

Erythrocyte Echinocytosis in Liver Disease

Role of Abnormal Plasma High Density Lipoproteins

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Abstract

Echinocytes were frequently found in patients with liver disease when their blood was examined in wet films, but rarely detected in dried, stained smears. When normal erythrocytes (discocytes) were incubated with physiologic concentrations of the abnormal high density lipoproteins (HDL) from some jaundiced patients, echinocytosis developed within seconds. Other plasma fractions were not echinocytogenic. There was a close correlation between the number of echinocytes found in vivo and the ability of the corresponding HDL to induce discocyte-echinocyte transformation. On incubation with normal HDL, echinocytes generated in vitro rapidly reverted to a normal shape, and echinocytes from patients showed a similar trend. Echinocytosis occurred without change in membrane cholesterol content, as did its reversal, and was not caused by membrane uptake of lysolecithin or bile acids. Abnormal, echinocytogenic HDL showed saturable binding to $\sim 5,000$ sites per normal erythrocyte with an association constant of 10^8 M^{-1} . Nonechinocytogenic patient HDL and normal HDL showed only nonsaturable binding. Several minor components of electrophoretically separated erythrocyte membrane proteins bound the abnormal HDL; pretreatment of the cells with trypsin or pronase reduced or eliminated binding. Echinocytosis by abnormal HDL required receptor occupancy, rather than transfer of constituents to or from the membrane, because cells reversibly prefixed in the discoid shape by wheat germ agglutinin, and then exposed to abnormal HDL, did not become echinocytes when the HDL and lectin were successively removed. Binding did not cause dephosphorylation of spectrin. We conclude that the echinocytes of liver disease are generated from discocytes by abnormal HDL, and we infer that the shape change is mediated by cell-surface receptors for abnormal HDL molecules.

Introduction

The initial assessment of erythrocyte morphology in liver disease is usually based on examination of dried, stained blood films.

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Unfortunately, drying may cause shape artefacts to which wet preparations and scanning electron microscopy (SEM)¹ are not subject. "Target" cells are common in stained smears from jaundiced patients, but result from distortion because they are codocytic (bell-shaped) in wet films. In 1968, Grahn and colleagues (1) found spiculated erythrocytes in wet blood films from patients with liver disease; they were not evident in dried, stained specimens. This observation has largely been disregarded. They pointed out that these cells (which we shall call echinocytes—see Methods) were also present in "spur cell anemia," a hemolytic disorder seen occasionally in severe alcoholic liver disease and characterized by the presence of acanthocytes² (1-4). Acanthocytes are bizarre, irregularly spiculated cells recognizable in stained smears, as well as in wet films.

Grahn et al. (1) found that the serum from blood containing echinocytes caused echinocytosis of normal erythrocytes; serum from blood that contained acanthocytes also induced formation of echinocytes, but not of acanthocytes. They therefore argued that an additional factor acting in vivo might be necessary to produce acanthocytes and suggested that the spleen might be involved. They were unable to identify the serum fraction(s) responsible for the formation of echinocytes.

Hui and Harmony (5) produced echinocytes by incubating normal erythrocytes with isolated normal low density lipoproteins (LDL). The active constituent was apparently apolipoprotein B (apoB), inasmuch as cyclohexanedione blockage of the arginine residues of apoB reduced echinocytogenic potency (6). The high density lipoprotein (HDL) of patients with liver disease is often enriched in apoE, which competes with the apoB of normal LDL for "apoB,E-receptors" on the surface of nucleated cells (7). We have therefore investigated whether abnormal HDL from jaundiced patients, like normal LDL, would cause echinocytosis. We found echinocytes in patients with liver disease; morphologically similar cells were formed rapidly when normal erythrocytes were incubated with abnormal HDL from these patients. Echinocytosis did not result from addition or extraction of membrane components; the important factor seemed to be reversible, saturable binding of abnormal HDL particles to the erythrocyte surface.

Methods

Patients. HDL were isolated from 18 inpatients with liver disease of varying severity (Table I). Diagnostic methods included liver biopsy,

1. *Abbreviations used in this paper:* apo, apolipoprotein; LPDS, lipoprotein-deficient serum; SEM, scanning electron microscopy.

2. These irregularly spiculated erythrocytes have also been termed "spur cells" and "spur-shaped cells" (2, 3) or "burr cells" (1); we prefer to use the nomenclature of Brecher and Bessis (9), as described in Methods, and call them "acanthocytes."

Table 1. Clinical Data for Patients at the Time of Isolation of Their HDL

| Patient | Age (years) | Sex | Diagnosis | Bilirubin (5-17)* $\mu\text{mol/liter}$ | Aspartate transaminase (5-40) IU/liter | Alkaline phosphatase (35-130) IU/liter | Albumin (30-50) g/liter | Hemoglobin (11.5-16.5) g/dl | HDL apolipoprotein composition† | | % echinocyte II and III | |
|---------|-------------|-----|---------------------------|---|--|--|-------------------------|-----------------------------|---------------------------------|----------------|-------------------------|-------------|
| | | | | | | | | | % apoE | % unidentified | In blood§ | HDL induced |
| J.C. | 54 | F | Primary biliary cirrhosis | 205 | 330 | 1,550 | 36 | 12.0 | 20.8 | 10.8 | ND | 55 |
| A.L. | 53 | M | Cryptogenic cirrhosis | 57 | 17 | 5 | 30 | 11.8 | 12.5 | 25.3 | ND | 9 |
| R.B. | 54 | M | Alcoholic hepatitis | 11 | 132 | 15 | 42 | 13.6 | 5.0 | 0 | ND | 8 |
| J.R. | 57 | F | Primary biliary cirrhosis | 555 | 116 | 231 | 46 | 11.6 | 23.3 | 5.0 | ND | 100 |
| M.M. | 23 | F | Chronic active hepatitis | 51 | 71 | 12 | 43 | 12.7 | 4.0 | 0 | ND | 2 |
| C.C. | 83 | M | Carcinoma pancreas | 279 | 158 | 1,045 | 28 | 10.6 | 9.7 | 0 | 0 | 0 |
| M.W. | 57 | F | Chronic active hepatitis | 18 | 51 | 67 | 38 | 14.1 | 8.0 | 25.5 | 0 | 0 |
| M.H. | 57 | M | Carcinoma pancreas | 31 | 21 | 606 | 33 | 11.0 | 10.3 | 13.5 | 3 | 2 |
| B.L. | 72 | F | Primary biliary cirrhosis | 42 | 57 | 444 | 34 | 14.2 | 6.8 | 1.0 | 3 | 0 |
| J.S. | 37 | F | Subacute hepatic necrosis | 133 | 62 | 237 | 20 | 11.1 | 20.0 | 0 | 15 | 8 |
| S.F. | 52 | M | Cryptogenic cirrhosis | 32 | 33 | 119 | 25 | 8.9 | 19.2 | 11.9 | 17 | 6 |
| L.M. | 38 | M | Alcoholic hepatitis | 620 | 130 | 278 | 28 | 9.8 | 19.2 | 0 | 17 | 30 |
| R.C. | 24 | M | Hodgkin's disease | 450 | 352 | 950 | 31 | 12.5 | 10.1 | 7.1 | 19 | 58 |
| M.A.-M. | 58 | F | Cryptogenic cirrhosis | 19 | 21 | 72 | 42 | 9.9 | 13.0 | 0 | 40 | 32 |
| P.K. | 36 | M | Alcoholic cirrhosis | 420 | 116 | 354 | 22 | 13.2 | 30.0 | 3.0 | 64 | 60 |
| A.S. | 32 | F | Lupoid hepatitis | 395 | 445 | 147 | 22 | 10.6 | 10.1 | 0 | 69 | 100 |
| M.F. | 65 | F | Alcoholic cirrhosis | 249 | 61 | 125 | 33 | 4.3 | 5.2 | 0 | 84 | 76 |
| M.T. | 49 | F | Alcoholic cirrhosis | 187 | 36 | 159 | 31 | 9.9 | 10.1 | 0 | 86 | 100 |

ND, not determined. * Normal range. † Apolipoproteins from patient HDL were separated by isoelectric focusing and relative amounts were measured by densitometry. Unidentified apolipoproteins refer to the two glycoproteins with pI values of 6.3 and 6.0 (see Results). § The percentage of patient erythrocytes that were echinocytes II and III was determined in wet films by differential counting of 200 cells in three to four fields using light microscopy. || Washed, normal erythrocytes (6×10^9 cells/ml of Hanks' solution) were incubated for 1 min at 37°C with freshly isolated HDL from the patients (final concentration 500 μg of protein/ml).

cholangiography, and/or surgery. Five patients had obstructive jaundice (three intrahepatic, two extrahepatic): thirteen had parenchymal liver disease and two of them (M.F. and M.T.), both with hemolytic anemia, showed acanthocytes in dried blood smears. In the last 13 patients investigated, erythrocytes were examined in wet films and by SEM. Reversibility of echinocytosis was studied in 30 additional patients, mostly alcoholic cirrhotics, selected because >10% of their cells were echinocytes. HDL were isolated from three patients (K.N., M.J., and S.J.) with abetalipoproteinemia, and their erythrocyte morphology was studied by SEM. These patients lack apoB-containing lipoproteins and their clinical details have been reported previously (8). Normal subjects were healthy medical and laboratory staff.

Reagents. Human serum albumin (<0.005% fatty acids) and all other materials were from Sigma Chemical Co., Poole, England, except Hanks' balanced salt solution (Flow Laboratories Ltd., Irvine, Scotland), Cab-O-Sil and Merck silica gel (BDH Chemicals Ltd., Poole, England), and pronase (Calbiochem-Behring Corp., San Diego, CA).

Lipoproteins. Very low density lipoproteins (VLDL, $d < 1.006$ g/ml), LDL (1.019–1.063), HDL (1.063–1.21) and lipoprotein-deficient serum (LPDS, $d > 1.25$) were isolated by differential ultracentrifugation and washed once with buffer of the appropriate density (7). The lipoproteins were used within a day or two of isolation and were passed through a 0.2- μ m filter before addition to erythrocyte suspensions; techniques used for compositional analysis, labeling with 125 I, and chemical modification have been described previously (7).

Erythrocyte isolation, morphology, and analysis. Erythrocytes were separated by centrifugation at 4°C of venous blood anticoagulated with 10 U/ml heparin. They were washed three times with Hanks' solution, brought to a hematocrit of ~50% and fixed for 30 min at 0°C by adding an equal volume of 1% glutaraldehyde in Hanks' solution. Morphology was examined by light microscopy of wet films and frequently by SEM (Philips 501). When whole blood was fixed, erythrocyte shape appeared identical to that in washed preparations.

Use of SEM has allowed reclassification of erythrocyte shape and a better insight into the relationship between spiculated forms (9). In liver disease four "spiculated" erythrocyte shapes can be recognized in addition to normal biconcave discs (discocytes). These are discs with protuberances around the edge (echinocytes I), flat cells with spicules over the surface (echinocytes II), spherical or ellipsoidal cells with a large number of spicules evenly distributed over the surface (echinocytes III), and cells with a bulbous body and a small number of irregularly spaced spicules, generally with knobby, club-shaped ends (acanthocytes) (Fig. 1). These shapes may generally be distinguished by light microscopy of wet films,

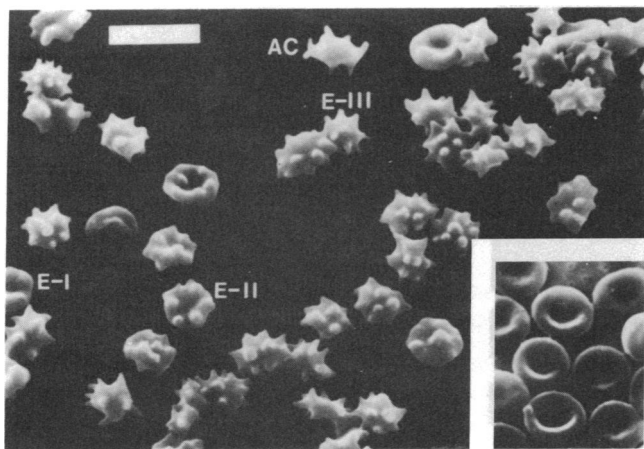


Figure 1. Scanning electron micrograph of erythrocytes from a patient (M.F., Table I) with alcoholic cirrhosis and associated hemolytic anemia. Examples of acanthocytes (AC), and type I (E-I), type II (E-II), and type III (E-III) echinocytes are labeled. (Inset) Discocytes from a normal subject. Calibration bar = 10 μ m.

but we suggest that the identification of acanthocytes should rest on SEM. Using light microscopy the proportion of echinocytes II and III in a cell population was determined by counting 200 cells in three to four fields; the observer was unaware of the source of material.

Erythrocyte and lipoprotein lipids were extracted with isopropanol-chloroform and chloroform-methanol, respectively, and the concentrations of individual phospholipids measured as described previously (10, 11). Total bile acids were determined by the technique of Barnes and Chitranukroh (12).

General incubation procedures. Washed erythrocytes (usually 10 μ l containing 6×10^9 cells/ml of Hanks' solution) were incubated with 4 vol of lipoprotein or reagent. Lipoprotein concentrations are expressed as milligram of protein per milliliter, and all concentrations of lipoproteins or reagents refer to the final incubation mixture. Incubations were stopped by fixation with 1 vol of 1% glutaraldehyde or, if addition of a second reagent was required, by diluting with 50 vol of Hanks' solution, followed by immediate centrifugation and a second wash. Further details are given in legends to our tables and figures. Washed erythrocytes were enriched in cholesterol using cholesterol-rich phosphatidylcholine dispersions (13).

Binding studies. Washed erythrocytes (25 μ l of a 10% suspension) were added to 250 μ l of varying concentrations of 125 I-labeled lipoprotein with 0–40 mg/ml human serum albumin and incubated for 40 min at room temperature with periodic agitation. Cells were separated from unbound lipoprotein, by brief centrifugation of the suspension (200 μ l) through a barrier (50 μ l) of dibutylphthalate, and bound 125 I was measured. Determinations were generally made in quadruplicate and blanks (without erythrocytes) were subtracted; the correction was ~10%.

To identify binding components, membrane proteins were first fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (14). The protein zones were electrophoretically transferred to a nitrocellulose membrane and nonspecific binding sites blocked by agitation for 24 h in 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2 mM sodium azide, pH 7.5, containing 25 mg/ml bovine serum albumin (15). The buffer was then replaced by 125 I-labeled patient HDL at 0.1–1.0 mg of protein/ml in the same buffer. After 2 h of gentle agitation at room temperature the nitrocellulose membrane was rinsed, dried, and left in contact with preflashed Kodak X5 film at –70°C for up to 4 h. Duplicate lanes of the original SDS gel were stained with Coomassie blue.

Washed erythrocytes (10% hematocrit) were subjected to proteolysis by incubating for 1 h at 37°C with 0.5 mg/ml of trypsin, chymotrypsin, or pronase in 5 mM sodium phosphate, 150 mM NaCl, pH 7.4. The cells were washed, membranes were prepared, and their protein compositions were analyzed by SDS-gel electrophoresis in 0.1 M Tris-Bicine *N,N*-bis(2-hydroxyethyl)glycine, pH 8.1 (15). The "dot-blot" technique was used to establish whether binding of patient HDL still occurred; membrane protein was spotted onto washed nitrocellulose, incubated with radioiodinated HDL and the amount of binding measured.

Other studies. For measurement of phosphorylation changes in the spectrin of the membrane cytoskeleton, erythrocytes were prelabeled with 32 P (16) and incubated with lipoproteins. Cells were removed at intervals and their membrane proteins were separated by electrophoresis (14) and stained with Coomassie Brilliant Blue G. Relative concentrations of spectrin were estimated by dye elution with 70% (vol/vol) formic acid and the 32 P content by Cerenkov counting.

Erythrocytes (10^7 /ml) were treated with wheat germ agglutinin (20 μ g/ml), incubated with abnormal HDL or other echinocytic agents for 1 min at 37°C, and washed three times; bound lectin was displaced by addition of 20 mM *N*-acetylglucosamine (17). Erythrocytes were also treated with the oxidant diamide to introduce disulphide bonds predominantly between spectrin chains (18). Cross-links were removed by addition of 15 mM dithiothreitol.

Results

Erythrocyte morphology. SEM was used to study erythrocytes from 30 patients with liver disease, all with at least 10% echino-

cytes II and/or III in wet films (mean 47%, range 10–98%). Fig. 2 *A* and *B* exemplify the morphologic variation at the lower (14%) and upper (83%) ends of the range. Discocytes and echinocytes I, II, and III could be seen in most preparations; specimens with the highest degree of morphologic abnormality contained mainly type II echinocytes and those with the least mainly type I. No acanthocytes were seen in this group of 30 patients.

Two of the 18 patients (M.T. and M.F., Table I), from whose blood HDL was isolated, were alcoholic cirrhotics with a hemolytic anemia and “spur cells.” Acanthocytes were evident by SEM (Fig. 1 and Fig. 2 *C*) but comprised only 4–6% of the total erythrocyte population; most cells were echinocytes II or III. Erythrocytes in the other 16 patients showed variable echinocytosis but no acanthocytes in wet films or by SEM; in only two (A.S. and S.F.) were spiculated cells reported on routine examination of dried, stained smears. Light microscopy of wet films allowed discrimination of the various types of echinocytes (Fig. 3 *A*) and acanthocytes (Fig. 3 *C*). Like Grahn et al. (1), we found almost no type II or III echinocytes in dried, stained films (Fig. 3 *B*), but acanthocytes were readily recognized (Fig. 3 *D*).

In all three patients with abetalipoproteinemia small proportions of acanthocytes were present, but most cells were echinocytes II and III (Fig. 2 *D* and *E*).

Effects of liver disease HDL on normal erythrocytes. Normal erythrocytes were incubated for 1 min at 37°C with HDL from jaundiced patients at a concentration similar to that in plasma (500 µg/ml) (19, 20); echinocytes I, II, or III appeared in the suspension (Table I, Fig. 4). With some patient HDL, only

echinocytes I and II were formed (Fig. 4 *A*); with HDL from others (A.S., M.T., and J.R., Table I) complete conversion to type II or III echinocytes occurred (Fig. 4 *B*). The ability of a patient’s HDL to transform normal erythrocytes correlated with the proportion of echinocytes in the patient’s blood ($r = 0.92$, $P < 0.001$; Table I). Less than 1% of normal cells became echinocytes when incubated with HDL from normal subjects. Echinocytosis occurred within the time required to stop the reaction (>5 s); no further changes were seen with incubation for up to 4 h. The degree of echinocytosis was dependent on HDL concentration but independent of temperature, giving the same result at 0°C and at 37°C after a 1-min incubation (Fig. 5).

Several patients’ VLDL, LDL, HDL, and LPDS were isolated and incubated with normal erythrocytes for 1 min at 37°C. Only the HDL and LPDS fractions were echinocytogenic at concentrations equal to those in the original plasma. LPDS was much less effective than HDL but may still have contained lipoprotein components, derived artefactually by ultracentrifugation (21), because it became inactive on treatment with Cab-O-Sil. VLDL, present at low concentration in severe parenchymal liver disease (20), became echinocytogenic only when concentrated above 1 mg/ml. Patient LDL was inactive except with longer incubations (>1 h) and higher concentrations (>2 mg/ml). Suspending the washed cells in normal plasma or normal LPDS before addition of abnormal HDL caused only minor reduction in the number of echinocytes produced. When active patient HDL was incubated overnight at 4°C with normal LPDS and recovered by ultracentrifugation, it retained its echinocytogenic ability; nor-

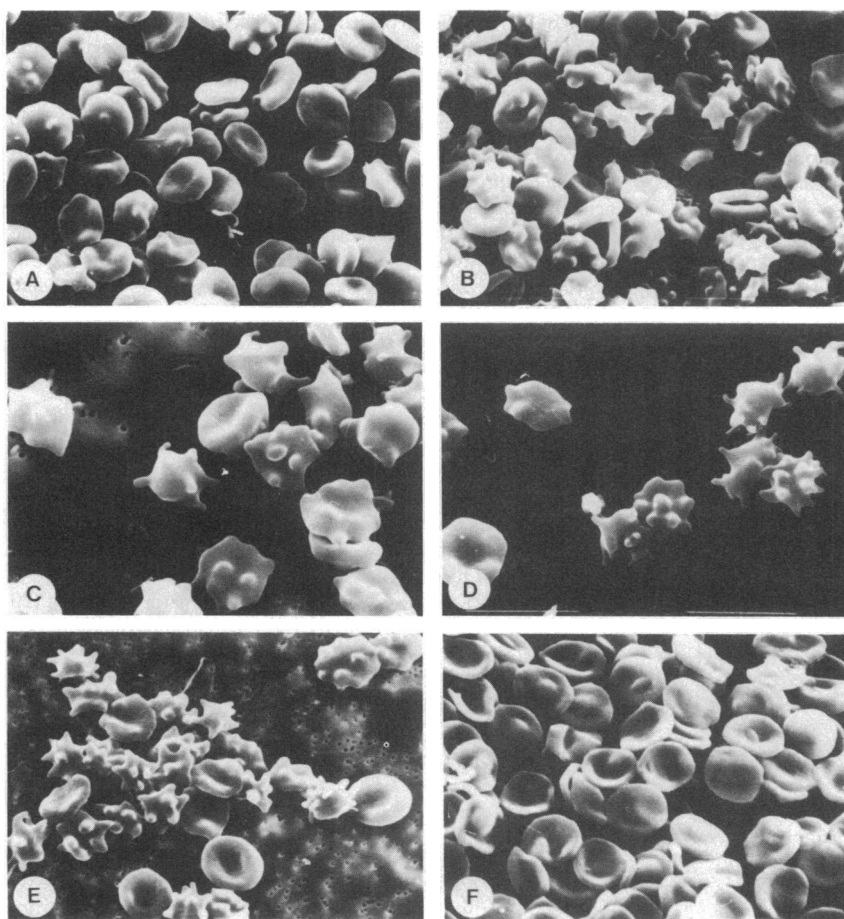


Figure 2. Scanning electron micrographs of erythrocytes from patients with liver disease or abetalipoproteinemia, and of cholesterol-enriched normal erythrocytes. (*A* and *B*) Examples of the various types of echinocytes seen in liver disease. The acanthocytes of liver disease (M.T., Table I) and abetalipoproteinemia (K.N. and S.J., Reference 8) are illustrated in *C*, *D*, and *E*, respectively. (*F*) Erythrocytes enriched in cholesterol by incubation in vitro with a cholesterol-rich phospholipid dispersion (cholesterol/phospholipid molar ratio = 1.38 compared with 0.85 in control cells).

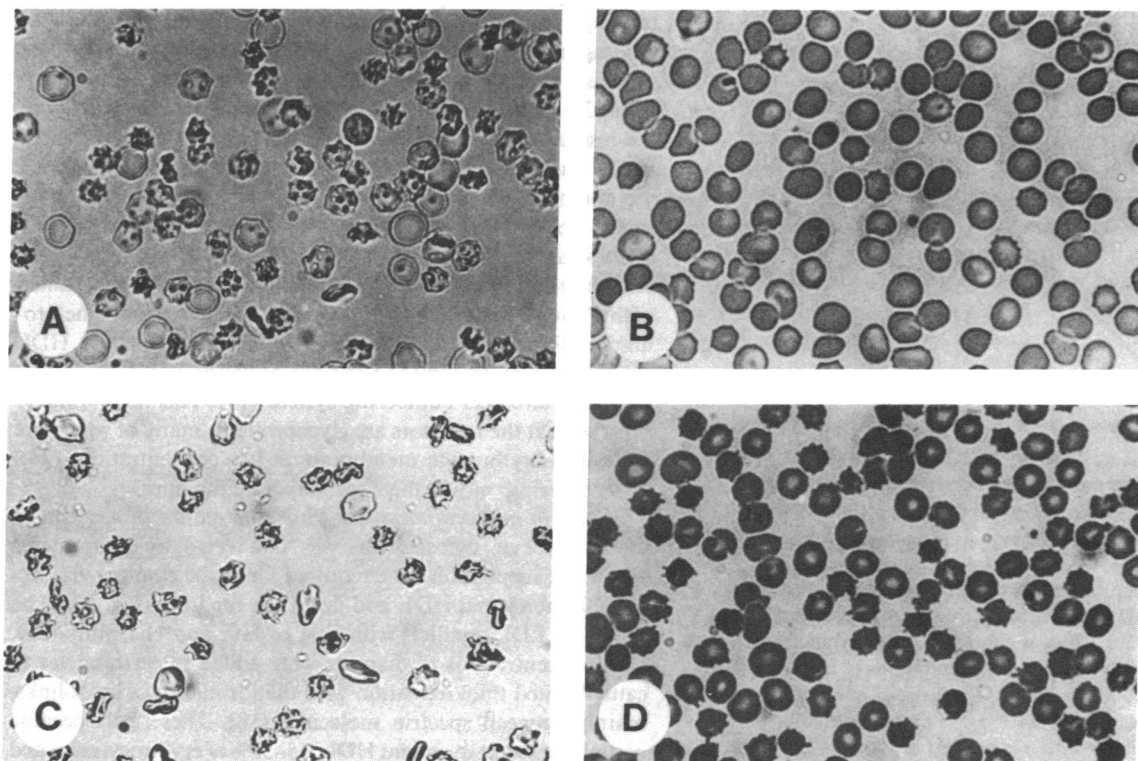


Figure 3. Light microscopy of wet films and dried stained blood smears from patients with liver disease. (A) Echinocytes are clearly visible when wet films are examined from patient P.K. (Table I) but are

rarely seen in dried smears (B). In a patient with "spur cell" anemia (M.T., Table I) acanthocytes can be distinguished from echinocytes in wet films (C) and are also recognized in dried films (D).

mal HDL did not acquire shape-transforming properties after incubation with patient LPDS.

Reversibility of the discocyte-echinocyte transformation. Echinocytes formed in seconds retained their abnormal shape on prolonged incubation (up to 4 h) with the patient HDL, and also showed little reversal after washing and subsequent reincubation in Hanks' solution for up to 8 h. When they were incubated with normal HDL there was complete reversal to normal erythrocyte morphology within seconds (Fig. 6). Normal LPDS induced only partial reversal even at high concentrations.

Reversibility of echinocytes found in liver disease. Normal HDL (8 mg/ml) was added to suspensions of erythrocytes from

30 patients with liver disease, whose blood contained at least 10% echinocytes II and III. In each case there was a rapid reduction in the percentage of type II and III echinocytes (Fig. 6 and Fig. 7). Complete reversal was rarely achieved, possibly because the cells had adapted to their new morphology during prolonged exposure to the echinocytic agent and reversed only slowly when the agent was removed (22). Excess normal HDL also ameliorated the echinocytosis of cells from the two patients with spur cell anemia but the proportion of acanthocytes was unchanged, suggesting that acanthocytosis was irreversible.

Binding studies. Lipoprotein binding by erythrocytes reached equilibrium within 30 min, but the small number of sites per

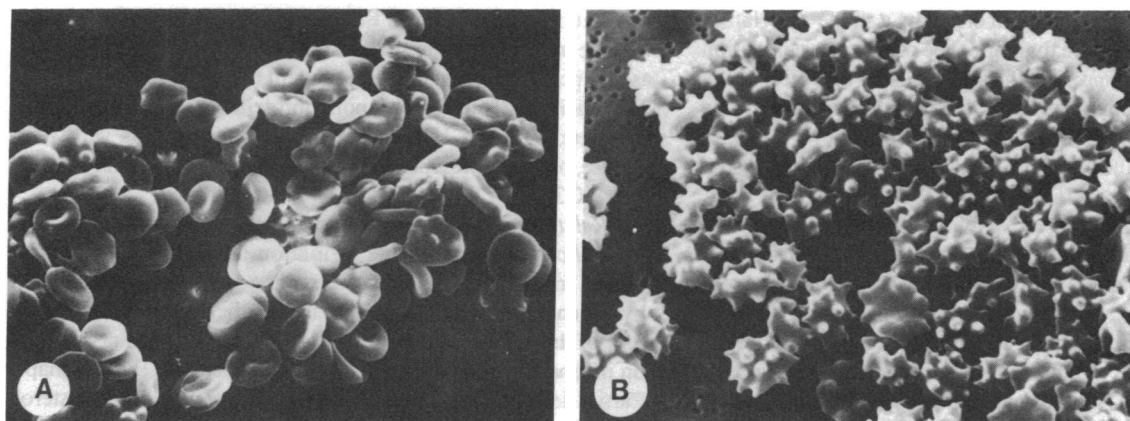


Figure 4. Ability of HDL from patients with liver disease to transform normal erythrocyte into echinocytes. Normal, washed erythrocytes (6×10^9 cells/ml Hanks' buffer) were incubated for 1 min at 37°C with freshly isolated HDL (final concentration $500 \mu\text{g}$ of protein/ml) from patients S.F. (A) and A.S. (B).

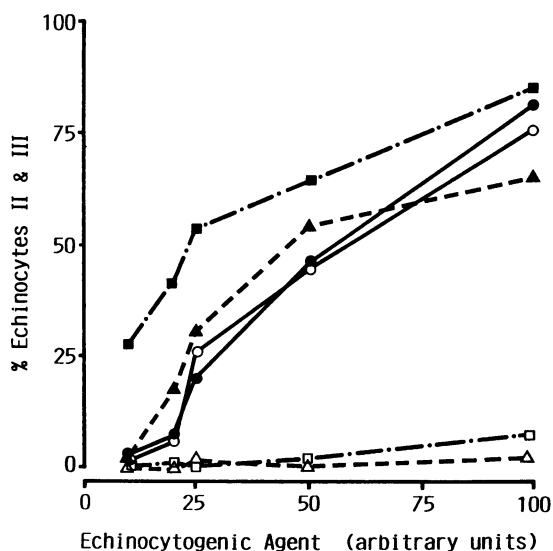


Figure 5. Transformation of normal erythrocytes to echinocytes at 0°C and 37°C. Normal, washed erythrocytes (6×10^9 cells/ml Hanks' buffer) were incubated for 1 min at either 0°C (open symbols) or 37°C (solid symbols) with increasing concentrations of freshly isolated HDL from patient M.A.-M. (○, ●; 100 U = 1 mg of protein/ml), lysolecithin-enriched plasma (□, ■; 100 U = 430 nmol lysolecithin/ml plasma) or plasma enriched with bile salts (△, ▲; 100 U = 2.5 μmol total bile acids/ml plasma). The cells were then fixed and the percentage of echinocytes II and III determined in wet films by differential counting of 200 cells in three to four fields using light microscopy.

cell limited precision of measurement. Strongly echinocytogenic HDL (patient A.S., Table I) was bound by erythrocytes in a saturable manner when added in increasing concentration (Fig. 8). Saturation of sites was achieved at an HDL concentration of ~40 nM (10 μg/ml), at which point conversion to erythrocytes II and III had reached a plateau at ~85%. Labeled HDL was displaced by unlabeled HDL, and counts were also progressively lost by repeated washing of the cells. These findings are consistent with a reversible binding process, and assuming independent, noninteracting sites, the binding curve can be satisfactorily fitted by a single binding process with an association constant of $\sim 1 \times 10^8 \text{ M}^{-1}$ (inset to Fig. 8). There appeared to be 4,000–5,000 sites per cell. Defatted albumin reduced the amount of abnormal HDL bound, but a large part was not displaced (Fig. 9). Thus, despite the fit (within experimental error) by a single association constant, there may be more than one type of site; one population being specific, the other nonspecific and available for competition by albumin. Such studies required large amounts of HDL and only one other potent HDL was examined (patient M.T., Table I); this also gave a binding profile consistent with the presence of saturable sites. By contrast normal HDL, and patient HDL with little or no echinocytogenic activity, did not appear to bind to the sites. No saturable binding was detected, and instead there was a continuous uptake of lipoprotein in the manner of a partition process over the wide concentration range tested (0–6,000 nM, data not shown). Normal LDL, which did induce erythrocyte shape changes on prolonged incubation, also showed no saturable binding. This disagrees with the report of Hui et al. (6).

The dot-blot technique indicated that binding of radioiodinated patient HDL could be affected by proteolytic pretreatment of erythrocytes and pronase treatment largely eliminated

binding. However, trypsin caused only partial reduction of binding and chymotrypsin was ineffective, possibly because these proteases degrade only certain of the externally accessible proteins (23) or because cleaved fragments remain associated with the residue of the parent molecule (24). When labeled patient HDL was incubated with the electrophoretically separated membrane proteins several bands were detected by autoradiography. These were weak, presumably because the small number of sites had relatively modest association constants, and did not obviously correspond to major membrane proteins when stained with Coomassie blue or with periodate-Schiff reagent. The pronase-sensitive membrane proteins which bound patient HDL were approximately in the positions of bands 3, 4.9 (or 5), and 6 in the Fairbanks numbering system (14). This heterogeneity suggests that the receptors are glycoproteins, many of which are present in erythrocyte membranes at low concentrations (25), some differing only in their carbohydrate moieties.

Spectrin and echinocytosis. Phosphorylation of spectrin in the erythrocyte cytoskeleton was unaffected by lipoproteins within the incubation time required for shape changes viz. seconds for abnormal HDL and up to 1 h for LDL (Fig. 10). The result for LDL conflicts with that of Hui and Harmony (26). Low concentrations of diamide were added to erythrocytes to cause limited thiol oxidation with the formation of cross-links, mainly between spectrin molecules (18). These cells became partially resistant to patient HDL; 45–50% of erythrocytes treated with diamide remained discocytic after addition of abnormal HDL compared to <10% of untreated cells. The protective effect of diamide was reversed on reduction of the cross-links with dithiothreitol.

Apolipoproteins and echinocytosis. The proportion of apoE in HDL from our 18 patients was higher ($13.2 \pm 7.3\%$, mean \pm SD, range 4.0–30.0%) than that in HDL from 10 normal subjects ($3.9 \pm 1.0\%$, range 2.6–5.4%; $P < 0.001$), but there was no significant correlation with echinocytogenic potency ($r = 0.26$, $P > 0.05$; calculated from Table I). Agents that prevent apoE binding by apoB,E receptors on nucleated cells, e.g., protamine (200 μg/ml) or heparin (10 mg/ml), or cyclohexanedione modification of apolipoprotein arginine residues (7), did not impede echinocytosis. Moreover, discocytes from patients with homozygous familial hypercholesterolaemia, which lack apoB,E receptors, were readily transformed to echinocytes. HDL from three patients with abetalipoproteinemia also contained excess apoE ($10.5 \pm 0.8\%$, range 9.6–11.1%) but were nonechinocytogenic at concentrations up to 1.5 mg/ml; presumably other factors are more important for echinocytosis in these patients, such as the increased sphingomyelin content (8) in the cell membrane. Two additional, more basic apolipoproteins, previously described (7), were found in HDL from nine of the patients with liver disease, but their presence did not reflect echinocytogenic activity ($r = -0.31$, $P > 0.05$).

Relation of changes in erythrocyte shape to erythrocyte cholesterol content. Echinocyte formation by patient HDL, as expected from the rapidity of the process, was not accompanied by net cholesterol uptake (Table II). Furthermore, the lipid content of these echinocytes, and those of patients, did not change on incubation with normal HDL and reversion to normal shape. Partial delipidation of patient HDL removed 92% of cholesterol, 24% of phospholipid, and essentially all of the cholesteryl ester and triglyceride, but did not affect echinocytogenic potency. We found, in agreement with others (13, 27, 28), that normal erythrocytes became broader and flatter on enrichment with mem-

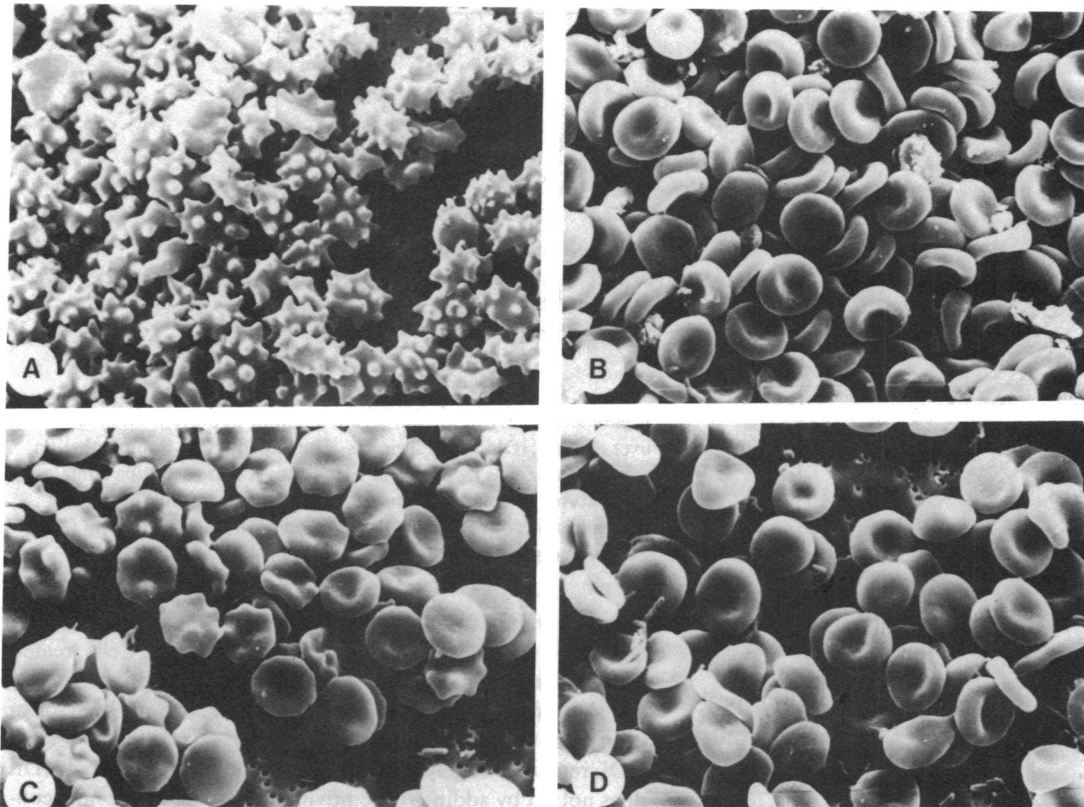


Figure 6. Reversibility of echinocytes produced by incubation of normal red cells with HDL isolated from jaundiced patients and of echinocytes found in liver disease. Normal erythrocytes were transformed to echinocytes II and III by HDL from patient A.S. as described in Fig. 4 (A). After washing, these echinocytes were incubated for 1 min at

37°C with normal HDL (final concentration 8 mg of protein/ml) to give complete reversal to discocytes (B). Echinocytes from a patient with alcoholic cirrhosis also returned towards a normal erythrocyte shape when similarly incubated with normal HDL; the percentage of echinocytes II and III present were reduced from 14% (C) to zero (D).

brane cholesterol by equilibration with cholesterol-rich liposomes (Fig. 2 F); many showed peripheral distortion as in type I echinocytes. Type II echinocytes were rare, and probably resulted from prolonged incubation, in that they also appeared in controls.

Amphipaths and echinocyte formation. Small amounts of amphipathic anions or other ligands cause echinocytosis by partitioning mainly into the outer leaflet of the membrane bilayer and expanding its area relative to the inner leaflet (29–31). We studied two physiologic amphipaths, bile salts and lysolecithin, to determine whether they could be the active components of abnormal HDL. Echinocytosis by bile salts or lysolecithin, and its reversal by addition of normal HDL, was rapid and the echinocytes produced resembled by SEM those generated by patient HDL. However, HDL-induced echinosytosis could not be explained by detectable increases of these amphipaths in active HDL; freshly isolated echinocytic HDL contained no more lysolecithin than normal HDL (1–2% of total phospholipid) and had a low total bile acid content (2–9 nmol/mg protein) when compared to inactive HDL from four patients with extrahepatic obstructive jaundice (11–17 nmol/mg protein). Furthermore, we found the echinocytogenic effects of bile salts, lysolecithin and patient HDL to differ. Normal plasma was enriched with either lysolecithin (by incubation at 37°C) or bile salts (by adding equal amounts of sodium taurocholate and sodium glycochenodeoxycholate) until it produced 65–85% type II and III echinocytes on 1 min of incubation at 37°C. In contrast to patient

HDL, far less echinocytosis occurred when the incubations were repeated at 0°C (Fig. 5). Echinocytes formed by exposure to bile salts reverted to discocytes after three washes with Hanks' solution; those generated by lysolecithin or by patient HDL did not revert. Washed echinocytes produced by lysolecithin acquired a more normal shape after 7–8 h of incubation, presumably because lysolecithin progressively entered the inner membrane leaflet (31), but those produced by patient HDL did not reverse. VLDL, LDL, HDL, and LPDS were prepared from plasma enriched with lysolecithin or bile salts and washed once by ultracentrifugation, and their volumes were adjusted to that of the original plasma sample. Only the LPDS fractions were found to be echinocytogenic; the HDL produced <1% type II and III echinocytes.

These results seem to us to exclude lysolecithin and bile salts as the active constituents of echinocytogenic HDL. To assess whether other, so far unrecognized, amphipaths might be present we (reversibly) fixed a erythrocyte suspension in the discocytic shape with wheat germ agglutinin (17). Aliquots were incubated either with plasma enriched in lysolecithin or in bile salts (prepared as above) or with abnormal HDL. In each case echinocytosis was markedly inhibited, the number of type II and III echinocytes produced being reduced from 65–85% to 3–10%. Reagents were removed by washing three times with 50 vol of Hanks' buffer (without albumin) and then the bound lectin was dissociated by addition of *N*-acetylglucosamine. Cells exposed to lysolecithin became predominantly echinocytic; as expected,

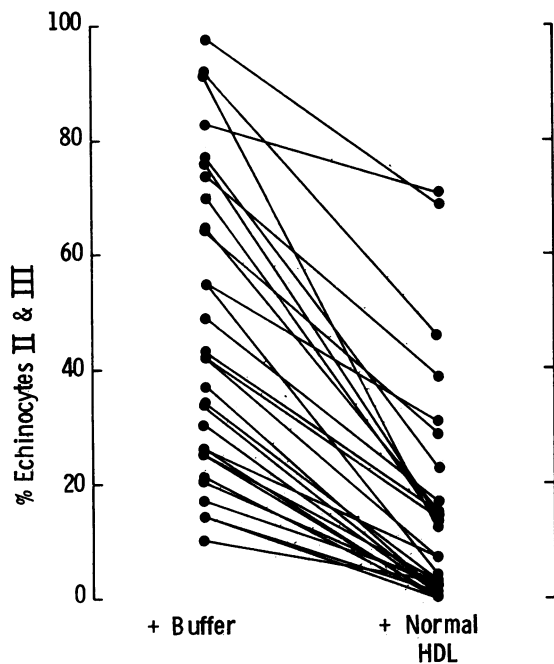


Figure 7. Reversibility of echinocytes found in liver disease by normal HDL. Suspensions of washed erythrocytes (6×10^9 cells/ml Hanks' buffer) containing at least 10% of echinocytes II and III were obtained from 30 patients with liver disease, mainly alcoholic cirrhotics. One portion of the cells was incubated with normal HDL (final concentration 8 mg of protein/ml) for 1 min at 37°C and another with Hanks' buffer. The cells were then fixed and the percentage of echinocytes II and III determined in wet films by differential counting of 200 cells in three to four fields using light microscopy.

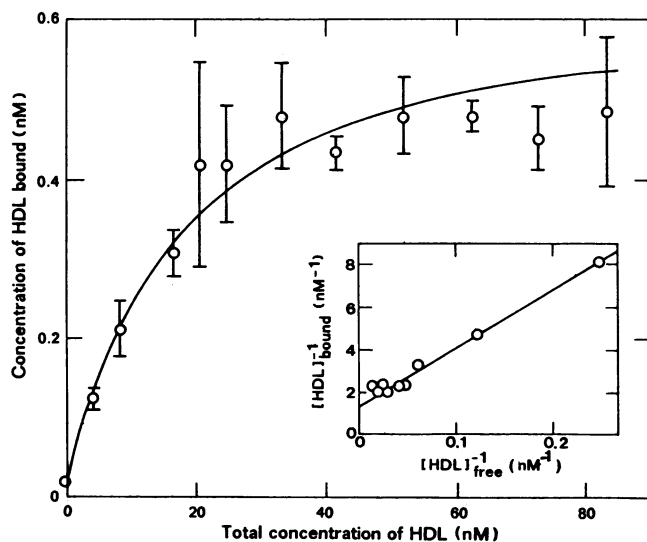


Figure 8. Saturable binding of ^{125}I -labeled liver disease HDL by normal erythrocytes. Cells were incubated for 40 min at room temperature with increasing concentrations of HDL from a patient with lupoid hepatitis (A.S., Table I). Each point is the mean of four determinations; bars are 1 SD. The molecular weight of the HDL was assumed to be 0.25×10^6 and the curve (—) is calculated for 4,500 binding sites per erythrocyte and $K_a = 1 \times 10^8 \text{ M}^{-1}$. *Inset:* double reciprocal plot of $1/[\text{HDL}]_{\text{bound}}$ vs. $1/[\text{HDL}]_{\text{free}}$.

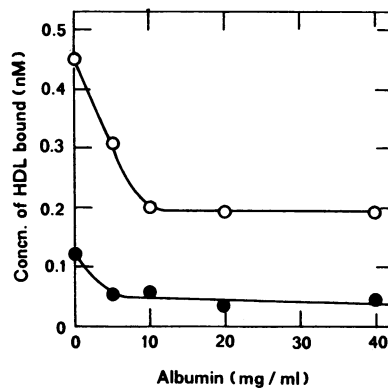


Figure 9. Inability of human serum albumin to prevent binding of liver disease HDL by erythrocytes. The patient HDL and incubation conditions were as described in Fig. 8 except that each tube contained up to 40 mg of defatted human serum albumin per milliliter. Final concentrations of patient HDL were either $31 \mu\text{g}$ of protein/ml (○) or $14 \mu\text{g}$ of protein/ml (●).

the membrane of lectin-fixed cells took up the amphipath and retained it during washing and lectin dissociation. Cells treated with bile salts remained discocytic, because washing reverses bile salt-induced echinocytosis (unlike that induced by lysolecithin or by patient HDL, see above). Cells exposed to abnormal HDL remained largely discocytic, suggesting that abnormal HDL does not act by adding (or removing) lipophilic membrane constituents; it must occupy its receptors to be echinocytogenic.

Discussion

The literature on erythrocyte shape in liver disease is confused by inconsistent terminology. The term "spur cell," which should be reserved for acanthocytes, has often been used for spiculated cells, seen *in vivo* or generated *in vitro*, that are in fact echinocytes (as defined by Brecher and Bessis [9]). Spur cell anemia, (*i.e.*, with acanthocytes) is rare, but we have frequently found echinocytes in jaundiced patients when their blood was examined in wet films; unlike acanthocytes, they were not usually evident on routine examination of stained smears. The protein or lipopro-

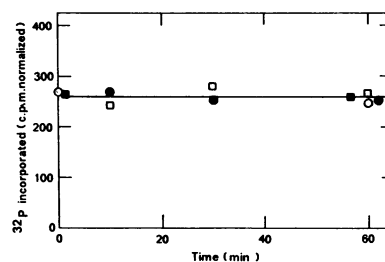


Figure 10. Inability of liver disease HDL, or normal LDL and normal HDL, to dephosphorylate spectrin in normal erythrocytes. Erythrocyte phosphorus was labeled by preincubation with $^{32}\text{P}_i$ and then incubated for up to 1 h with buffer (○), HDL from a patient (M.T., Table I) with alcoholic cirrhosis (□; 0.5 mg of protein/ml), normal HDL (■; 1.0 mg of protein/ml) or normal LDL (●; 1.0 mg of protein/ml). Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis (14) and the bands corresponding to spectrin were removed. Their ^{32}P content was measured by Cerenkov counting and the values were normalized to a constant spectrin concentration as estimated by dye elution of the stained bands.

Table II. Unaltered Erythrocyte Lipid Composition after HDL-induced Changes in Erythrocyte Shape

| Erythrocyte source | Cholesterol | Phospholipid | % Total lipid phosphorus | | | | | | |
|---|-------------|--------------|----------------------------|----------------------------|-----|------|------|------|-----|
| | | | LL§ | SM | PI | PS | L | PE | PA |
| | | | nmol/10 ⁷ cells | nmol/10 ⁷ cells | % | % | % | % | % |
| Normal cells | 3.55 | 4.08 | 1.1 | 27.1 | 0.6 | 13.5 | 29.1 | 27.5 | 2.1 |
| Transformed cells* | 3.54 | 4.17 | 1.0 | 26.9 | 0.5 | 12.8 | 29.6 | 27.4 | 1.8 |
| Transformed cells reversed with normal HDL* | 3.68 | 4.10 | 1.1 | 27.8 | 0.5 | 12.9 | 29.4 | 26.3 | 2.0 |
| Patient cells | 5.68 | 4.32 | 0.8 | 18.9 | 0.7 | 11.8 | 43.7 | 22.2 | 1.9 |
| Patient cells incubated with normal HDL‡ | 5.86 | 4.44 | 0.9 | 18.6 | 0.5 | 12.4 | 42.9 | 22.5 | 2.2 |

* Washed, normal erythrocytes (6×10^9 cells/ml Hanks' solution) were incubated for 1 min at 37°C with freshly isolated HDL from patient M.T. (final concentration 250 μ g of protein/ml). All cells were transformed to echinocytes II or III. After washing, one portion of the transformed cells was incubated for 1 min at 37°C with normal HDL (8 mg/ml) to give complete reversal to discocytes. Similar results were found in a further experiment using HDL from patient A.S. ‡ Washed erythrocytes (6×10^9 cells/ml Hanks' solution) from patient A.S. were incubated for 1 min at 37°C with normal HDL (8 mg/ml) to reduce the percentage of type II or III echinocytes (from 69% to 21% of the cells). The experiment was repeated twice using erythrocytes from two additional patients (M.T. and M.F.); the same conclusions were drawn (data not shown). § Abbreviations: LL, lysolecithin; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; L, lecithin; PE, phosphatidylethanolamine; PA, phosphatidic acid.

tein fraction(s) present in liver disease plasma that is responsible for echinocyte and/or acanthocyte formation has not previously been identified (1, 32, 33) but our results establish that the echinocytogenic agent can be isolated with abnormal HDL.

In liver disease, erythrocyte membranes tend to acquire excess cholesterol from abnormal circulating lipoproteins (34, 35), but this is unlikely to explain echinocytosis, inasmuch as spiculated cells have been found unaccompanied by elevation in membrane cholesterol content (32) and vice versa (36). The shape of cells loaded with cholesterol in our experiments, and in others (13, 27, 28), bore little resemblance to the echinocytes of jaundiced patients and could not be reversed by brief incubation with normal HDL. Cholesterol enrichment was evidently unrelated to echinocytosis induced in vitro by abnormal HDL, for the latter occurred in seconds, without increase in membrane cholesterol, and could also be induced by cholesterol-depleted HDL.

Two physiologic amphipaths producing echinocytes, lysolecithin and bile salts, were excluded as the active constituents of patient HDL; echinocytogenesis by these agents, and the properties of the resulting echinocytes, differed in several ways from those induced by abnormal HDL. Moreover, our experiments with wheat germ agglutinin tend to exclude other, so far unrecognized, amphipaths as echinocytic components of patient HDL, even were there evidence of their existence; in any case, the different properties of the echinocytes generated by known amphipaths renders such a mechanism unlikely. We conclude that echinocytogenesis involves the occupation of binding sites on the cell surface by abnormal HDL. How this causes the imbalance of area between the membrane leaflets implied by echinocytosis (22, 37–39) is uncertain, although cytoskeletal changes (though not dephosphorylation of spectrin) may be involved, in that partial cross-linking of spectrin molecules by diamide inhibited echinocytosis. Removal of abnormal HDL by washing did not reverse the echinocytosis, possibly because a slow passive adaptation by the cytoskeleton (22, 38, 39) is required for reversal in the absence of normal HDL.

Our evidence suggests that abnormal HDL acts via saturable receptors, numbering $\sim 5,000$ per cell. Because binding and rapid echinocytosis occurred within the same concentration range, and

because other plasma lipoproteins that do not show saturable binding were inactive, it seems reasonable to conclude that receptor binding and shape change are causally related. The receptors involved are unlikely to be the 160,000-mol wt apoB,E surface-receptors present in nucleated cells inasmuch as these have not been found in mature red cells (40). Moreover, neither agents that prevent binding of lipoproteins by apoB,E receptors, nor the use of erythrocytes from a patient with a genetic deficiency of apoB,E receptors on their nucleated cells, impeded echinocytosis by abnormal HDL. Our tentative conclusion that the receptors are glycoproteins is based on their susceptibility to proteolysis and their heterogeneity; proof will require additional experimental evidence. Their function is also obscure, although other cell types have surface receptors which bind lipoprotein molecules without internalization (41). They may, as suggested for insulin receptors (42), remain from an earlier stage of erythroid cell development.

We have not yet identified the constituent(s) of abnormal HDL required for receptor binding and echinocytosis, although the retention of echinocytogenic activity after partial delipidation suggests an apolipoprotein as the active component. If this is a minor part of the total protein, then the association constant will be considerably higher than is apparent from the binding curves. Liver disease HDL is markedly heterogeneous (19, 20, 43) and our present aim is to isolate a population of potent, homogeneous HDL molecules and to identify the active component.

What is the pathophysiologic consequence of erythrocyte shape changes in liver disease? Erythrocyte survival is often diminished in patients with liver disease, but is not usually a major clinical problem. Target cells are thought to have a normal life span (34), but acanthocytosis in liver disease is associated with marked hemolysis and predominant splenic destruction, although anemia is not a prominent feature of abetalipoproteinemia or of certain other acanthocytic disorders (44). Echinocytes might be expected to have a shortened life as the spleen removes many cells of abnormal shape. Powell et al. (45) considered that only in patients with spur cells did morphologic alterations lead to the premature removal of cells (though their scanning electron micrograph of a spur cell showed an echinocyte). The hemoglo-

bin levels in our patients did not correlate closely with the number of echinocytes in their blood. However, hemoglobin concentration in liver disease is altered by many factors (34) and the degree of hemolysis is best assessed by direct measurement.

Are echinocytes the precursors of acanthocytes as suggested by Grahn et al. (1) and Cooper et al. (46) and what is the mechanism for the conversion? The preponderance of echinocytes in our patients with spur cell anemia and with abetalipoproteinemia is consistent with such a precursor-product relationship. However, we believe the suggestion (13, 46) that, in liver disease, the conversion occurs in the spleen as a consequence of cholesterol enrichment must be viewed with caution. Evidence for a splenic conversion is derived from a single case (46) whereas, in other disorders, splenectomy can itself produce acanthocytosis (47). The relevance of cholesterol enrichment to acanthocytogenesis must also be questioned, as in abetalipoproteinemia the increase in cell cholesterol content is only marginal. It seems, therefore, that in liver disease the echinocytic shape, induced by abnormal HDL, may be a more critical factor to the conversion than the reductions in membrane fluidity (35) and cell deformability (13) caused by excess cholesterol. Clearly, additional studies are needed, both to establish the relationship between erythrocyte survival in liver disease and cell morphology and membrane lipid composition, and to understand in greater detail factors regulating erythrocyte shape in general.

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