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INTERACTION OF PLASMA LIPOPROTEINS WITH ERYTHROCYTES

I. ALTERATION OF ERYTHROCYTE MORPHOLOGY

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Summary

Intact erythrocytes incubated in the presence of low density lipoproteins (LDL) undergo a time-dependent morphologic transformation from biconcave discs to spherocytes within 4 h. No shape change is observed when erythrocytes are incubated with high density lipoproteins (HDL). The LDL-induced change in erythrocyte morphology occurs without concomitant leakage of hemoglobin from the cell or depletion of intracellular ATP; no change in the distribution of the major lipids of the erythrocyte membranes was detected. The alteration of morphology does require attachment of LDL to the erythrocyte surface. The LDL-induced morphologic alteration is inhibited by HDL, but not by serum albumin. HDL prevent the attachment of LDL to the cell membrane; however, the HDL subfractions, HDL_2 and HDL_3, are only partially effective. These data suggest that normal erythrocyte morphology and cell function may depend on the concentration and composition of the circulating lipoproteins.

Introduction

Plasma lipoproteins are the major macromolecular complexes which transport lipids in the circulation. The major class of lipoproteins which transports cholesterol is that of the low density lipoproteins (LDL) [1]. It is well established that elevated LDL cholesterol is associated with the development of coronary heart disease [2—4]. On the other hand, a high level of HDL appears to inhibit the development of premature atherosclerosis [5,6]. Moreover, lipoproteins have an important role in the regulation of lipid metabolism at the extracellular and intracellular levels [7,8]. The processes of lipid transport,

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Abbreviations: LDL, low density lipoproteins (*d* 1.019—1.063); HDL, high density lipoproteins (*d* 1.063—1.21) which are subclassified as HDL_2 (*d* 1.063—1.124) and HDL_3 (*d* 1.124—1.21) [1,22].

regulation of intracellular lipid metabolism, and the accumulation of lipoproteins by tissues require interaction between lipoproteins, cell surfaces and tissue constituents [9—11]. LDL bind with both high and low affinity to the plasma membrane of a number of cell types [12—16]. Studies from various laboratories have also indicated that HDL bind to cell surfaces [11,17]. In some cell types [11,17,18], HDL reduce the cellular uptake of LDL, particularly that due to low affinity binding.

In measuring the response of cultured cells to added lipoproteins, it is difficult to distinguish lipoprotein-induced alterations at the cell surface from effects due to cellular uptake and degradation of the lipoproteins. Thus, in the present communication we have utilized the mature erythrocyte, a cell type which does not take up and degrade lipoproteins, to study the effects of lipoproteins on cell morphology and physiology. Shore and Shore [19] have reported that VLDL and LDL activate a specific Mg^{2+}-ATPase situated in the erythrocyte membrane. In addition, LDL promote agglutination of the cells by concanavalin A [20]. While mecahnisms for these alterations are not known, it is thought that the initial event is an interaction of the lipoprotein at the cell surface. In this study we have attempted to isolate cell membrane events by investigating the interaction of LDL and HDL with intact, mature human erythrocytes. Our findings show that lipoproteins affect the morphology of intact erythrocytes. In addition, we have determined the influence of these lipoproteins on the osmotic sensitivity of the cells and on the concentration of intracellular ATP, two criteria important for normal erythrocyte morphology and function.

Methods

Isolation of erythrocytes. Human blood was collected in EDTA from fasting healthy male donors and was used immediately without storage. Erythrocytes were isolated by centrifugation at 2000 × *g* for 10 min; the plasma and buffy coat were removed by aspiration. The erythrocytes were further purified by the procedure of Poon and Simon [21]. The isolated cells were washed 3 times in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM KCl.

Isolation and characterization of lipoproteins. Lipoproteins were isolated from the freshly collected plasma of fasting human donors by sequential ultracentrifugal flotation in KBr [22] at 15°C using a Beckman 50.2 Ti rotor. VLDL and any intermediate density lipoproteins were removed after centrifugation at *d* 1.019 for 18 h at 50 000 rev./min. To obtain LDL, the plasma density was increased to 1.063 and the solution was centrifuged for 18 h at 50 000 rev./min. KBr was subsequently added to LDL-free plasma to raise the density to 1.21, and the density-adjusted plasma was centrifuged for 24 h at 47 000 rev./min to float lipoproteins of the high density class. Densities were measured with a hydrometer. The purity of each lipoprotein fraction was assessed by electrophoresis on agarose (1%) and cellulose acetate, and by immunochemical analysis using antibodies prepared against the purified lipoproteins and selected apolipoproteins (AI, AII, B). LDL did not react with antibodies raised against human HDL, nor with antiapolipoprotein AI or antiapolipoprotein AII. A very faint precipitin line was observed when HDL were analyzed by immuno-

TABLE I

CHEMICAL COMPOSITION OF THE PLASMA LIPOPROTEINS

LDL and HDL were isolated and analyzed as described in Methods. The chemical composition was determined before and after incubation for 4 h at 37°C with intact erythrocytes. Reported values are subject to an error of ±3%.

Lipoprotein	Composition (mg/mg lipoprotein)						
	Protein	Phospholipid	Protein: phospholipid *	Cholesterol	Cholesteryl esters	Total cholesterol: phospholipids *	Glyceride
LDL							
before incubation	0.21	0.22	1.05	0.09	0.36	2.05	0.11
after incubation	0.21	0.22	1.06	0.09	0.36	2.04	0.12
HDL							
before incubation	0.51	0.25	0.5	0.05	0.15	0.80	0.05
after incubation	0.50	0.25	0.5	0.04	0.15	0.78	0.05

* The ratios are weight/weight.

diffusion using antiLDL; HDL did react with antiHDL, antiapolipoprotein AI and antiapolipoprotein AII. The lipid composition (phospholipid, triglyceride, cholesterol, cholesteryl esters) of each lipoprotein class was within the nornal range (ref. 23 and Table I). Lipids were extracted from the lipoproteins at 4°C in diethyl ether/ethanol (3 : 1, v/v) according to the method of Scanu and Edelstein [24]. Lipoprotein samples were dialyzed exhaustively against 150 mM NaCl before use.

Analytical procedures. Protein concentration was determined by a modified method of Lowry et al. [25] using 1% sodium dodecyl sulfate (SDS); bovine serum albumin was used as standard. Total triglyceride was determined enzymically [26] using the Triglycerides Test Combinations Kit (Boehringer Mannheim). Total phospholipid was measured as phosphorus by the method of Bartlett [27]. Cholesterol and cholesteryl esters were determined by the method of Roeschlau et al. [28] using the Cholesterol Test Combination Kit (Boehringer Mannheim). Individual phospholipids were separated by thin-layer chromatography on silica gel 1B2 (J.T. Baker Chemical Co.) in a solvent system of chloroform/acetone/methanol/acetic acid/water (10 : 4 : 2 : 2 : 1, v/v); after localization with iodine, the phospholipids were scraped from the TLC plate and analyzed by the method of Bartlett [27].

ATP was extracted from the erythrocyte suspensions with 10% $HClO_4$ at 4°C for 1 h. The samples were then centrifuged at 4°C for 15 min at 20 000 × *g*. The supernatant solutions were decanted, neutralized with 1 M KOH, and the resulting precipitates removed by centrifugation at 20 000 × *g* for 20 min. The amount of ATP in the sample was determined by the phosphoglycerate kinase assay of Adam [29].

Incubation of erythrocytes with lipoproteins. Erythrocytes were resuspended with an equal volume of 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and glucose, adenine and $CaCl_2$ at final concentrations of 10 mM, 1 μM

and 1 mM, respectively. As indicated, LDL were added to a final concentration of 2.5 mg/ml; HDL were included at a final concentration of 0.25 or 4.5 mg/ml. When added, bovine serum albumin (Sigma Chemical Co., Fraction V) or defatted human serum albumin (Miles Laboratories, Inc.; <0.1 mol fatty acid per mol of albumin) was included in the incubation mixture at a final concentration of 50 mg/ml. In all experiments, the maximum incubation time was 4 h at 37° C. At the end of the incubation, erythrocytes were pelleted by centrifugation and were washed three times in 150 mM NaCl. LDL and HDL were reisolated from the supernatant fraction by ultracentrifugation in KBr between *d* 1.020—1.063 and 1.063—1.210, respectively. Densities were measured with a hydrometer.

Erythrocyte morphology. Erythrocyte morphology was assessed by scanning electron microscopy. The cells were fixed at 4° C for 1 h by the addition of an equal volume of 1% glutaraldehyde in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4. After extensive washing with distilled water, the packed erythrocytes were dehydrated with acetone and were vacuum evaporated ($1 \cdot 10^{-5}$ Torr). The specimens were then coated with light carbon and gold-palladium (60 : 40), and the cells were examined with an ETEC-Autoscann electron microscope. Alternatively, erythrocyte morphology was observed by light microscopy. After fixation in 1% glutaraldehyde, a few drops of the erythrocyte suspension were placed on glass slides for observation.

Osmotic sensitivity. Erythrocytes were washed extensively and resuspended to 20% by volume in 150 mM NaCl. Osmotic fragility was measured by adding 70 µl of the cell suspension to 5 ml of a series of hemolyzing solutions of different salt concentrations. After incubation at 23° C for 30 min, the extent of hemolysis was determined by measuring the absorbance at 576 nm of the supernatant solution following centrifugation [30]. Cell suspensions diluted in distilled water served as a standard for total hemoglobin.

Determination of the composition of red cell membrane. Washed erythrocytes were lysed and membrane ghosts prepared by the method of Dodge et al. [31]. Packed ghosts were resuspended in 5 mM sodium phosphate, pH 8.0, to give approx. 0.5 mg/ml protein. Membrane lipids were extracted by the chloroform-methanol procedure described by Waku and Lands [32].

LDL binding to erythrocytes. Binding of LDL to erythrocytes was assayed with [*Me*-^{3}H]LDL. The radiolabeled lipoprotein was prepared by adding 65.5 µmol formaldehyde to 80 mg LDL at 4° C. After 2 min, 10 µl NaB^3H_4 (New England Nuclear; 50 mCi/ml) was then added. The solution was mixed for 2 min after which an additional 20 µl NaB^3H_4 were added. The methylated LDL was dialyzed at 4° C against 4 l 150 mM NaCl to remove the unreacted reagents. Only 1.5% of the radioactivity associated with the lipoprotein could be extracted by chloroform/methanol (2 : 1, v/v). Incubation of [*Me*-^{3}H]LDL with erythrocytes was carried out as described above. After the incubation period, erythrocytes were washed three times with 150 mM NaCl and digested with periodic acid overnight. The samples were treated with H_2O_2 prior to the addition of 15 ml Aquasol.

Results

Influence of lipoproteins on the morphology of erythrocytes

The majority of erythrocytes incubated for 4 h at 37°C in the absence of lipoproteins remain intact and retain the native biconcave disc form (Fig. 1A); approx. 5% of the control cells are crenated. However, when erythrocytes are incubated with LDL, the morphology is significantly altered. As shown in Fig. 1B, both echinocytes and spherocytes are present. The time dependence of this morphologic alteration was assessed by light microscopy (Fig. 2). The photographs show that the transformation occurs after a 2 h incubation of LDL with erythrocytes. Incubation of erythrocytes with LDL (2.5 mg/ml) for periods greater than 5 h or with higher concentrations of LDL results in hemoglobin release. When erythrocytes are incubated with HDL (0.25 mg/ml) the cells remain biconcave discs for 4 h (Figs. 1C and 1D) and longer. Furthermore, exposure to an 18-fold higher concentration of HDL (4.5 mg/ml) or to serum albumin (50 mg/ml, defatted and non-defatted) does not induce alterations of erythrocyte morphology (micrographs not shown). Figs. 1 and 2 are representative of the results of numerous experiments. Since it is difficult to appreciate the statistical aspects of cell morphology after viewing single photographs, Table II was prepared to indicate the relative populations of discocytes, echinocytes, and spherocytes present in the various incubation mixtures described pictorially in Figs. 1 and 2.

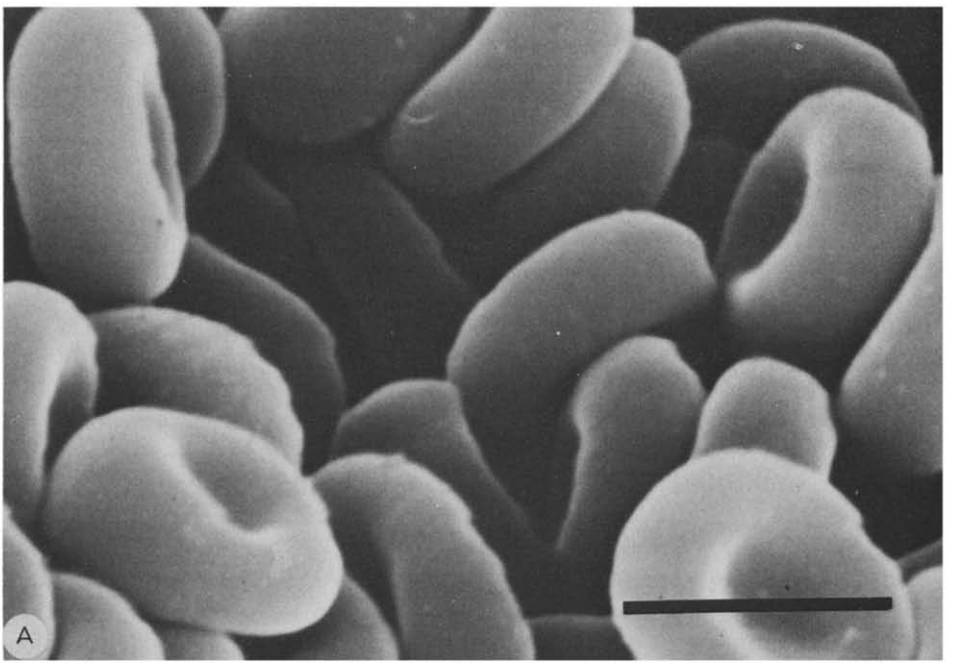
Lipoprotein and erythrocyte membrane lipid composition

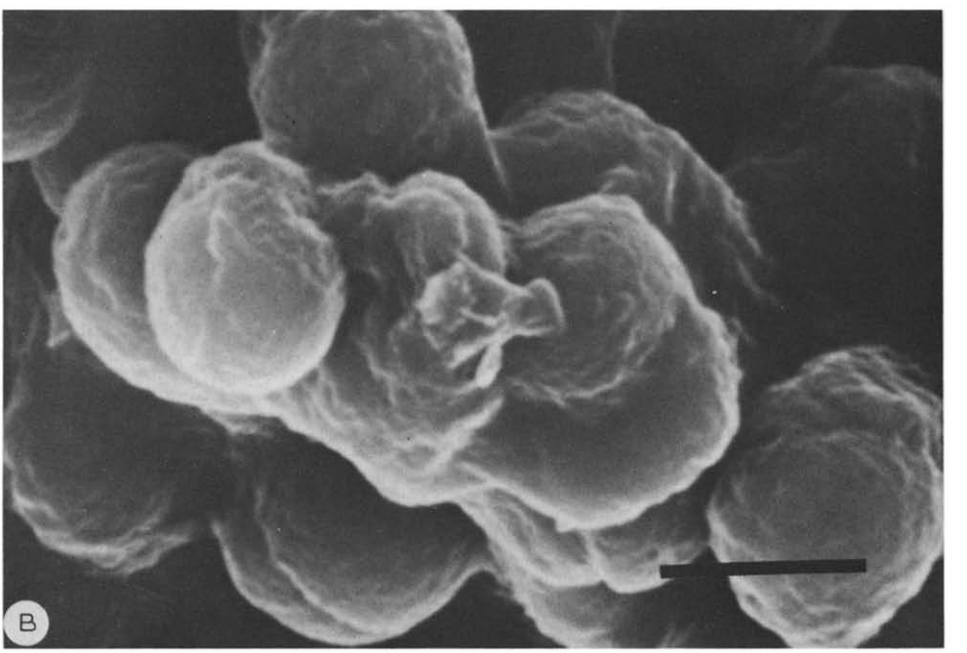
Since unesterified cholesterol and phospholipids of plasma lipoproteins exchange with the lipids of the erythrocyte membrane [33,34], it was of interest to determine if the LDL-induced shape alteration might be due to the loss or gain of membrane lipid constituents. As shown in Table III, there are no significant differences in the phospholipid to cholesterol ratio of ghosts prepared from erythrocytes incubated in the presence or absence of lipoproteins: the mol ratio of phospholipid : cholesterol is 1.5 ± 0.01. No net change in the concentration of the major phospholipids of the cell membrane could be detected. Moreover, the composition of the plasma lipoproteins is unaltered by incubation with erythrocytes (Table I). The reisolated lipoproteins float at the respective densities required to float the original particles: 1.063 for LDL and 1.21 for HDL. Comparison of LDL with reisolated LDL and of HDL with reisolated HDL reveals that the ratios protein : phospholipid and cholesterol : phospholipid are identical. Typically, greater than 90% of the added lipoproteins are recovered following incubation with erythrocytes.

Lipoproteins contain trace amounts of non-esterified fatty acids and of monoacyl phosphatidylcholine, both of which are known to alter the morphology of erythrocytes and to induce cell lysis [35,36]. The concentrations of these constituents in our system are below values which we are able to determine accurately (note, for example, the values of monoacyl phosphatidylcholine reported in Table III). Since serum albumin complexes both lipids [37] and reverses lysin-induced sphering [35], we investigated the possibility that this protein prevents the LDL-induced shape change. Albumin, 50 mg/ml, added to the erythrocyte suspension coincident with LDL does not inhibit the morphologic alteration elicited by LDL.

Erythrocyte ATP

Human erythrocytes undergo morphological transformation from biconcave discs to crenated spheres upon the depletion of intracellular ATP [38]. There-





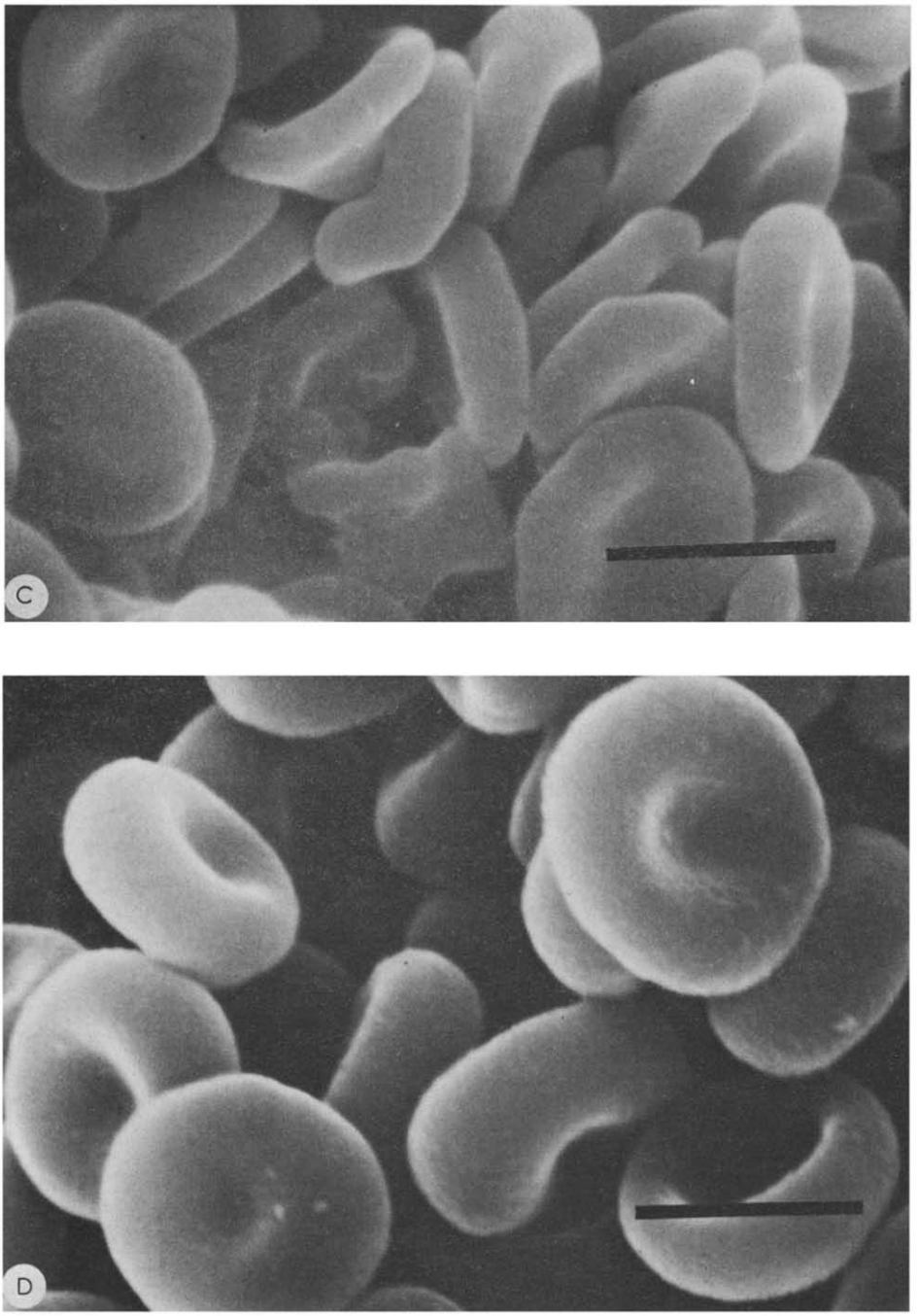
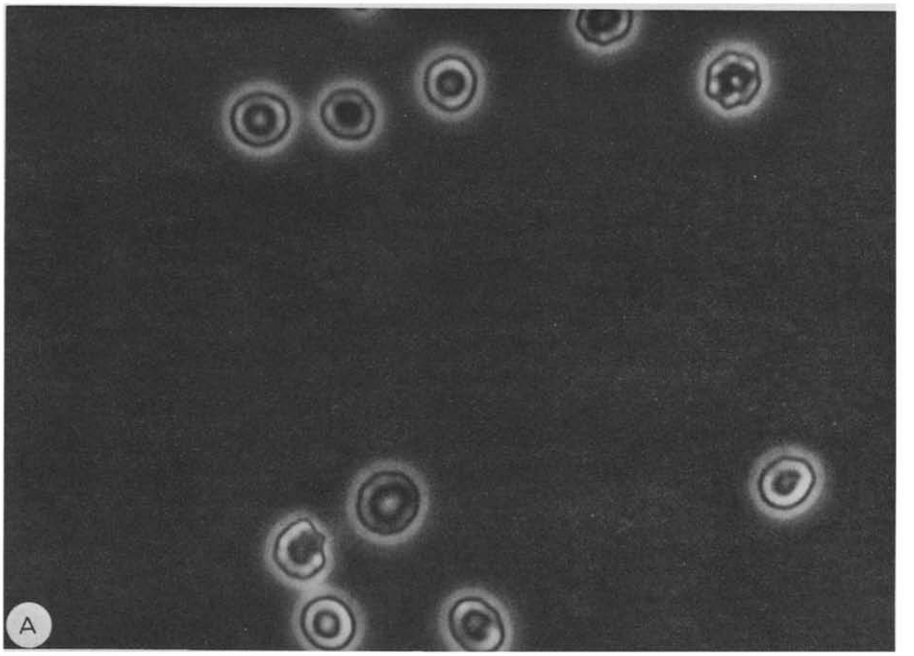
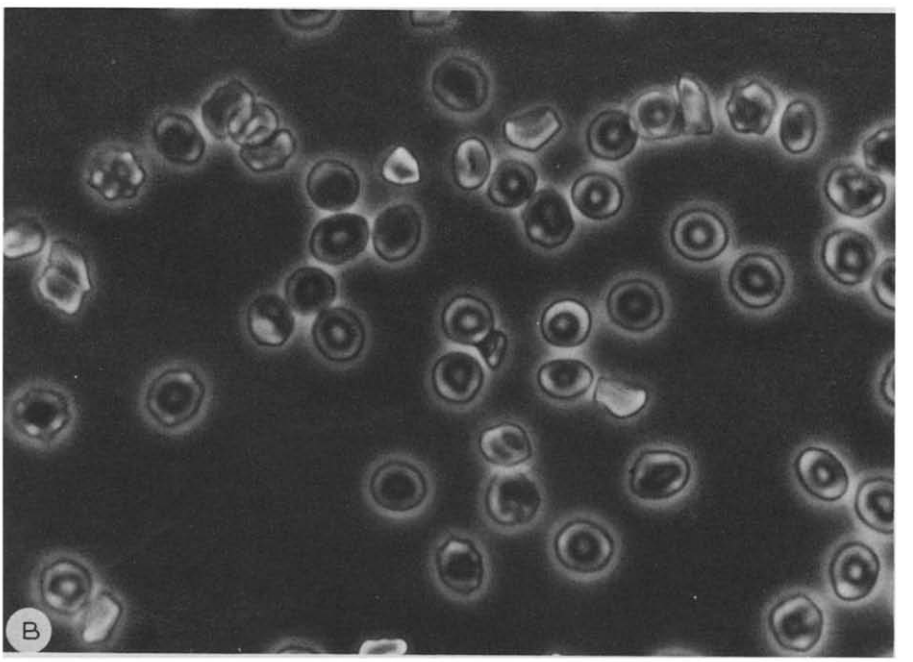


Fig. 1. Scanning electron micrographs of human erythrocytes. Erythrocytes were incubated in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM KCl, 10 mM glucose, 1 μM adenine, and 1 mM $CaCl_2$ for 4 h. (A) No lipoprotein added. (B) LDL added to 2.5 mg/ml. (C) HDL added to 0.25 mg/ml. (D) HDL_2 plus HDL_3 added in an equimolar ratio to 0.25 mg/ml. Samples were fixed with 1% glutaraldehyde, dehydrated with acetone, vacuum evaporated, and coated with light carbon and gold-palladium (60 : 40). Bar represents 5 μm.

fore, the concentration of intracellular ATP was determined in an attempt to correlate it with LDL-induced shape change. Neither LDL nor HDL affect the intracellular level of ATP. Erythrocytes incubated in the presence of LDL or HDL or in the absence of lipoproteins have an intracellular ATP concentration





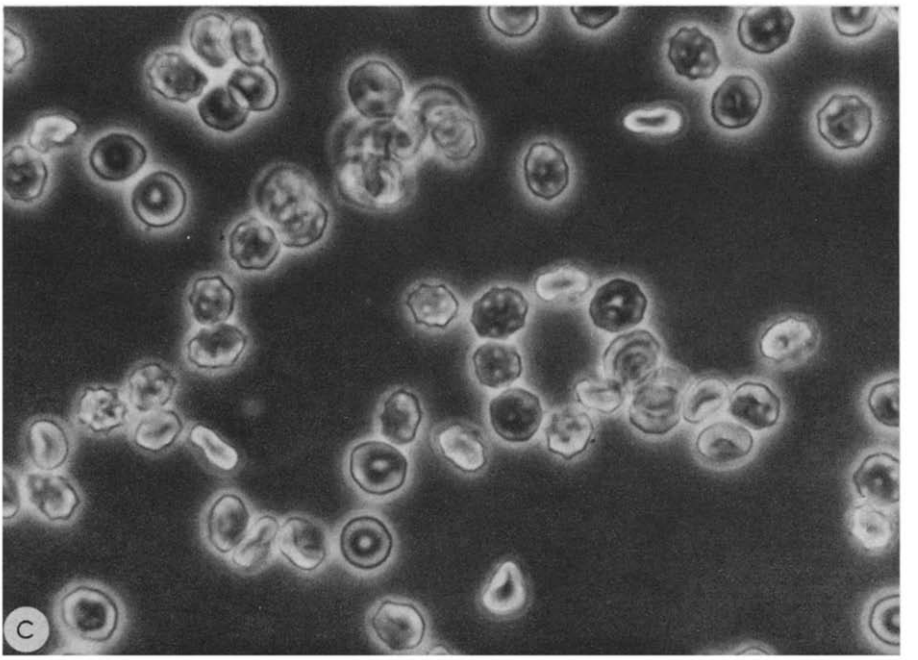


Fig. 2. Light microscope observations of erythrocytes incubated with LDL. Human erythrocytes were incubated with LDL in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM KCl, 10 mM glucose, 1 μM adenine, and 1 mM $CaCl_2$ for: A, 1 h; B, 2 h; C, 3 h. Samples were fixed with 1% glutaraldehyde before observation. Magnification 1500X.

of 40 ± 1 μmol/100 ml packed cells, a value in agreement with that reported by Nakao et al. [38] for erythrocyte populations containing approx. 5% crenated cells; a similar percentage of crenated cells was present in our control experiments (Fig. 1A).

Osmotic sensitivity

The effect of lipoproteins on the osmotic sensitivity of erythrocytes was investigated (Table IV). After incubation for 4 h in the presence of LDL (2.5 mg/ml), erythrocytes are more resistent to osmotic lysis than cells incubated in the absence of added lipoproteins. HDL (4.5 mg/ml) have little influence on the osmotic fragility of erythrocytes. Although the osmotic fragility of cells from different donors varies, the extent of the decrease in cell fragility in the presence of LDL is reproducible.

Protective effect of HDL

To pursue the possibility that HDL modify the LDL-induced morphologic transformation, cells were incubated in the presence of both classes of lipoproteins. Results depicted in Fig. 3A illustrate that, in the presence of HDL, LDL have no effect on erythrocyte morphology. Moreover, with both LDL and HDL in the incubation medium, HDL prevent the LDL-induced increased resistance of erythrocytes to osmotic lysis (Table IV). The protective effect of

TABLE II

QUALITATIVE STATISTICAL ASSESSMENT OF ERYTHROCYTE MORPHOLOGY

Erythrocytes were incubated at 37°C. Cells were fixed by addition of an equal volume of 1% glutaraldehyde as described in Methods. Morphology was assessed by scanning electron microscopy and by light microscopy. Morphological type was scored as follows: ++++, 100%; +++, 75%; ++, 50%; +, 25%; —, 0%. The data of Table II correspond to Fig. 1 and 2 of the text.

Incubation	Morphology			
	Time (h)	Discocyte	Echinocyte	Spherocyte
Erythrocytes, control	1	++++	—	—
	2	++++	—	—
	3	++++	—	—
	4	++++	trace	—
Erythrocytes plus LDL	1	++++	—	—
	2	+++	+	—
	3	+	+++	—
	4	+	++	+
Erythrocytes plus HDL	1	++++	—	—
	2	++++	—	—
	3	++++	—	—
	4	++++	—	—
Erythrocytes plus HDL and LDL	1	++++	—	—
	2	++++	—	—
	3	++++	—	—
	4	++++	trace	—

TABLE III

EFFECT OF LIPOPROTEINS ON THE LIPID COMPOSITION OF THE ERYTHROCYTE MEMBRANE

Human erythrocytes were incubated with lipoproteins at the indicated concentration for 4 h at 37°C. After incubation, cells were washed three times with 150 mM NaCl and membrane ghosts were prepared. Membrane lipids were extracted and analyzed. Data represent the average of five samples in each case. Values reported as mg lipid/mg membrane protein; cholesterol values subject to ≤10% error; phospholipid values, to 5% error. The numbers in parentheses were taken from ref. 47.

Lipoproteins added	Lipid composition					
	Cholesterol	Total phospholipid	Phosphatidylcholine	Phosphatidylethanolamine	Sphingomyelin	Monoacyl phosphatidylcholine
None	0.19(0.22)	0.62(0.66)	0.18(0.19)	0.19(0.19)	0.17(0.16)	0.01(0.01)
LDL(2.5 mg/ml)	0.18	0.59	0.17	0.18	0.16	0.02
HDL(4.5 mg/ml)	0.19	0.60	0.18	0.18	0.16	0.01
LDL(2.5 mg/ml) plus HDL(4.5 mg/ml)	0.19	0.62	0.19	0.19	0.17	0.02

TABLE IV

OSMOTIC FRAGILITY OF ERYTHROCYTES

Human erythrocytes were washed and resuspended to 20% by volume. 70-μl aliquots of the cell suspension were added to 5 ml NaCl at either 500 or 525 mg/100 ml. Aliquots added to distilled water were used as standards for 100% hemolysis. The extent of hemolysis was determined by measuring the absorbance at 576 nm of the supernatant following sedimentation of unhemolyzed cells. Erythrocytes were incubated as described in the legend to Fig. 1 with or without lipoproteins

Addition	Hemolysis (%)	
	NaCl (500 mg/100 ml)	NaCl (525 mg/100 ml)
No lipoprotein	70.0	55.0
4.5 mg/ml HDL	72.0	55.5
2.5 mg/ml LDL	60.0	42.5
4.5 mg/ml HDL + 2.5 mg/ml LDL	68.5	55.5

HDL is at least partially abolished by subfractionation of this lipoprotein class. When either HDL_2 or HDL_3 are incubated for 4 h with LDL and erythrocytes, echinocytes are observed (Figs. 3C and 3D). However, this morphologic change is not identical to that which results from incubation of erythrocytes for 4 h with LDL alone (Fig. 1B) since no spherocytes are observed. HDL_2 or HDL_3 alone have no effect on erythrocyte morphology. Complete inhibition of the morphologic change is achieved with 'reconstituted' HDL consisting of HDL_2 and HDL_3 (molar ratio 1 : 1) (Fig. 3B), indicating that the methods for separation of HDL subspecies do not result in the loss of constituents essential for inhibiting LDL-induced alteration of erythrocyte morphology.

The protective effect of HDL against LDL-induced morphologic changes

TABLE V

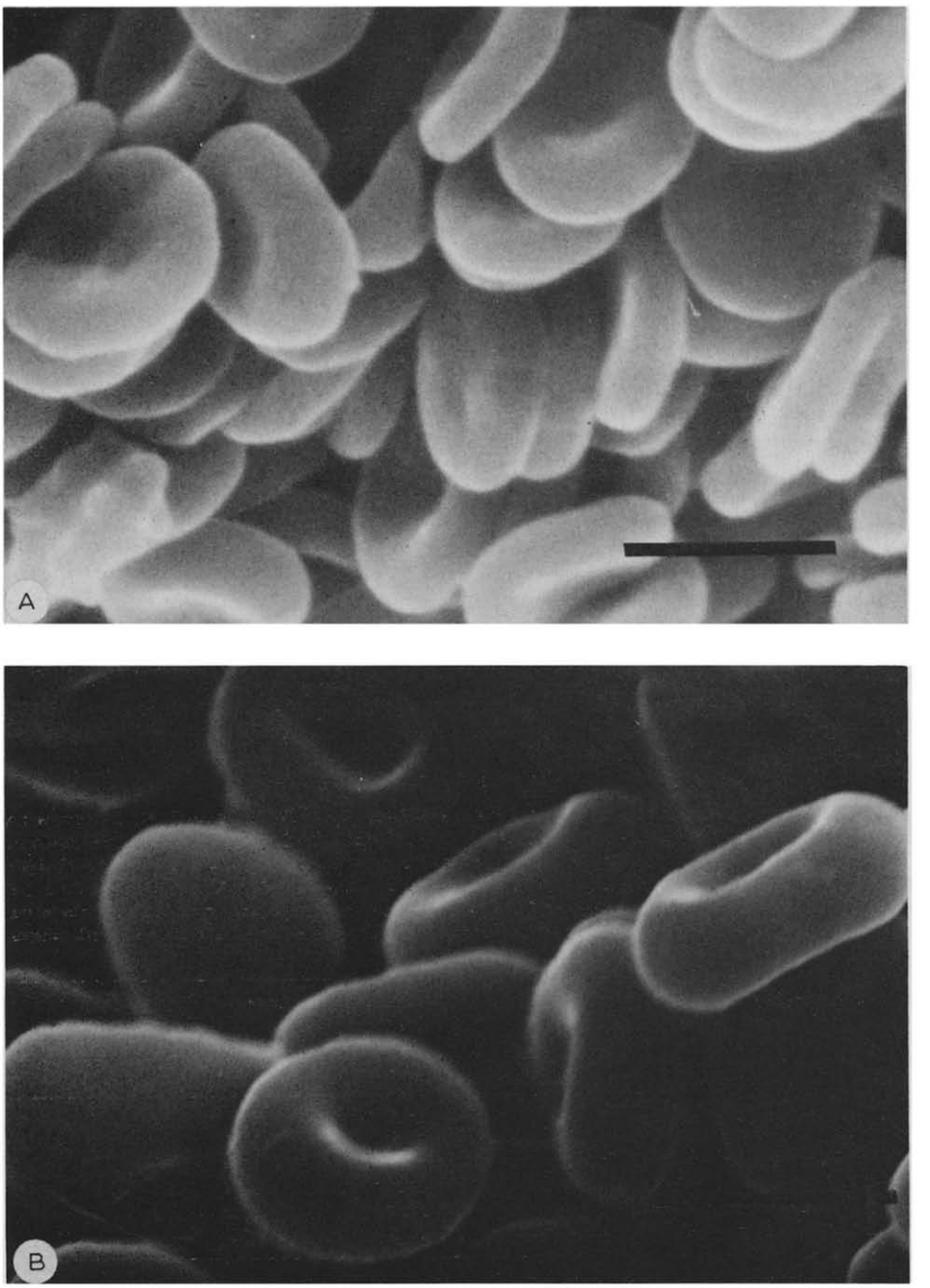
BINDING OF LOW DENSITY LIPOPROTEINS TO ERYTHROCYTES

[*Me*-^{3}H]LDL was prepared by adding 65.5 μmol formaldehyde and NaB^3H_4 to 80 mg LDL. Lipoproteins were incubated with erythrocytes in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM KCl, 10 mM glucose, 1 μM adenine, and 1 mM $CaCl_2$. After incubation, samples were washed three times with 150 mM NaCl and then digested overnight with periodic acid. Radioactivity was determined by liquid scintillation in 15 ml Aquasol after decolorization by H_2O_2, and is reported as percentage of the total radioactivity after incubation in the control experiments. Reported values are the average of five experiments, with ranges ±5%.

Lipoproteins in medium	Percent of maximum binding *	
	30 min	60 min
[*Me*-^{3}H]LDL (2.5 mg/ml)	62.5	100
[*Me*-^{3}H]LDL (1.25 mg/ml)	47.8	61.0
[*Me*-^{3}H]LDL (1.25 mg/ml) plus LDL (2.5 mg/ml)	27.3	37.4
[*Me*-^{3}H]LDL (2.5 mg/ml) plus HDL (4.5 mg/ml)	17.0	17.2
[*Me*-^{3}H]LDL (2.5 mg/ml) plus HDL_2 (4.5 mg/ml)	22.2	62.2
[*Me*-^{3}H]LDL (2.5 mg/ml) plus HDL_3 (4.5 mg/ml)	20.7	61.3

* Maximum LDL binding is 10^6 lipoproteins per cell.

may be attributed to the inhibition by HDL of LDL attachment to the cell membrane. To test this possibility, erythrocytes were incubated with [*Me*-^{3}H]-LDL. As shown in Table V, there is binding of the labeled lipoprotein to the cells; unlabeled LDL competitively inhibit the binding of radiolabeled LDL.



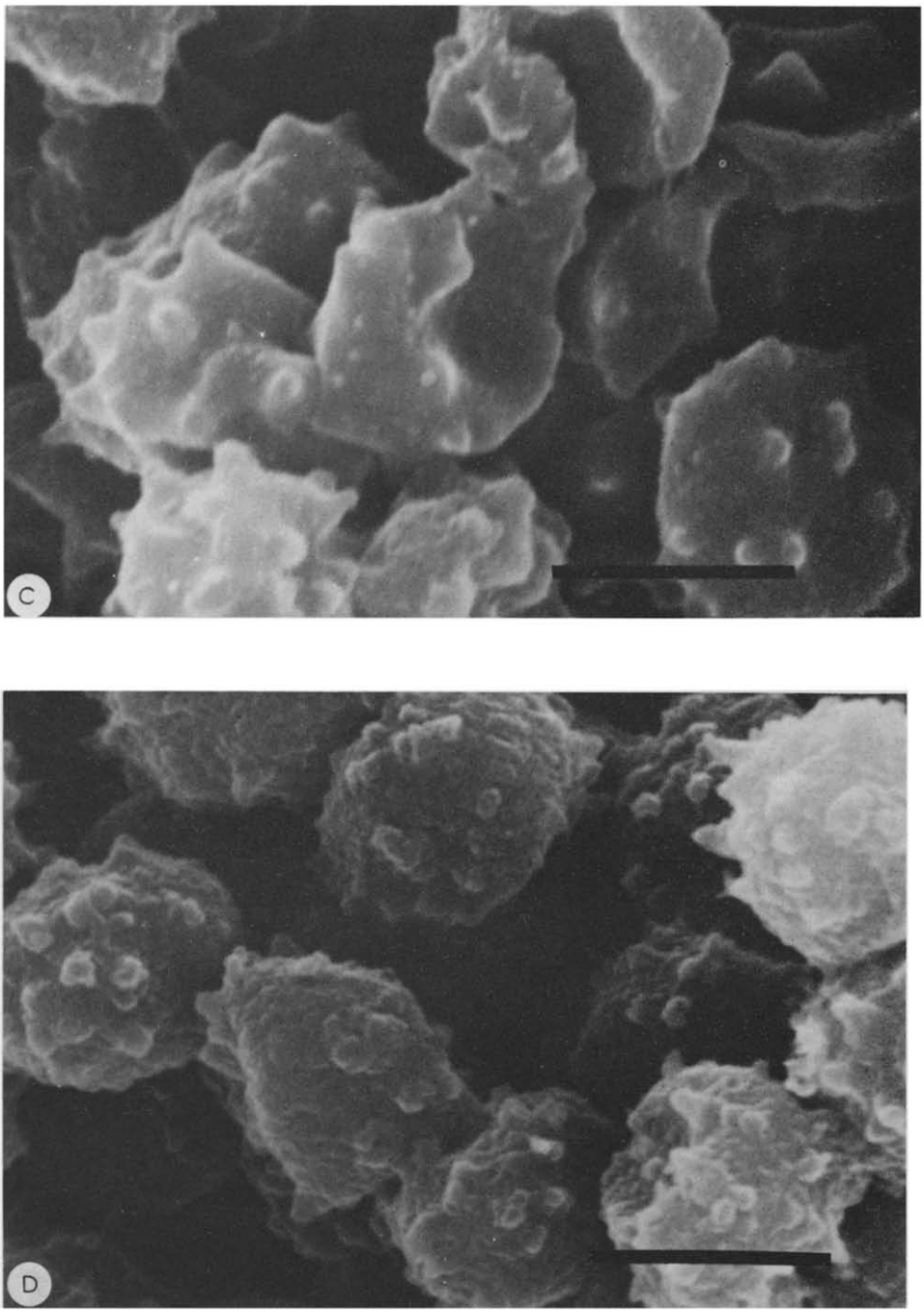
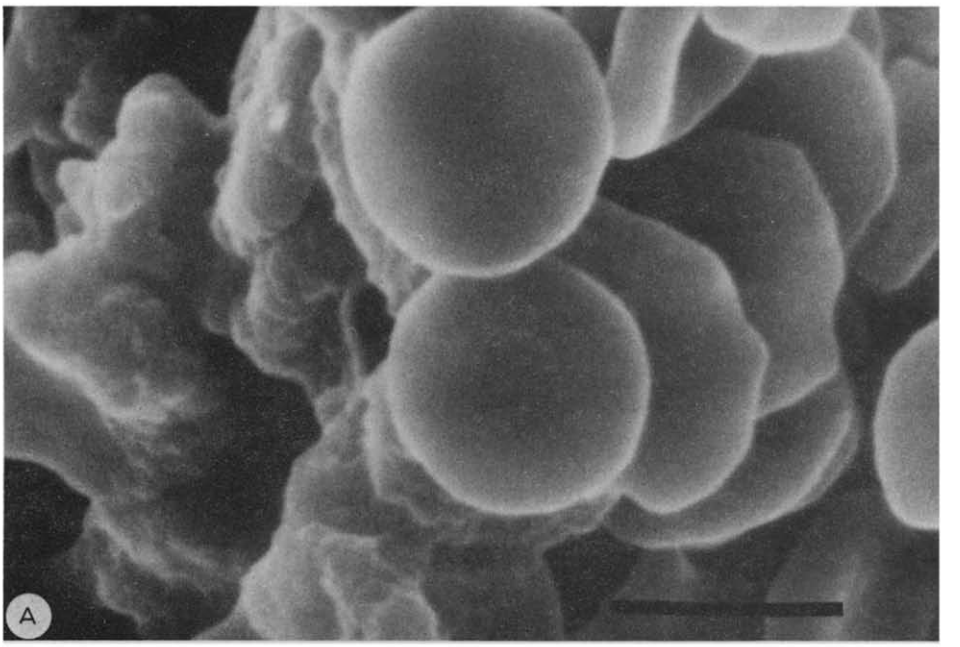
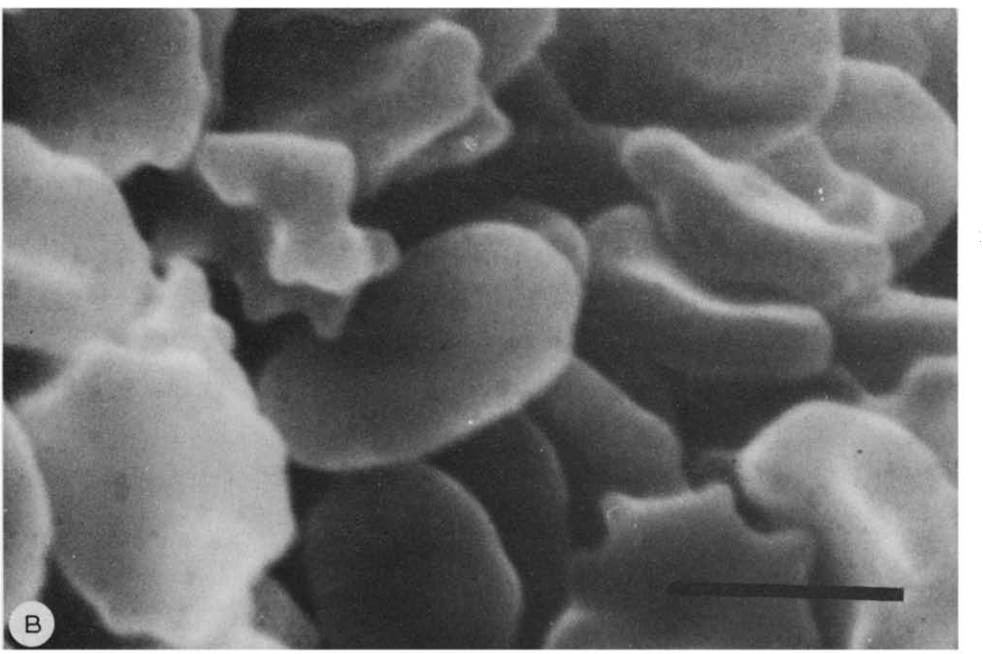


Fig. 3. Scanning electron micrographs of erythrocytes incubated with both LDL and HDL. Experimental conditions were identical to those as described under Fig. 1. (A) Erythrocytes incubated with LDL and HDL. (B) Erythrocytes incubated with LDL, HDL_2 and HDL_3. (C) Erythrocytes incubated with LDL and HDL_2; (D) Erythrocytes incubated with LDL and HDL_3. Bar represents 5 μm.

When [*Me*-^{3}H]LDL are incubated with erythrocytes in the presence of HDL, the amount of tritium associated with the erythrocyte fraction decreases dramatically (Table V). On the other hand, at comparable concentrations





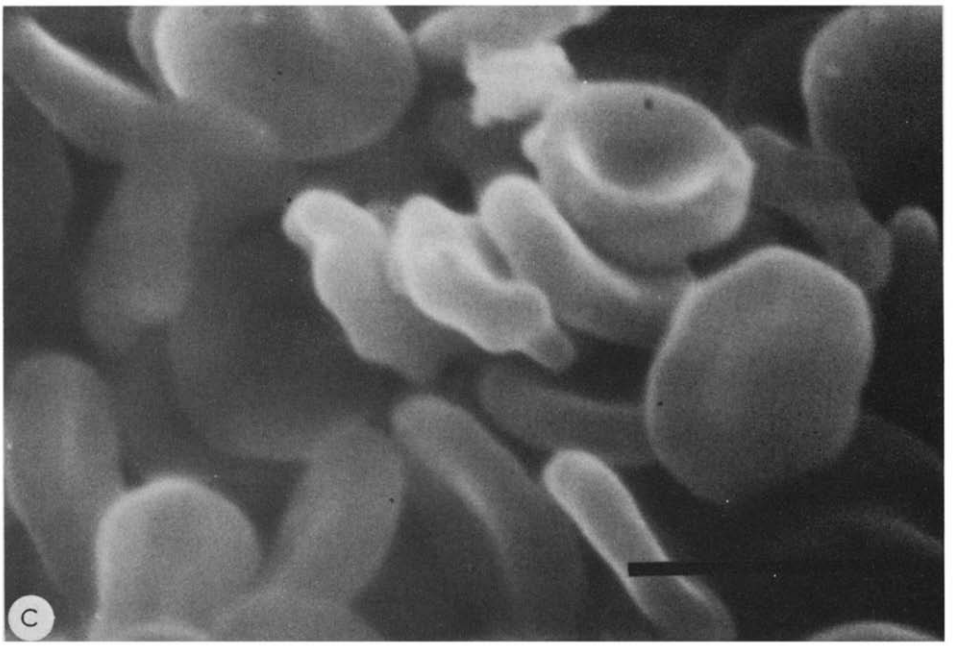


Fig. 4. Scanning electron micrographs illustrating the effect of various agents on the LDL-induced alteration of erythrocyte morphology. Incubation conditions were identical to those as described for Fig. 1. (A) Erythrocytes incubated with LDL for 3 h. (B) Erythrocytes incubated with LDL for 3 h and then HDL for an additional 1 h. (C) Erythrocytes incubated with LDL for 3 h and then with heparin (10 units/ml). Bar represents 5 μm.

HDL_2 or HDL_3 only partially inhibit the binding of LDL to the erythrocyte surface (Table V).

Reversibility of LDL-induced erythrocyte shape alterations

The ability of HDL and of heparin to reverse the morphologic alteration induced by LDL was investigated. Due to the fact that hemolysis occurs after 5 h total incubation with LDL, reversibility was investigated subsequent to a 3 h incubation of cells with LDL. HDL (4.5 mg/ml) or heparin (10 units/ml) were then added to the LDL-erythrocyte suspension and the mixtures were incubated an additional 1 h at 37°C. Erythrocytes were isolated and their morphology assessed by scanning electron microscopy. Results shown in Figs. 4A and 2 support the conclusion that a 3 h incubation with LDL is sufficient to produce a substantial fraction (about 75%) of crenated cells. When HDL are added after 3 h incubation of erythrocytes with LDL, further LDL-induced shape change is inhibited; no further alteration resulting in spherocytes was detected (Fig. 4B). However, HDL fail to restore biconcave disc morphology and the cell population remains 75% echinocytes, 25% discocytes. Heparin, however, reversed the LDL-induced morphologic alteration as evidenced by the reappearance of cells of normal biconcave disc morphology (Fig. 4C); 60 min after heparin addition 40% echinocytes and 60% discocytes are present.

Discussion

The primary consequence of the lipoprotein-erythrocyte interaction has been assumed to be lipid exchange [33,39], a view supported by studies of erythrocytes from individuals who lack LDL (abeta-lipoproteinemia) [40] or HDL (Tangier disease) [41] or who are deficient in the plasma enzyme lecithin : cholesterol acyltransferase [42] and from individuals who suffer from severe hepatocellular dysfunction [43]. The sequence of events resulting in exchange and/or net transfer of lipids between lipoproteins and erythrocytes is unknown; it is feasible that lipoproteins have a homeostatic role in erythrocyte function to which lipid exchange is ancillary. Such a hypothesis is indicated by results reported herein.

Erythrocyte morphology is dramatically altered by LDL as illustrated in Figs. 1 and 2. During the initial stages of the incubation with LDL, cells with scalloped edges are evident; after 4 h incubation at 37°C, a majority of the erythrocytes are echinocytes and spherocytes. Since erythrocytes were fixed with glutaraldehyde prior to microscopic observation, it is possible that the actual appearance of the cells is a consequence of the fixation procedure. Nevertheless, it is a fact that erythrocytes exposed to LDL respond to identical experimental manipulation in a manner distinct from erythrocytes incubated in the absence of lipoproteins. A decrease in osmotic fragility accompanies transformation from disc to sphere. The LDL-induced changes in erythrocyte morphology occur without concomitant leakage of hemoglobin from the cell and they cannot be attributed to depletion of intracellular ATP. Significantly, we have demonstrated that morphologic alteration is not due to an altered lipid content of the erythrocyte membrane (Table III). The cholesterol and phospholipid content of the erythrocyte membrane is not increased during the 4 h incubation period with LDL indicating that net lipid transfer does not occur, a conclusion confirmed by the fact that the lipid composition of LDL is not altered by incubation with erythrocytes. Furthermore, the distribution of erythrocyte phospholipids remains constant. The fact that LDL-induced morphologic transformation is not prevented by serum albumin (see, for example, refs. 35 and 44) indicates that the effect of LDL is not due to the transfer of monoacyl phosphatidylcholine or fatty acids, agents which induce disc → sphere alterations [35,36]. However, we cannot exclude the possibility that LDL produce minimal localized changes in lipid composition of the membrane resulting in a change of cell shape. We demonstrate in the succeeding manuscript [45] that LDL reduce the extent of phosphorylation of spectrin, a cytoskeletal protein intrinsic to the erythrocyte membrane and responsible for controlling cell shape and deformability [46]. This suggests a specific target in the membrane for LDL.

The morphologic transformation is a direct consequence of LDL binding. Based on data presented in Table V, approx. 10^6 [*Me*-^{3}H]LDL particles bind to the surface of each erythrocyte. In separate experiments employing [^{125}I]LDL (>99% ^{125}I associated with the protein moieties) and LDL containing radiolabeled cholesteryl esters, we also determine that about 10^6 LDL particle bind per cell (Hui, D.Y. and Harmony, J.A.K., unpublished data). Furthermore, we can visualize LDL-hemocyanin complexes at the cell surface by electron micro-

scopic techniques (Hui, D.Y., Johansen, M.G. and Harmony, J.A.K., unpublished data).

HDL do not alter erythrocyte morphology, nor do they decrèase the osmotic fragility of the cells. Lipoprotiens of the high density class, if added simultaneously with LDL, inhibit LDL-induced alteration of erythrocyte morphology, apparently by preventing the attachment of LDL to the cell membrane. Thus, HDL protect the erythrocyte from a potentially detrimental interaction with LDL. If added after the cells have been exposed to LDL for 3 h, HDL cannot reverse the LDL-specific alteration. We are intrigued by the inability of the HDL subfractions HDL_2 and HDL_3 to afford complete protection of the erythrocytes from LDL. Both subspecies inhibit the LDL-induced alteration. However, neither HDL_2 or HDL_3 is as effective as total HDL in inhibiting the binding of LDL to the cells. Although HDL_2 and HDL_3 seem to effectively prevent LDL binding in the early incubation phase, a relatively high amount of labeled LDL is associated with the cells in the presence of HDL_2 or HDL_3 after 1 h incubation. Reconstituted HDL (HDL_2 plus HDL_3) is as effective as the original HDL in protecting the erythrocytes. These observations suggest that interaction between components in the two subfractions is required for maximum protection.

Our observation that LDL influence the properties of the membrane of intact erythrocytes is unique. The alteration appears to be the result of binding of LDL to preexisting sites on the membrane in contrast to the high-affinity, LDL-specific binding sites induced on the 'surfaces' of nucleated cells by cholesterol deprivation [9—15]. Thus, the amount of LDL required to alter erythrocyte morphology and which allows detection of binding is about 20-fold higher than that required to saturate the high-affinity LDL receptors on the fibroblast membrane. In addition, HDL do not compete well with LDL for the high-affinity receptors. On the other hand, heparin, which reverses the binding of LDL to fibroblasts [8] also removes [^{125}I]LDL from the erythrocyte surface (Hui, D.Y. and Harmony, J.A.K., unpublished data) and allows the cell to return to normal disc morphology. Our data further indicate that normal erythrocyte morphology and, therefore, cell function may depend on the concentration and composition of the circulating lipoproteins. It is premature to stipulate the physiological significance of these findings. Our data cannot yet explain why erythrocytes from individuals with abeta-lipoproteinemia have spiculated morphology and why individuals with Tangier disease have erythrocytes of normal morphology [40]. The actual significance of these findings is to suggest a new focus for investigators attempting to elucidate the role of lipoproteins in normal and pathologic states.

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