Effects of plasma lipoproteins from control and cholesterol-fed guinea pigs on red cell morphology and cholesterol content: an in vitro study

Christian Sardet, Helen Hansma, and Rosemarie Ostwald

Department of Nutritional Sciences, University of California, Berkeley, California 94720

Abstract When guinea pigs are fed cholesterol, the cholesterol content of their red cells increases progressively, a large number of cells become spurred, and a hemolytic anemia develops. Unesterified cholesterol is readily transferred from plasma, HDL, or LDL of cholesterol-fed, anemic guinea pigs to normal red cells in vitro. This transfer is reversible and is proportional to the concentration of unesterified cholesterol in the incubation medium. Red cells loaded in vitro with cholesterol develop spurs identical with those on red cells in the circulation of cholesterol-fed, anemic guinea pigs. Neither the cholesterol content nor the morphology of normal red cells is altered by incubation in control plasma or in concentrated control lipoproteins. Plasma infranates (d > 1.21 g/ml) of either group do not cause spurring of control red cells. We conclude: (*a*) that accumulation of cholesterol by guinea pig red cells in vitro requires an increased concentration of unesterified cholesterol in lipoprotein rather than an increased concentration of normal lipoproteins, and (*b*) that an increased cholesterol content in guinea pig red cell membranes is necessary for their abnormal morphology. The flux of cholesterol between cholesterol-loaded cells and plasma from cholesterol-fed guinea pigs is three times greater than that between control red cells and control plasma, and the fractional exchange rates are altered.

Supplementary key words phospholipids · red cell ghosts · [^{3}H]cholesterol exchange · spur cells

We have previously reported that the chemical composition and morphology of guinea pig red cells were altered in response to a diet containing 1% cholesterol (1). The red cell cholesterol level increased to twice its normal value during the first 2 months;[1] thereafter, both cholesterol and phospholipid levels increased and a hemolytic anemia developed (1, 2). Red cell morphology was altered within 2 wk in guinea pigs fed the cholesterol-containing diet, and the red cells from anemic guinea pigs were severely spiculed (2).

Changes in the lipid composition of red cells have been observed in several other species: rabbits fed cholesterol (3), rats fed orotic acid (4), and humans with various pathological conditions (5–8). Hypercholesteremia in man does not necessarily increase red cell cholesterol (5, 9), and cholesterol accumulation in red cell membranes has been observed in humans and rats with hypocholesteremia (4, 5, 10). Factors other than the concentration of plasma cholesterol, per se, must therefore affect the cholesterol content of the red cell membrane.

Changes in the composition of red cells are generally accompanied by morphological changes, although no direct cause and effect relationship has been demonstrated (5–8). In several cases, the cholesterol content of normal red cells could be increased by incubating them in plasma from patients or animals with abnormal red cells (5–8). The mechanisms of these changes are not well understood.

We are reporting results of studies in which attempts were made to determine which factors in the plasma of cholesterol-fed, anemic guinea pigs (chol guinea pigs) are responsible for the increase in red cell cholesterol and

[1] Cholesterol designates unesterified cholesterol.

Abbreviations: control guinea pigs, guinea pigs fed the control diet; chol guinea pigs, guinea pigs that became anemic as a result of ingestion of a cholesterol-containing diet; HDL, high density lipoproteins (d 1.07–1.21 g/ml); LDL, low density lipoproteins (d 1.01–1.05 g/ml); chol (plasma, lipoprotein, HDL, LDL), plasma or plasma lipoproteins from cholesterol-fed, anemic guinea pigs (chol guinea pigs); control (plasma, lipoprotein, HDL, LDL), plasma or plasma lipoproteins from guinea pigs fed the control diet (control guinea pigs); UC, unesterified cholesterol; PL, phospholipid; LCAT, lecithin:cholesterol acyltransferase.

the accompanying change in red cell morphology. Our results show that isolated plasma lipoproteins of chol guinea pigs can transfer part of their unesterified cholesterol to normal red cells in vitro and that the altered red cell morphology is associated with this increase in membrane cholesterol.

MATERIALS AND METHODS

Sources of red cells, plasma, and plasma lipoproteins

Young, male, albino guinea pigs (Simonsen, Gilroy, Calif.) weighing 200–250 g were fed a semisynthetic diet with or without the addition of cholesterol to a level of 1% (11). They were autopsied when the red blood cell count of the cholesterol-fed guinea pigs dropped from a normal value of 5–6 $\times$ 10^6 cells/mm^3 to less than 3.5 $\times$ 10^6 cells/mm^3, usually after 10–15 wk on diet. The animals were fasted overnight and anesthetized with sodium pentobarbital (Diabutal; Diamond Laboratories, Des Moines, Iowa). The blood was taken by open-chest heart puncture in the presence of ACD[2] or EDTA (0.5 ml of 4% EDTA, disodium salt, per 20 ml of blood). Red cells and plasma were separated by centrifugation (1300 *g* for 15 min at 4°C) and were used immediately, as described below. Plasmas from control and chol guinea pigs were used to prepare the following lipoprotein fractions: total plasma lipoproteins (d < 1.21 g/ml), LDL (d 1.010–1.050 g/ml), HDL (d 1.070–1.21 g/ml), and infranate (d > 1.21 g/ml). The lipoprotein fractions were isolated by standard flotation techniques (12), and chol LDL vesicles were isolated by gel filtration of chol LDL (13). The isolation and characterization of these lipoproteins have been described in a previous communication (13).

Preparation of plasma labeled with [^{3}H]cholesterol

Control and chol guinea pig plasmas were labeled with [1,3-^{3}H]cholesterol (47.9 Ci/mmole, New England Nuclear) essentially as described by Rose (14). A solution of 0.06% cholesterol in ethanol with a 1:200 dilution of the original specific activity was prepared as follows: 3.8 ml of unlabeled cholesterol in ethanol (50 μg/ml) was added to 0.1 ml of [^{3}H]cholesterol in benzene (8 μg/ml). The mixture was dried under nitrogen, and 0.3 ml ethanol was added. The ethanolic solution was added to fresh plasma just before incubation by gently mixing 2 μl of the solution per ml of plasma. Before being labeled, the plasmas were heated at 56°C for 30 min in order to inactivate lecithin:cholesterol acyltransferase, the enzyme which esterifies cholesterol in plasma (15).

Incubation of red cells in plasma

Fresh red cells were washed once with ACD solution[2] and suspended in the incubation medium[3] at a hematocrit of 10–20% in small Erlenmeyer flasks which were then tightly sealed. The incubations were started immediately in a Dubnoff shaker at 37°C. Aliquots for lipid analyses and hematocrit measurements were taken at different time intervals (up to 24 hr) and were immediately centrifuged to separate red cells and plasma. Red cells were washed three times in saline (0.145 M NaCl) and stored in a freezer until lipid extraction.

Incubation of red cells in plasma lipoproteins

Just before incubation, lipoprotein fractions at known concentrations were dialyzed for 24 hr at 4°C against 200 vol of buffered saline (pH 7.4) containing glucose, magnesium, and antibiotic.[4]

Fresh red cells were washed three times and resuspended in buffered saline.[4] A known amount of the red cell suspension was added to a constant volume of buffered saline containing either LDL, HDL, whole plasma lipoproteins, or d > 1.21 g/ml infranate at various concentrations. These incubations were carried out in polyethylene centrifuge tubes at a 20% hematocrit at 37°C in a Dubnoff shaker. After 0, 4, or 6 hr of incubation, red cells and plasmas were separated and the red cells were washed three times in saline and extracted as described below.

Preparation of red cell ghosts and counting of red cells and red cell ghosts

Red cell ghosts were prepared in Tris buffer (tris[hydroxymethyl]aminomethane, 10 mM, pH 7.4 [Sigma Chemical Co.], and $MgCl_2$, 1 mM) essentially according to the method of Dodge, Mitchell, and Hanahan (16). The number and size distribution of red cells and red cell ghosts were obtained by means of an electronic particle counter (Coulter Counter model ZBI; Coulter Electronics, Hialeah, Fla.) equipped with a channel

[2] ACD (acid–citrate–dextrose, pH 7.2, Cutter Laboratories, Berkeley, Calif.) was used as follows: 3 ml of a mixture of ACD (60 ml) + 5% dextrose (20 ml) + antibiotic (8 ml of a solution containing 10,000 units of penicillin/ml) were placed in a syringe, and blood was drawn to the 20-ml line. The plasma glucose concentration was thus approximately 1.7 mg/ml, and the penicillin concentration was 300 units/ml.

[3] Plasma–glucose–ACD mixture. For most experiments (except those involving ^{3}H-labeled plasma) the plasma was used without prior heat treatment. LCAT activity is extremely low in guinea pig plasma and preliminary studies have shown no significant differences in the results when fresh or preincubated plasma was used.[5]

[4] Buffer: NaCl (7.84 g/l), KCl (0.26 g/l), $MgCl_2$ (0.21 g/l), Na_2HPO_4 (1.00 g/l), KH_2PO_4 (0.20 g/l), and antibiotic–antimycotic (10 ml/l, Grand Island Biological Co., Grand Island, N.Y.). Nine parts of buffer were added to one part 5% dextrose (sterile solution, Cutter Labs).

analyzer (Coulter Channelyzer). Red cell ghosts were fixed in Hayem's solution ($HgCl_2$, 2 mM; Na_2SO_4, 13 mM; and NaCl, 85 mM) before counting.

Analytical procedures

Hematocrits were determined in a microcapillary centrifuge (model MB, International Equipment Co.). The lipids of washed red cells and red cell ghosts were extracted as described by Rose and Oklander (17), those of plasma as described earlier (11), and those of plasma lipoproteins by the methods of Folch, Lees, and Sloane Stanley (18). Lipid phosphorus was measured by the procedure of Bartlett (19), and phospholipid values were obtained by multiplying phosphorus values by 25. Cholesterol was determined after digitonide precipitation (20) by the method of Zlatkis, Zak, and Boyle (21). The radioactivity of [^{3}H]cholesterol was determined in a liquid scintillation spectrometer (model LS 100, Beckman Instruments) after the digitonide precipitate was dissolved in methanol (22). PPO (2,5-diphenyloxazole, Eastman Kodak Co.), 0.5% in toluene, was used as a scintillator, and [^{3}H]cholesterol in toluene was used as an internal standard. Protein determinations were carried out according to Lowry et al. (23), with bovine serum albumin (fraction V, Armour Pharmaceutical Co.) as the standard.

Calculations

We analyzed the exchange of [^{3}H]cholesterol between red cells (RBC) and plasma (Pl), using the equations that define the movement of the label in a closed, two-compartment system as described by Solomon (24). They were of the type:

$$(F)t = \frac{k_{RBC-Pl}}{k_{RBC-Pl} + k_{Pl-RBC}} - \left(\frac{k_{RBC-Pl}}{k_{RBC-Pl} + k_{Pl-RBC}} \times e^{-(k_{RBC-Pl} + k_{Pl-RBC})t}\right)$$

in which $F(t)$ is the normalized specific activity of cholesterol in the initially unlabeled compartment as a function of time. In the case of the equation above, $F(t)$ = specific activity at time t in initially unlabeled compartment (RBC)/specific activity at time 0 in initially labeled compartment (Pl). The rates of exchange k_{RBC-Pl} and k_{Pl-RBC} were obtained from the semi-logarithmic plots of $F(t)$ vs. time and from the quantities of cholesterol in red cell and plasma compartments when isotopic equilibration was reached (24). Corrections for the net transfer of cholesterol from one compartment to the other, which occurred in some experiments, were made according to Solomon (24). Cholesterol fluxes in and out of red cells were calculated from the exchange rates and expressed in terms of molecules of cholesterol transferred per red cell per hour of incubation.

Morphological observations

Nonwashed red cells were examined by phase-contrast light microscopy in a Zeiss microscope immediately before and after incubation in the different media described above. A small aliquot of the incubation mixture was suspended in fresh control plasma or Hayem's solution (final hematocrit $\sim$ 3%). A drop of the suspension was placed on a clean microscope slide, covered with a cover slip, and sealed with paraffin. Double-blind observations were immediately conducted, and the red cells were photographed (Polaroid camera with type 47 film).

RESULTS

In another communication we have described in detail the effects of dietary cholesterol on the plasma lipids and lipoprotein composition in the guinea pig (13). Control guinea pig plasma contained only traces of HDL. With cholesterol feeding and the resulting hemolytic anemia, there was a large increase in HDL, and liposome-like particles (LDL vesicles) appeared in the LDL fraction. Both of these lipoproteins had abnormally high percentages of unesterified cholesterol (13).

In vitro exchange of cholesterol between red cells and plasma

When control red cells were incubated in control plasma, their unesterified cholesterol and phospholipid content remained within normal limits (Table 1). The small decrease in cholesterol content was accompanied by a comparable decrease in PL content, suggesting a small systematic error in sample measurement or the effects of the small amount of LCAT activity present in guinea pig plasma.[3]

When the same control red cells were incubated in the plasma from a cholesterol-fed, anemic guinea pig (chol plasma), however, their cholesterol content increased while their phospholipid content remained unchanged (Table 1). As a result, the molar ratio of cholesterol to phospholipid was increased well above the normal equimolar ratio. Red cell ghosts prepared before and after incubation had lipid values close to those of the corresponding red cells, demonstrating that the newly acquired cholesterol was located in the membrane (Table 1).

Fig. 1 shows the uptake of cholesterol by normal red cells incubated in chol plasma, as a function of time. Equilibration took place rapidly and appeared to be complete within 8–12 hr. The red cells were not saturated with cholesterol at this time, because when they were

TABLE 1. Lipid composition of guinea pig red cells before and after incubation in plasma from control and chol guinea pigs

	Unesterified Cholesterol		Phospholipids		Moles Cholesterol/ Moles Phospho-lipids
	$\mu g/cell \times 10^7$	$moles/cell \times 10^{10}$	$\mu g/cell \times 10^7$	$moles/cell \times 10^{10}$	
Control red cells	1.24 ± 0.05[a]	3.21	2.66 ± 0.16	3.24	0.99
Ghosts	1.10	2.85	2.66	3.31	0.86
Control red cells incubated in control plasma	1.10 ± 0.02[a]	2.85	2.51 ± 0.07	3.05	0.93
Ghosts	1.08	2.80	2.65	3.31	0.84
Control red cells incubated in chol plasma	1.74 ± 0.10[a]	4.50	2.47 ± 0.29	2.99	1.51
Ghosts	1.94	5.02	2.54	3.18	1.58

Control red cells were incubated 5 hr in fresh control or chol plasma (not heat-inactivated) in the presence of glucose (1.7 mg/ml) and antibiotic (300 units of penicillin/ml). Triplicate incubations were conducted at a 20% hematocrit. Before and after incubation period, red cells were isolated and counted, lipids were extracted, and amounts of cholesterol and phospholipids were determined. Values presented here are means ± SD from triplicate incubations of the same red cells.

[a] Means are significantly different at $P < 0.01$ from other values in the same vertical column. Red cells from two out of three incubations were used to prepare red cell ghosts, which were counted, and their lipids were quantitated. Values presented are means of data from duplicate ghost preparations. Only traces of cholesteryl esters were detected in red cell and ghost lipid extracts.

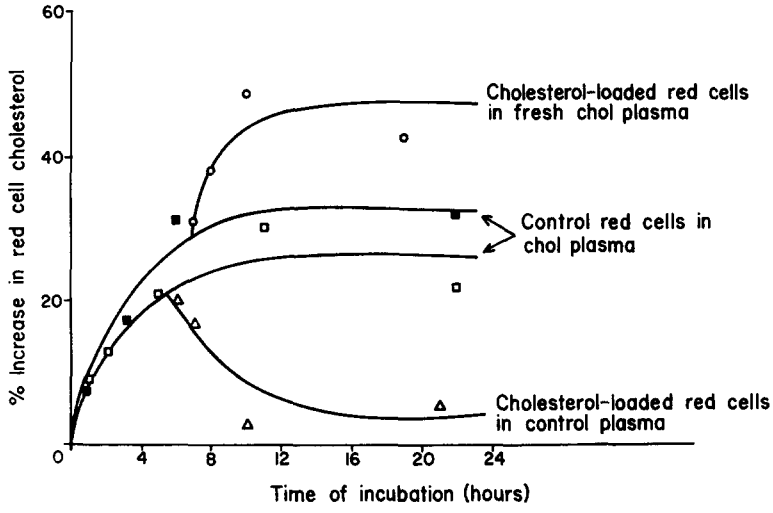

FIG. 1. Changes in red cell cholesterol during in vitro incubations in control or chol plasma. Control red cells were incubated at a 10–20% hematocrit in the presence of glucose and antibiotic. Aliquots were taken at time intervals indicated on abscissa. Red cell cholesterol (mg of cholesterol/100 ml of packed cells) is plotted as: % increase = 100 (cholesterol in red cell at time t − cholesterol in red cell at time 0)/cholesterol in red cell at time 0. Cholesterol-loaded red cells were obtained as indicated, after incubation in chol plasma. They were washed, centrifuged, and resuspended in fresh chol plasma or control plasma. Two to four experiments of each type were performed.

transferred to a fresh aliquot of the same chol plasma, they acquired additional cholesterol until a new equilibrium was reached (Fig. 1). Cholesterol uptake was shown to be reversible: cholesterol-loaded cells lost most of the acquired cholesterol when incubated in control plasma (Fig. 1).

The increase in red cell cholesterol was positively correlated with the absolute amount of unesterified cholesterol in the plasma of incubation (Fig. 2). The cholesterol transferred from chol plasma to control red cell membranes represented 8–16% of the plasma unesterified cholesterol.

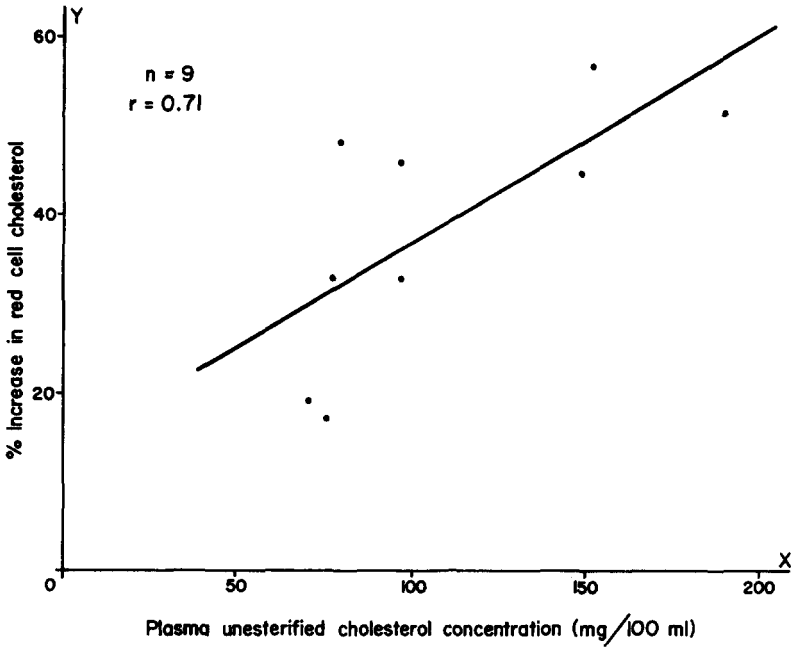

FIG. 2. Effect of plasma cholesterol concentration on uptake of cholesterol by red cells. Control red cells were incubated in plasma of various cholesterol concentrations at a 10–20% hematocrit. Chol plasmas were obtained from nine chol guinea pigs when their red blood cell counts dropped below $3.5 \times 10^6/mm^3$. The correlation ($r = 0.71$) was obtained by linear regression analysis of the data (linear regression equation: $y = 13.7 + 0.23x$); % increase in red cell cholesterol, see legend to Fig. 1.

We studied the kinetics of this cholesterol transfer using labeled plasma or red cells. Fig. 3 shows the loss and uptake of [^{3}H]cholesterol by plasma and red cells under different conditions. In most experiments red cell and plasma cholesterol reached similar specific activities within 24 hr of incubation. When control red cells were incubated in chol plasma, however, isotopic equilibrium was not reached within that period. We attributed this to the very large size of the plasma cholesterol compartment. (Chol plasma had 80–200 mg of UC/100 ml of plasma and 600–1300 μg of UC/ml of incubation medium compared with 8–16 mg/100 ml of plasma and 70–90 μg/ml of incubation medium for control plasma.) The fractional exchange rates and cholesterol fluxes could be calculated only for those experiments in which there was little or no net cholesterol transfer and in which an isotopic steady state was reached, i.e., control red cells incubated in control plasma (Fig. 3, *A* and *B*) and cholesterol-loaded red cells incubated in chol plasma (Fig. 3*F*). Table 2 shows that the cholesterol flux in both directions was much faster between cholesterol-loaded red cells and chol plasma than between control cells and control plasma. The data also indicate that the fractional exchange rate from cholesterol-loaded red cells to chol plasma was higher than from control cells to control plasma (k_{RBC-Pl} chol > k_{RBC-Pl} control), while the reverse was true for the fractional exchange rate of cholesterol from plasma to red cells (k_{Pl-RBC} chol < k_{Pl-RBC} control).

In vitro exchange of cholesterol between red cells and lipoproteins

Fig. 4 shows the in vitro transfer of unesterified cholesterol from isolated lipoproteins (d < 1.21 g/ml) to control red cells. The red cells maintained their original cholesterol concentration when they were incubated with control lipoproteins even when the lipoproteins were concentrated threefold, so that concentration of unesterified cholesterol was three times that of normal plasma. In contrast, chol lipoproteins transferred cholesterol to red cells. There was a strong correlation between the unesterified cholesterol concentration of chol lipoproteins and the percentage increase in red cell cholesterol even when the lipoproteins were present at lower concentrations than in chol plasma. The data therefore show that at comparable concentrations of unesterified cholesterol, transfer to red cells occurred only from chol lipoproteins and not from control lipoproteins. The amount of cholesterol acquired by red cells increased in a general way with an increasing ratio of unesterified cholesterol to protein in the chol lipoproteins. The statistical significance of this relationship could, however, not be assessed from the presently available data.

Table 3 and Fig. 5 show the transfer of cholesterol to red cells by individual chol lipoproteins. Chol HDL always transferred a larger percentage of its unesterified cholesterol to red cells than did chol LDL. Chol LDL

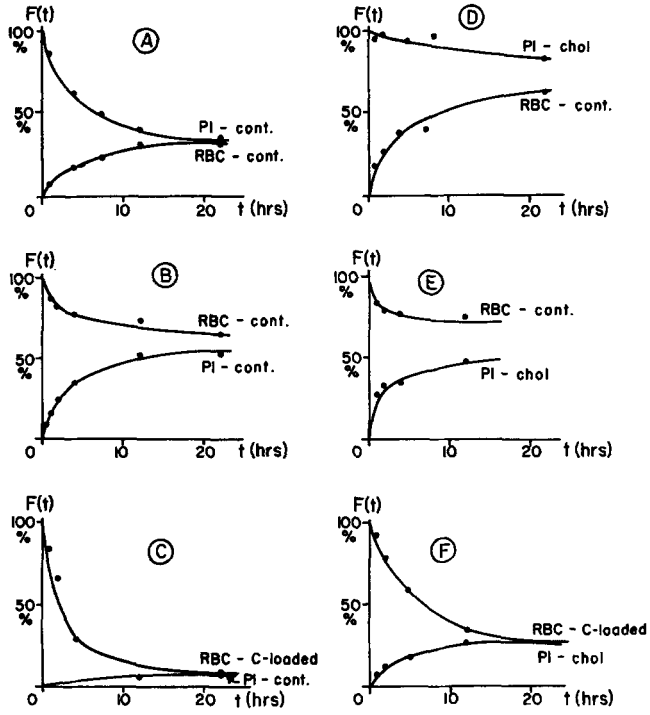

FIG. 3. Loss and uptake of [^{3}H]cholesterol by red cells and plasma as a function of time. $F(t)$ is normalized cholesterol specific activity: $F(t) \simeq 100$ (specific activity at time t in initially unlabeled compartment/specific activity at time 0 in initially labeled compartment). Representative curves are presented for each type of incubation experiment. Number of experiments carried out for each type: *A–C*, 2; *D*, 5; *E*, 1; *F*, 3. Heat-inactivated plasma labeled with [^{3}H]cholesterol was prepared as described by Rose (14). Labeled cholesterol-loaded red cells were obtained by incubating control red cells with labeled chol plasma for 6–8 hr. Washed red cells were incubated at a 10–20% hematocrit. Aliquots were removed from incubation mixture at time intervals indicated on abscissa. Cholesterol specific activity was determined in both red cells and plasma. Total amount of cholesterol in red cells and/or plasma was determined in representative experiments.

has two populations, 1000-Å vesicles and 250-Å particles (13). The vesicle population transferred a large percentage of its unesterified cholesterol to red cells, and thus resembled chol HDL more than whole chol LDL (Table 3). The transfer of cholesterol from lipoprotein to red cells tended to approach a limit with increasing concentration of lipoprotein cholesterol in the medium (Fig. 5). This limit was greater for chol HDL than for chol LDL and was therefore not due to a saturation of the red cells with cholesterol.

Red cell morphology

Observation of red cell shape is difficult in guinea pigs because their red cells are easily distorted by common methods of handling, i.e., washing, centrifuging, etc. For example, some of the control cells suspended in control plasma exhibited wavy perimeters (Fig. 6*A*). In Hayem's solution, however, control red cells consistently had a very regular biconcave appearance (Fig. 6*C*). Chol red cells, on the other hand, had a thorny, spur-cell appearance when observed in control plasma (Fig. 6*B*), Hayem's solution (Fig. 6*D*), or their own plasma (not shown). We took advantage of the unambiguous difference in morphology between control and chol red cells in Hayem's solution to assess the effects of in vitro changes in membrane cholesterol content on red cell morphology.

Control red cells retained a normal appearance after 5 hr of incubation in control plasma or in control LDL (Fig. 6*E*). In contrast, control red cells acquired spurs when incubated in either chol plasma or chol lipoproteins (Fig. 6, *F* and *H*). Incubation of control red cells in lipoprotein-free plasma infranates ($d > 1.21$ g/ml) from either control or chol guinea pigs did not alter the normal biconcave shape (Fig. 6*G*). Therefore, the factor(s) which causes spurs in guinea pig red cells is present in chol plasma and chol lipoproteins but not in control plasma and control lipoproteins or in infranates from control or chol plasma.

We attempted to relate red cell cholesterol content and morphology unambiguously by reducing the cholesterol content of spurred red cells. Control red cells loaded with cholesterol by in vitro incubation and chol red cells were incubated in control plasma. Although the red cell cholesterol decreased to only 5–10% above normal levels, many cells still exhibited an abnormal morphology.

DISCUSSION

In vitro alterations of red cell cholesterol

We have shown that the cholesterol content of red cell membranes from guinea pigs fed a control diet can be increased by in vitro incubations with plasma, HDL, or LDL from cholesterol-fed, anemic guinea pigs, but not with plasma or lipoproteins from control guinea pigs regardless of the concentration of lipoprotein unesterified cholesterol in the medium. The increase in red cell cholesterol after incubation was reversible and was proportional to the concentration of unesterified cholesterol in the chol plasma or chol lipoproteins of the incubation medium.

We suggest that these results indicate that the accumulation of cholesterol by red cells requires the presence of abnormal lipoproteins, not just an increased concentration of normal lipoproteins. The most important compositional change in chol lipoprotein is probably an increased cholesterol concentration. Our data do not exclude the possibility that other factors influencing the cholesterol transfer in this system may be present.

Similar results have been reported in other systems. Cooper, Garcia, and Trey (25) have shown that lipo-

TABLE 2. Exchange of [^{3}H]cholesterol between guinea pig red cells and plasma[a]

Type of Experiment	Cholesterol Concentration[b] Red Cells	Plasma	k_{Pl-RBC}[c]	k_{RBC-Pl}[c]	F_{Pl-RBC}[d]	F_{RBC-Pl}[d]
	$\mu g/ml$	$\mu g/ml$				
Control red cells incubated in control plasma	116	72[e]	14.6	9.4	6.2	6.4
	212	89[e]	17.5	4.3	6.2	3.7
	52[e]	75	10.9	15.8	7.6	7.6
	183[e]	94	18.1	8.7	8.3	8.3
Mean ± SD			15.3 ± 3.3[f]	9.6 ± 4.7[g]	7.1 ± 1.0[g]	6.5 ± 2.0[g]
Cholesterol-loaded red cells incubated in chol plasma[h]	232[e]	620	8.5	18.6	26.5	22.0
	288[e]	1590	5.0	26.1	44.0	43.0
	335[e]	1180	7.7	24.1	44.0	39.0
Mean ± SD			7.1 ± 1.8[f]	22.9 ± 3.9[g]	38.2 ± 10.1[g]	34.7 ± 11.1[g]

[a] Experimental conditions were those described in Fig. 3. The data at $t = 4$ hr were used to calculate the kinetic parameters.

[b] μg of UC/ml of incubation mixture prior to incubation (t_0).

[c] k_{Pl-RBC} and k_{RBC-Pl} are the fractional exchange rates of cholesterol, i.e., the percentage of plasma cholesterol exchanging with red cells and of red cell cholesterol exchanging with plasma, respectively, per hour.

[d] F_{Pl-RBC} and F_{RBC-Pl} are the cholesterol fluxes (molecules of cholesterol $\times 10^7$/red cell/hr) from plasma to red cells and from red cells to plasma, respectively.

[e] Compartment initially labeled with [^{3}H]cholesterol.

[f] Differences between means are statistically significant at $P < 0.02$.

[g] Differences between means are statistically significant at $P < 0.01$.

[h] Cholesterol concentration of red cells after incubation was 15–35 μg/ml higher than before incubation; plasmas showed a comparable decrease.

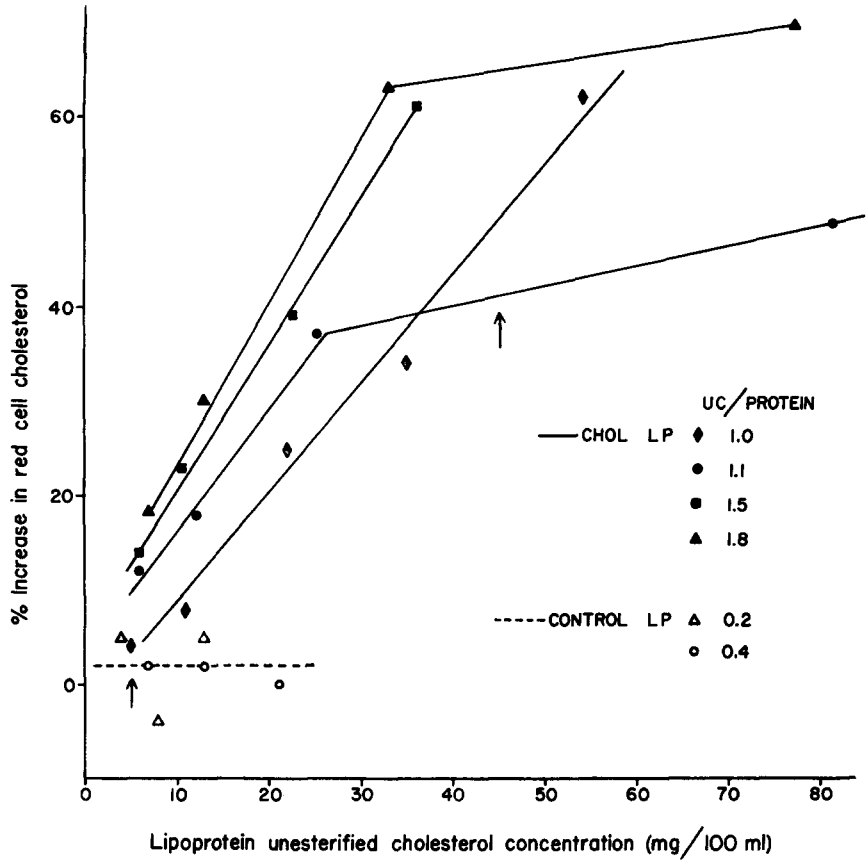

FIG. 4. Effect of unesterified cholesterol concentration in lipoproteins on uptake of cholesterol by guinea pig red cells. Control red cells were incubated for 8 hr at a 20% hematocrit (in presence of glucose, 1.7 mg/ml, and antibiotic, 300 units of penicillin/ml) with plasma lipoproteins (d < 1.21 g/ml) isolated from control plasma (– – –) or from chol plasma (——). Four chol lipoprotein preparations and two control lipoprotein preparations were used in these experiments. Similar results were obtained after 4 hr of incubation. Percentage increase in red cell cholesterol is expressed as in Fig. 1. UC/protein = weight ratio of unesterified cholesterol to protein of the individual plasma lipoprotein samples. Arrows indicate mean original concentration of lipoprotein UC in plasma (control, 5.0 mg/100 ml; chol, 45.1 ± 18.5 mg/100 ml).

TABLE 3. Transfer of cholesterol from chol LDL and HDL to control red cells in vitro

Lipoprotein in Medium	Unesterified Cholesterol Concentration	UC/Protein[a]	Increase in Red Cell Cholesterol	Lipoprotein Cholesterol Transferred to Red Cells
	mg/100 ml		%	%
HDL (C)	250	1.10	57.2	14
HDL (D)	66	0.80	31.1	24
HDL (E)	61	0.68	28.9	23
HDL (F)	39	0.52	26.8	34
HDL (G)	18	0.53	11.6	30
HDL (H)	7	0.51	2.9	15
LDL (C)	383	5.3	28.9	4
LDL (E)	175	3.5	28.9	7
LDL (F)	164	3.5	25.3	8
LDL (D)	133	4.9	24.6	9
LDL (H)	131	1.8	18.1	7
LDL (G)	117	1.8	22.4	10
LDL (E)	88	3.5	10.8	8
LDL (D)	76	4.9	9.4	8
LDL vesicles (F)	33	6.7	12.7	22

Control red cells were incubated for 5 hr at a 25% hematocrit in chol LDL or chol HDL isolated from six different chol guinea pigs. Chol HDL concentrations used were 3 times the physiological concentrations. Chol LDL concentrations were 0.75–1.5 times the physiological concentrations. HDL and LDL with same letter in parentheses were isolated from the same plasma. LDL vesicles were obtained by gel chromatography (13). Duplicate incubations were conducted with LDL vesicles.

[a] Weight ratio: mg of unesterified cholesterol/mg of protein.

protein alterations precede increased red cell cholesterol in lithocholic acid-fed monkeys. High concentrations of normal human lipoproteins do not alter red cell cholesterol either in vivo (types I and IV hyperlipoproteinemia [5, 9]) or in vitro (26). In contrast, the abnormal lipoproteins of patients with LCAT deficiency (8) or obstructive jaundice (6) can transfer cholesterol to normal red cells in vitro, and the red cells of these patients have elevated cholesterol levels. Patients with spur-cell anemia also have high red cell cholesterol levels, and their plasmas can transfer cholesterol to normal human red cells (7).

There was no increase in red cell phospholipid, similar to other incubation studies in which erythrocytes were kept metabolically viable (5, 27, 28). Consequently, the UC/PL ratio of these membranes was greater than 1. These cholesterol-loaded red cells and ghosts (Table 1) had compositions very similar to those of nonanemic guinea pigs fed cholesterol for 6 wk (29). Red cells and ghosts from cholesterol-fed, anemic guinea pigs had an increased amount of PL as well as of UC. This is probably due to the high percentage of circulating reticulocytes (10–35% of total red cells),[5] which are known to have a greater amount of PL than do mature erythrocytes.

The kinetics of [^{3}H]cholesterol exchange showed that cholesterol exchanged rapidly and completely between

[5] Ostwald, R., C. Sardet, M. Akers, H. Hansma, and J. Kroes. Unpublished observations.

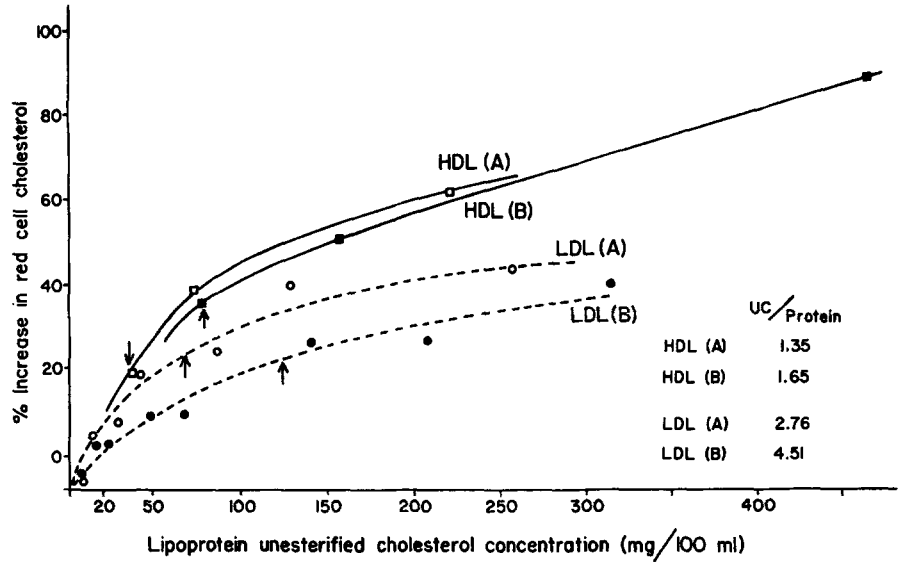

FIG. 5. Effect of concentrations of unesterified cholesterol in chol HDL and LDL on transfer of cholesterol to control red cells. Percentage increase in red cell cholesterol is expressed as in Fig. 1. Incubation conditions were essentially those described in Table 3. Chol HDL and LDL used were not the same as those shown in Table 3. Arrows indicate their original concentrations in plasma.

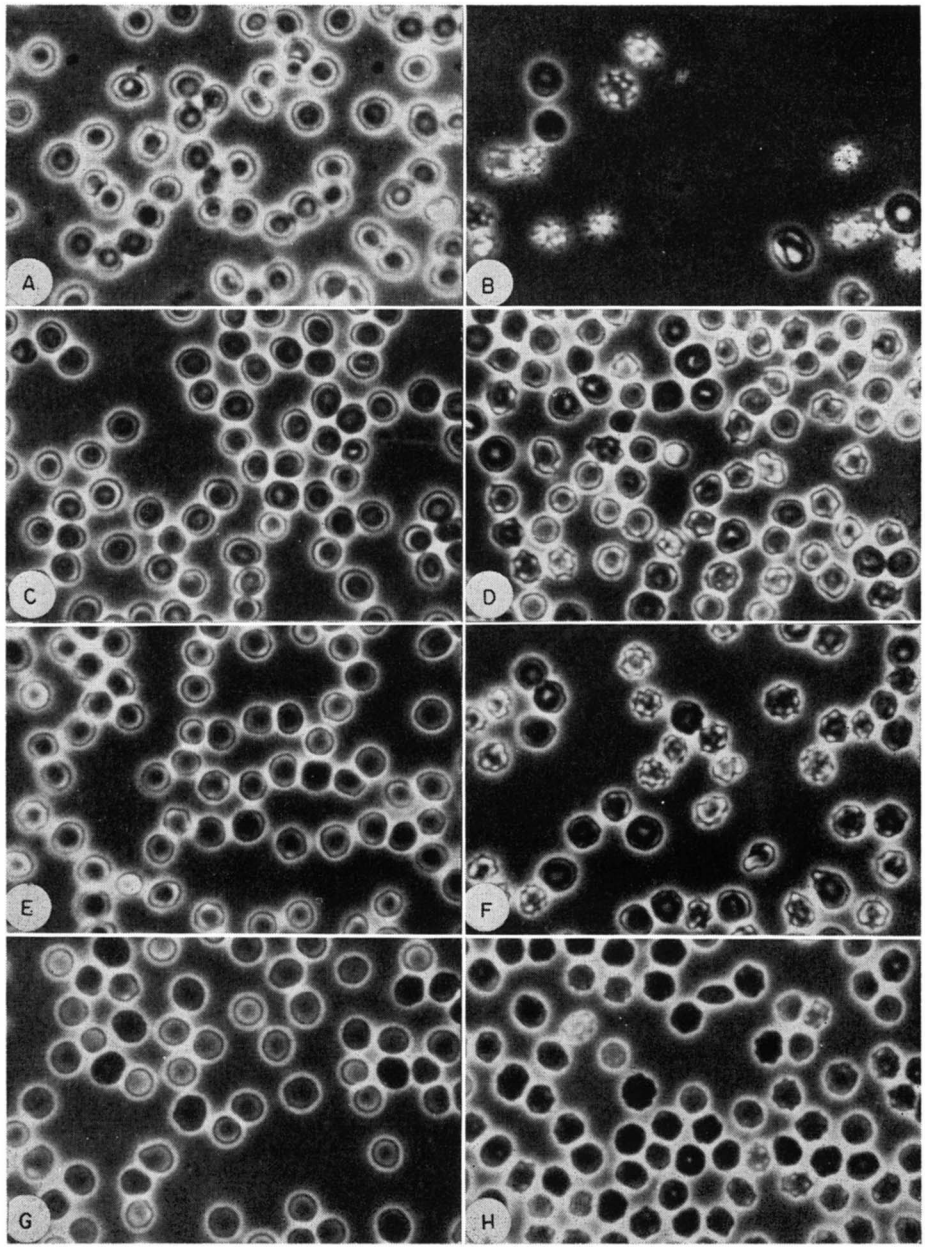

FIG. 6. Morphology of guinea pig red cells by phase-contrast microscopic observation. *A*, control red cells suspended in control plasma; *B*, red cells from chol guinea pigs, suspended in control plasma; *C*, *D*, *E*, *F*, *G*, *H*, red cells suspended in Hayem's solution; *C*, control red cells; *D*, red cells from a chol guinea pig; *E*, control red cells incubated 5 hr in control plasma; *F*, control red cells incubated 5 hr in chol plasma; *G*, control red cells incubated 5 hr in d > 1.21 g/ml infranate from chol plasma; *H*, control red cells incubated 5 hr in chol HDL (HDL [A] in Fig. 5).

guinea pig plasma and red cells, as in most other mammalian species (5, 26), except when chol plasma was incubated with control red cells. The cholesterol fluxes and fractional exchange rates in the control guinea pig system were similar to those reported by Quarfordt and Hilderman for humans (26).

Cholesterol fluxes between chol plasma and cholesterol-loaded red cells were much larger than those of control plasma and red cells. The fractional exchange rate of plasma cholesterol (k_{Pl-RBC}) was decreased and that of red cells (k_{RBC-Pl}) was increased, compared with the control rates. These results are reasonable for a system in which the red cell cholesterol content and the plasma lipoprotein concentration are increased. It has been shown for the cholesterol exchange between human plasma lipoproteins and red cells that the fractional exchange k_{Pl-RBC} decreased as the concentration of lipoprotein increased, possibly because there was a higher

probability of lipoprotein–lipoprotein collision (26). The fractional exchange of k_{RBC-Pl} of cholesterol-loaded guinea pig red cells increased, perhaps because there were more lipoprotein particles present for collisions or because there was more cholesterol per unit membrane area, although Quarfordt and Hilderman report that these factors were not limiting in the human system (26).

Alterations in cholesterol flux have been reported for several other systems. Human red cells lose cholesterol when incubated in plasma that has been depleted of unesterified cholesterol by the action of LCAT; Murphy (30) has shown that this was due to a decreased flux of cholesterol from plasma to red cells. McBride and Jacob (4) reported that the presence of abetalipoproteinemia plasma reduces cholesterol efflux from cholesterol-loaded human red cells when compared with that of normal plasma. Cholesterol flux and fractional exchanges can be increased by adding acetone to an incubation mixture containing human red cells and LDL (26, 31), whereas there was no change in either parameter when HDL was substituted for LDL (26). The authors suggested that perhaps the hydrophobic binding of cholesterol was decreased, thus facilitating its diffusion in the red cell–LDL collision complex; but the charge repulsion between HDL and red cells increased, since the dielectric constant of the incubation medium was lowered. It is possible that the large fluxes between guinea pig chol plasma and cholesterol-loaded red cells and the large k_{RBC-Pl} are due in part to decreased charge repulsions between the "cholesterol-coated" lipoproteins and red cells. Chol HDL has in fact been shown to have a slower electrophoretic mobility than control HDL (13). However, the electrophoretic mobilities of LDL and red cells were the same for control and cholesterol-fed guinea pigs.[5]

Red cell morphology and cholesterol content

When control guinea pig red cells were incubated with isolated chol lipoproteins, they acquired cholesterol and developed spurs similar to those of red cells circulating in cholesterol-fed guinea pigs; when control red cells were incubated with control lipoproteins or chol plasma infranates ($d > 1.21$ g/ml) they retained a normal shape. We suggest that an increase in membrane cholesterol is sufficient to cause spur formation in this in vitro system. There is some support for this hypothesis, because in many other cases of spurred or spiculed red cells the red cell cholesterol content has been found to be increased (3–5, 25). There are, however, reasons why the causal relationship between increased membrane cholesterol and morphological change for in vivo systems has been questioned. (*a*) The types of morphological alterations are not the same in different cases. Target cells have been observed, for instance, in patients with obstructive jaundice and LCAT-deficiency (6, 32), whereas spur cells have been observed in hepatocellular disease, abetalipoproteinemia (4, 5), and as a consequence of dietary orotic acid (rats), cholesterol (rabbits), and lithocholic acid (monkeys) (3, 4, 25). The role of cholesterol in maintaining normal red cell shape and the mechanism by which excess cholesterol causes alterations in normal shape are not known. Cooper and Jandl (6) have shown that both types of cells have an increased surface area/volume ratio and Murphy (33) has suggested that changes in the UC/PL ratio might be expected to cause changes in the surface tension parameters of the cell which could be responsible for shape changes. Target cells commonly have increased amounts of both membrane cholesterol and phospholipids, but in spur cells the phospholipid content usually remains unchanged. Consequently, the UC/PL ratio is more often abnormal in spur cells than in target cells, which might be one reason for their different morphology. It is also interesting to note that these two types of abnormal cells are sometimes interconvertible. For instance, both types of cells have been reported in cholesterol-fed rabbits (4), and McBride and Jacob reported that in orotic acid-fed rats red cells progressed from target forms to spur cells (4). (*b*) The amount of excess red cell cholesterol does not seem to be quantitatively related to the degree of morphological abnormality. For instance, the percentage of spiculed cells in a patient with obstructive jaundice varied greatly from day to day, while red cell cholesterol did not (7). Membrane cholesterol increases in hereditary abetalipoproteinemia, about 15–20% (34, 35), are far less than in spur-cell anemia, about 50–75% (5, 7), yet abnormalities in red cell morphology are reported to be quite similar. These anomalies are, however, difficult to interpret because the quantitation of morphological abnormality is not simple, and different authors may not have used the same scoring method. In lithocholic acid-fed monkeys, spurring precedes increased membrane cholesterol by several days (25), indicating that in some cases there must be factors other than membrane cholesterol content or UC/PL ratio which affect red cell morphology. (*c*) Unloading of excess cholesterol from guinea pig red cells did not proportionally reverse the morphological change. In other cases, however, the morphological changes were reversible both in vivo and in vitro (6, 7, 36). It is quite possible that the spurring of guinea pig red cells in vitro is irreversible even if it is caused by cholesterol loading or that the 5–10% of additional cholesterol remaining after unloading may still be sufficient to cause spurs.

We are currently investigating whether the cholesterol content of guinea pig red cells is quantitatively corre

lated with the degree of spur formation and whether lipoprotein or red cell bile acid levels change in response to dietary cholesterol.

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