.org by guest, on June 18, 2012

normal, and almost fourfold lower than those typical of cirrhotics lacking spur cells. Determination of the plasma apoA-I:apoA-II ratio further emphasized the importance of the A-II protein since it revealed a marked and statistically significant difference between the two groups of cirrhotic patients on the one hand, and between the cirrhotic subjects presenting with spur cells and the normolipidemic, healthy group on the other. Indeed, this ratio was three- to fourfold higher in spur cell plasma (Table 2). In a similar manner, the ratio of apoA-II:apoB levels in patients with spur cells distinguished them from both the second cirrhotic group and from control subjects, this ratio being about threefold lower in spur cell plasma in both instances.

These initial observations on whole plasma suggested profound perturbations of the structure and metabolism of high density lipoproteins in cirrhotic subjects exhibiting spurred erythrocytes, and such a postulate was entirely consistent with data on HDL subclasses obtained upon gradient fractionation. Thus, despite the essentially normal chemical composition of HDL_3 in spur cell plasma, its apoA-II content was markedly depressed relative to that of apoA-I. Furthermore, the absolute concentration of apoA-II in spur cell HDL_3 was significantly lower than that in individuals with alcoholic cirrhosis but lacking spur cells, as well as that in normals.

ApoA-II concentrations were reduced to similarly low levels in spur cell HDL_2 as in HDL_3 (i.e., <3 mg/dl), giving rise to a dramatic elevation in the apoA-I:apoA-II ratio (9.8:1) in this subclass, as compared to values in the normal and second cirrhotic groups (2.5:1 and 1.9:1, respectively). Absolute levels of apoA-I in HDL_2 and HDL_3 were increased over normal in all cirrhotic individuals irrespective of red cell morphology. As judged by the electrophoretic patterns in TMU/alkaline urea gels and in isoelectric focusing gels (Figs. 2 and 3), the proportions of apoE may have been moderately elevated in both HDL_2 and HDL_3 from the spur cell subjects as compared to the corresponding fractions from controls. ApoC-II and apoC-III levels appeared considerably diminished in both HDL_2 and HDL_3 in the former group, to the extent that in certain subjects, only the most acidic isoform of apoC-III (C-III-3) was detectable. Clearly then, the HDL_2 subclass, and to a lesser extent HDL_3, of spur cell plasma appears to consist primarily of particles containing exclusively apoA-I with a minor population possessing both apoA-I and apoA-II; this proposal is entirely consistent with the recent report of Cheung and Albers (39).

Furthermore, the major proportion of apoA-I was present in the HDL_2 density interval in spur cell plasma (40.6%), although this phenomenon was also apparent in our cirrhotic subjects lacking spur cells, in which HDL_2-apoA-I represented 31% of the total. By contrast, apoA-I in HDL_2 represented only 13.5% of total apoA-I in normals, in which HDL_3-apoA-I predominated (44.7%). It is also notable that the proportion of apoA-I in "heavy HDL_3" (14, 40) (subfraction IFc, d 1.140–1.154 g/ml) was markedly diminished in spur cell plasma, accounting for only 2.9% of total apoA-I as compared to 11% in "heavy HDL_3" of normal plasma. This finding may be explained by a decreased rate of secretion of such a particle from the liver and possibly intestine.

In addition to the enrichment in apoA-I, the HDL_2 of spur cell plasma was also distinct from normal in showing elevated contents of free cholesterol and triglyceride, and conversely, lower amounts of cholesteryl ester (Table 4). These features were not however unique to HDL_2 and were in fact shared by the HDL_3 and LDL of spur cell plasma. Moreover, the free cholesterol content of all gradient subfractions from spur plasma was increased, as it was in the corresponding fractions from our second cirrhotic group lacking such cells, though in all cases the elevation was less pronounced (Table 4). A similar finding was made by Cooper and colleagues (7) upon isolation from spur plasma of LDL (d 1.006–1.063 g/ml) and HDL (d 1.063–1.21 g/ml); they noted a greater FC/PL ratio in both. Such calculations revealed the FC/PL molar ratio in VLDL, LDL, HDL_2, and HDL_3 subfractions from our spur cell patients to be distinctly greater than those in the corresponding fractions from either normal plasmas or from those of cirrhotics lacking spurred erythrocytes (**Table 6**). We have not, however, detected the increased LDL levels seen by others (4), but rather a tendency to

TABLE 6. Molar ratios of free cholesterol and phospholipid contents in lipoproteins from alcoholic cirrhotic patients with or without spur cells and from control subjects

	Subfraction			
	VLDL	LDL	HDL_2	HDL_3
Cirrhotics with spur cells	1.23	1.15	0.61	0.25
Cirrhotics without spur cells	0.91	0.96	0.50	0.20
Controls	0.75	0.96	0.27	0.14

Data calculated from Table 4 and expressed as mol/mol. The molecular weight for phospholipid was taken as 790.

Downloaded from www.jlr.org by guest, on June 18, 2012

reduced amounts (Table 3); this finding readily distinguished our spur cell patients from cirrhotics lacking such cells, since LDL concentrations were higher than normal in the latter.

From the foregoing discussion, it is evident that subjects with alcoholic cirrhosis, irrespective of red cell morphology, share a number of qualitative and quantitative abnormalities in lipoprotein and apolipoprotein profile. These abnormalities primarily concern apoA-II, HDL_2, HDL_3 concentrations, the relative amounts of apoA-I and apoA-II in HDL_2 and HDL_3, the mean free cholesterol content of lipoprotein subfractions, and the free cholesterol/phospholipid ratio in lipoprotein subfractions. In all of the aforementioned instances, such alterations were more pronounced in spur cell plasmas, and only diminished LDL levels were unique to cirrhotic subjects presenting with spur cells.

What then are the possible origins of the modifications in lipoprotein profile detailed here? Firstly, we may reasonably assume that the capacity of the cirrhotic liver to synthesize and secrete nascent lipoprotein particles (VLDL and HDL) and lipolytic enzymes (LCAT and hepatic lipase) is reduced, as well as its ability to degrade certain of the lipoproteins (chylomicron and VLDL remnants, LDL, and HDL) formed in the circulation (41). Secondly, the rate of secretion of nascent lipoproteins from the intestine (chylomicrons, VLDL, and HDL) (41, 42) may also be reduced as a consequence of the decreased dietary intake often seen in individuals with alcoholic cirrhosis.

The diminished apoA-II and HDL_3 concentrations of plasma from cirrhotic subjects may therefore directly reflect their low hepatic and intestinal secretory activities, or alternatively, alterations in their catabolism. It is not, however, apparent why apoA-II should be primarily affected, and apoA-I (and apoB) to a lesser extent. Some light may be shed on one aspect of this important question when more is known of the relative hepatic and intestinal rates of secretion of these apoproteins in man.

The elevated free cholesterol and low cholesteryl ester contents of HDL_2 and HDL_3 in cirrhotic plasmas are presumably a direct consequence of low LCAT activity characteristic of liver disease (7, 43), but the precise relationship of such reduced cholesterol esterification to changes in red cell membranes remains controversial (7). Nonetheless, it is noteworthy that all of the lipoprotein subfractions of cirrhotic subjects with or without spur cells contained increased amounts of free sterol. Concomitantly, cholesteryl ester was uniformly reduced in lipoprotein subfractions of d 1.028–1.140 g/ml in both cirrhotic groups. These effects could be due not only to low levels of circulating LCAT protein and thus low rates of cholesterol esterification, but also to changes in substrate availability.

The high levels of HDL_2 in cirrhotic plasma apparently reflect the elevated lipoprotein lipase activity of chronic alcoholics (44), who effectively catabolize triglyceride-rich lipoproteins with the production of HDL_2 in increased amounts (45). This suggestion is entirely consistent with the absence of hypertriglyceridemia and the normal to low circulating VLDL levels seen in the present study. Moreover, a reduced synthesis and, thus, activity of hepatic lipase in cirrhotic subjects would also favor an increase in HDL_2 levels (46). Whether the failure of cirrhotics to degrade such HDL_2 results from a reduction in their apoE content due to diminished hepatic biosynthesis of this protein, and thus inability to interact with hepatic and extrahepatic apoB, E receptors required for cellular degradation of the particle, or whether such HDL_2 contains elevated amounts of apoE, thereby exceeding the removal capacity of the receptor degradation pathway, remains to be elucidated.

Finally, it is pertinent to note that earlier studies (4, 5, 7) have suggested that changes in the lipid composition and surface area of red cell membranes are secondary to alterations in circulating lipoproteins. In this context, we would suggest that while plasma HDL_3 and apoA-II levels may not be determinants per se of spur cell formation, they may constitute, together with the degree of acquired LCAT deficiency (43), useful predictive markers of the likelihood of any alcoholic cirrhotic patient to form spur cells.

G. D. was supported by a Fellowship from the Delegation Generale de la Recherche Scientifique et Technique and P.F. by a Research Fellowship from ICI-Pharma Ltd. P.B. and M.J.C. gratefully acknowledge the award of Grant no. HCEIA no. 1980-02 from the Haut Comité d'Etude et d'Information sur l'Alcoolisme in support of this work. We are most grateful to Dr. B. Fraitag for supplying some blood samples, to Dr. P. K. Weech for provision of purified human apoA-II, apoE, and apoC, and to Drs. T. P. Bersot and C. Ehnholm for stimulating discussion. Mlle. D. Lagrange gave excellent technical assistance. We are especially appreciative of the facilities provided by Dr. R. W. Mahley, Director, Gladstone Foundation Laboratories, San Francisco, California, in the preparation of the manuscript. Mrs. M. Prator kindly prepared the typescript.

Manuscript received 3 February 1983.

REFERENCES

1. Herbert, P. N., A. M. Gotto, and D. S. Fredrickson. 1978. Familial lipoprotein deficiency (abetalipoproteinemia, hypobetalipoproteinemia, and Tangier disease). *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, New York. 544–588.
2. Smith, J. A., E. T. Lonergan, and K. Sterling. 1964. Spur cell anemia: hemolytic anemia with red cells resembling acanthocytes in alcoholic cirrhosis. *N. Engl. J. Med.* **271:** 396–398.

Downloaded from www.jlr.org by guest, on June 18, 2012

3. Silber, R., E. Amorosi, J. Lhowe, and H. J. Kayden. 1966. Spur-shaped erythrocytes in Laennec's cirrhosis. *N. Engl. J. Med.* **275:** 639–643.
4. Cooper, R. A. 1969. Anemia with spur cells: a red cell defect acquired in serum and modified in the circulation. *J. Clin. Invest.* **48:** 1820–1831.
5. Cooper, R. A., E. C. Arner, J. S. Wiley, and S. J. Shattil. 1975. Modification of red cell membrane structure by cholesterol-rich lipid dispersions: a model for the primary spur defect. *J. Clin. Invest.* **51:** 3182–3192.
6. Cooper, R. A., D. B. Kimball, and J. R. Durocher. 1974. Role of the spleen in membrane conditioning and hemolysis of spur cells in liver disease. *N. Engl. J. Med.* **290:** 1279–1284.
7. Cooper, R. A., M. Diloy-Puray, P. Lando, and M. S. Greenbery. 1972. An analysis of lipoproteins, bile acids, and red cell membranes associated with target cells and spur cells in patients with liver disease. *J. Clin. Invest.* **51:** 3182–3192.
8. Cooper, R. A. 1980. Lipids of human red cell membrane: normal composition and variability in disease. *Semin. Hematol.* **7:** 296–322.
9. Sterling, K., H. G. Rose, J. A. Smith, and E. T. Lonergan. 1964. Abnormal erythrocyte cholesterol accumulation in spur cell anemia. *J. Clin. Invest.* **43:** 1235 (Abstract).
10. Ways, P., C. F. Reed, and D. J. Hanahan. 1963. Red cell and plasma lipids in acanthocytosis. *J. Clin. Invest.* **42:** 1248–1260.
11. Phillips, G. B. 1962. Quantitative chromatographic analysis of plasma and red blood cell lipids in patients with acanthocytosis. *J. Lab. Clin. Med.* **59:** 357–363.
12. McBride, J. A., and H. S. Jacob. 1970. Abnormal kinetics of red cell membrane cholesterol in acanthocytes: studies in genetic and experimental abetalipoproteinemia and in spur cell anemia. *Br. J. Haematol.* **18:** 383–397.
13. Flamm, J., and D. Schachter. 1982. Acanthocytosis and cholesterol enrichment decrease lipid fluidity of only the outer human erythrocyte membrane leaflet. *Nature.* **198:** 290–292.
14. Chapman, M. J., S. Goldstein, D. Lagrange, and P. M. Laplaud. 1981. A density gradient ultracentrifugal procedure for isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* **22:** 339–358.
15. Anthony, P. P., K. G. Ishak, N. C. Nayak, H. E. Poulsen, P. J. Scheuer, and L. H. Solin. 1978. The morphology of cirrhosis. Recommendations on definition, nomenclature, and classification by a working group sponsored by the World Health Organization. *J. Clin. Pathol.* **31:** 395–414.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
17. Roeschlau, P., E. Bernt, and W. Gruber. 1974. Enzymatische Bestimmung des Gesamt-Cholesterins im Serum. *Z. Klin. Chem. Klin. Biochem.* **12:** 403–407.
18. Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu. 1977. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta.* **79:** 93–98.
19. Skipski, V. P. 1972. Lipid composition of lipoproteins in normal and diseased states. *In* Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism. G. J. Nelson, editor. Wiley-Interscience, New York. 471–583.
20. Biggs, H. C., J. M. Erickson, and R. Moorehead. 1975. A manual colorimetric assay of triglycerides in serum. *Clin. Chem.* **21:** 437–450.
21. Forte, T. M., A. V. Nichols, and R. M. Glaeser. 1968. Electron microscopy of human serum lipoproteins using negative staining. *Chem. Phys. Lipids.* **2:** 396–408.
22. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. NY Acad. Sci.* **121:** 404–427.
23. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
24. Chapman, M. J., G. L. Mills, and J. H. Ledford. 1975. The distribution and partial characterization of the serum apolipoproteins in the guinea pig. *Biochem. J.* **149:** 423–436.
25. Cheung, M. D., and J. J. Albers. 1977. The measurement of apolipoprotein A-I and A-II levels in men and women by immunoassay. *J. Clin. Invest.* **60:** 43–50.
26. Pagnan, A., R. J. Havel, J. P. Kane, and L. Kotite. 1977. Characterization of human very low density lipoproteins containing two electrophoretic populations: double pre-beta lipoproteinemia and primary dysbetalipoproteinemia. *J. Lipid Res.* **18:** 613–622.
27. Forgez, P., and M. J. Chapman. 1982. A simplified and efficient procedure for the purification of apolipoprotein A-I from human serum high-density lipoprotein-3 by preparative isoelectric focusing on polyacrylamide gel beads. *J. Biochem. Biophys. Methods.* **6:** 283–296.
28. Karlson, C., H. Davies, J. Ohman, and U. B. Andersson. 1973. Analytical thin layer gel electrofocusing in polyacrylamide gel. LKB application note 75.
29. Marcel, Y. L., M. Bergseth, and A. C. Nestruck. 1979. Preparative isoelectric focusing of apolipoproteins C and E from human very low density lipoproteins. *Biochim. Biophys. Acta.* **573:** 175–183.
30. Nestruck, A. C., G. Suzue, and Y. L. Marcel. 1980. Studies on the polymorphism of human apolipoprotein A-I. *Biochim. Biophys. Acta.* **617:** 110–121.
31. Chapman, M. J., and S. Goldstein. 1976. Comparison of the serum low density lipoprotein and of its apoprotein in the pig, rhesus monkey, and baboon, with that in man. *Atherosclerosis.* **25:** 267–291.
32. Ouchterlony, O. 1964. Gel diffusion techniques. *In* Immunological Methods. J. F. Ackroyd, editor. Blackwell, London. 55.
33. Snedecor, G. W., and W. G. Cochran. 1971. Statistical Methods. Iowa State University Press, Ames, Iowa. 128.
34. Groszek, E., and S. M. Grundy. 1978. Electron microscopic evidence for particles smaller than 250 Å in very low density lipoproteins of human plasma. *Atherosclerosis.* **31:** 241–250.
35. Puppione, D. L., T. M. Forte, A. V. Nichols, and E. H. Strisower. 1970. Partial characterization of serum lipoproteins in the density interval 1.04–1.06 g/ml. *Biochim. Biophys. Acta.* **202:** 392–395.
36. Schmitz, G., and G. Assmann. 1982. Isolation of human serum HDL_1 by zonal ultracentrifugation. *J. Lipid Res.* **23:** 903–910.
37. Kostner, G. M. 1972. Isolation and characterization of lipoprotein B from high-density lipoproteins. *Biochem. J.* **130:** 913–917.
38. Weidman, S. W., J. B. Ragland, and S. M. Sabesin. 1982. Plasma lipoprotein composition in alcoholic hepatitis: accumulation of apolipoprotein E-rich high density lipoprotein and preferential reappearance of "light"-HDL during partial recovery. *J. Lipid Res.* **23:** 556–569.
39. Cheung, M. D., and J. J. Albers. 1982. Distribution of high

Downloaded from www.jlr.org by guest, on June 18, 2012

density lipoprotein particles with different apoprotein composition: particles with A-I and A-II and particles with A-I but no A-II. *J. Lipid Res.* **23:** 747–753.
40. Patsch, W., G. Schonfeld, A. M. Gotto, Jr., and J. R. Patsch. 1980. Characterization of human high density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **255:** 3178–3185.
41. Chapman, M. J. 1982. Lipoproteins and the liver. *Gastroenterol. Clin. Biol.* **6:** 482–499.
42. Green, P. H. R., and R. M. Glickman. 1981. Intestinal lipoprotein metabolism. *J. Lipid Res.* **22:** 1153–1173.
43. Sabesin, S. M., H. L. Hawkins, L. Kuiken, and J. B. Ragland. 1977. Abnormal plasma lipoproteins and lecithin-cholesterol acyltransferase deficiency in alcoholic liver disease. *Gastroenterology.* **72:** 510–518.
44. Nilsson-Ehle, P. 1979. Alcohol-induced alterations in lipoprotein lipase activity and plasma lipoproteins. *In* Metabolic Effects of Alcohol. P. Avogaro, C. R. Sirtori, and E. Tremoli, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. 175–186.
45. Taskinen, M. R., M. Valimaki, E. A. Nikkila, T. Kuusi, C. Ehnholm, and P. Ylikahri. 1982. High density lipoprotein subfractions and postheparin plasma lipases in alcoholic men before and after ethanol withdrawal. *Metabolism.* **31:** 1168–1174.
46. Kuusi, T., P. Saarinen, and E. A. Nikkila. 1980. Evidence for the role of hepatic endothelial lipase in the metabolism of plasma high density lipoprotein$_2$ in man. *Atherosclerosis.* **36:** 589–593.

Downloaded from www.jlr.org by guest, on June 18, 2012